Acta Physiol Plant (2017) 39:32
DOI 10.1007/s11738-016-2337-x

ORIGINAL ARTICLE

Identification of microRNAs from Zn-treated *Solanum nigrum* roots by small RNA sequencing

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Received: 8 June 2016 / Revised: 17 December 2016 / Accepted: 19 December 2016 / Published online: 24 December 2016

Abstract MicroRNAs (miRNAs) play key roles in regulating zinc (Zn) toxicity tolerance in plants. *Solanum nigrum* is a typical Zn/Cd-accumulating plant that has a high Zn/Cd tolerance. Despite their importance, no miRNAs have been identified from *S. nigrum* thus far. In this study, small RNA sequencing was used to identify Zn-responsive miRNAs in *S. nigrum* roots. We identified 176 differentially expressed miRNAs in *S. nigrum* roots in response to Zn toxicity. We also found that all these differentially expressed Zn-miRNAs simultaneously respond to the exogenous NO donor sodium nitroprusside (SNP), indicating that NO is involved in Zn-mediated miRNA expression in *S. nigrum*. These differentially expressed miRNAs are involved in regulating the following processes in *S. nigrum* roots: phenylpropanoid catabolic and metabolic processes; lignin catabolic and metabolic processes; and programmed cell death in response to ROS. These results suggest that these miRNAs play key roles in the Zn toxicity tolerance of *S. nigrum*.

Keywords Zinc toxicity · *Solanum nigrum* · High-throughput sequencing · MicroRNA

Introduction

Zinc (Zn) is indispensable micronutrients for plant growth and development, because it plays catalytic/co-catalytic structural roles in many enzymes (Wang et al. 2009). Thus, Zn is involved in many biological processes in plants. However, excess Zn in plants is harmful, because it can inhibit the uptake and distribution of mineral nutrition and disturb the metabolic processes, including photosynthesis and transpiration (Sharma et al. 2004). However, the adaptation mechanisms of Zn toxicity in plants remain unclear (Xu et al. 2010, 2011).

Nitric oxide (NO) plays a role in different signaling pathways, such as stomatal closure mediated by ABA, root morphogenesis regulated by auxin, wounding, and programmed cell death (PCD) (Correa-Aragunde et al. 2004). In a previous study, we determined that NO plays a role in the plant response to Zn toxicity (Xu et al. 2010, 2011; Li et al. 2013). However, the molecular mechanisms of the plant response to Zn toxicity that involves the NO signaling transduction pathway remain unclear.

MicroRNAs (miRNAs) play a role in modulating biological and metabolic processes, including the response to environmental stress and developmental processes (Song et al. 2010). Many miRNAs are evolutionarily conserved in plants (Jones-Rhoades et al. 2006). These conserved miRNAs may be used as a strategy for predicting novel miRNAs by homology searches in other species (Zhao

Communicated by E. Schleiff.

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Electronic supplementary material The online version of this article (doi:10.1007/s11738-016-2337-x) contains supplementary material, which is available to authorized users.

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et al. 2010). Several methods can be used to identify miRNAs, including computational methods using comparative genomics, microarray analysis, direct cloning using miRNA-enriched libraries, and high-throughput sequencing technologies.

MiRNAs modulate heavy metal tolerance in plants. Cadmium (Cd) toxicity upregulates the expression of *miR529* and *miR171*, whereas it downregulates the expression of *miR166* and *miR398* (Zhou et al. 2012). Ding et al. (2011) found that Cd-responsive miRNAs contain one or more heavy metal responsive elements (MRE) in their promoters. Cd toxicity downregulates the expression of *miR164*, *miR159*, and *miR167*, whereas it upregulates the expression of *miR156*. These miRNAs target MG (monothiol glutaredoxin), ABC (ATP-binding cassette) transporters, NRAMP transporter, and GGT (glutathione-c-glutamylcysteinyl transferase), indicating that these miRNAs are involved in modulating plant developmental response to heavy metal (Gupta et al. 2014; Li et al. 2013). However, miRNAs involved in Zn toxicity response are not known. In this study, we identified the Zn-responsive *Solanum nigrum* miRNAs by the small RNA sequencing technology. Potential mechanisms for the role of these Zn-responsive miRNAs in *Solanum nigrum* are discussed.

Materials and methods

Plants and treatments

Solanum nigrum seeds were sown on 1/2 MS medium (Sigma, St. Louis, MO, USA). Seven-day-old seedlings were transferred into Hoagland solution (Hoagland and Arnon 1950). Three-week-old seedlings were transferred to Hoagland solution with or without 200-μM $ZnCl_2$ or 100-μM NO donor sodium nitroprusside (SNP) for 2 days.

Isolation of small RNAs, library preparation, and deep sequencing

Total RNA was extracted from control, Zn-treated, and SNP-treated *S. nigrum* roots using TRIzol (Invitrogen) following the manufacturer's instructions (Wang et al. 2015). RNA samples from three independent biological replicates were mixed, and the small RNA fragments of 18–28 nt were then isolated and purified by 15% denaturing polyacrylamide gel electrophoresis. The small RNAs were converted to DNA by RT-PCR, then the DNA product (approximately 25 μg) was sequenced using the Solexa 1G Genome Analyzer (Shenzhen BGI, China). The low-quality tags and several types of contaminants were excluded from the 50-nt tags obtained from small RNA sequencing by data cleaning. Small RNAs (sRNAs) from high-throughput sequencing include siRNA, piRNA, snRNA, tRNA, rRNA, snoRNA, and miRNA. By comparing the sequences with those in databases and identifying the overlapping genome locations, small RNAs were annotated into different categories. Based on the miRNA results, the differentially expressed miRNAs were obtained between different treatments using a flexible method that depends on the specificity of the samples. Then, target prediction for the above-mentioned miRNAs was performed to confirm target sites. GO enrichment and KEGG pathway analysis were then annotated. Based on the above analysis, we obtained a clear biological information map of the miRNAs that regulate many key biological processes. The small RNA sequencing processing is shown in Fig. S1. All sequencing data were archived at Short Read Archive (SRA) of National Center for Biotechnology Information (NCBI) under accession no SUB1515222.

MiRNA prediction

This prediction strategy of potential miRNAs and their precursor sequences was implemented in the Mireap program developed by the BGI (http://sourceforge.net/projects/mireap/, Beijing Genome Institute, China) (Jin et al. 2015). The expression levels of known miRNA were compared to identify the differentially expressed miRNAs (Su et al. 2015).

Procedures for comparing miRNA expression

The expression levels of miRNA in the three samples (control, Zn-, and SNP-treated) were normalized to obtain the expression values in transcript per million (TPM). The normalization formula was: Normalized expression = Actual miRNA count/Total count of clean reads × 1,000,000 (Sun et al. 2014). The fold-change formula was: fold change = log 2 (treatment/control). The *P* value formula was (Zhu et al. 2016):

$$p(x|y) = \left(\frac{N_2}{N_1}\right)^y \frac{(x+y)!}{x!y!\left(1+\frac{N_2}{N_1}\right)^{(x+y+1)}} \quad \begin{matrix} C(y \le y_{\min}|x) = \sum_{y=0}^{y \le y_{\min}} p(y|x) \\ D(y \le y_{\max}|x) = \sum_{y \ge y_{\max}}^{\infty} p(y|x) \end{matrix}$$

The log 2 ratio plot and scatter plot were then generated. The process of data analysis was shown in Fig. S2. The criteria for target prediction are according to the suggestion of Schwab et al. (2005) and Allen et al. (2005), as well as those described by Shi et al. (2013).

Mature miRNA stem-loop qRT-PCR

The Stem-loop qRT-PCR was performed as previously described (Li et al. 2013). All qRT-PCR were replicated three times using templates prepared from three independent samples (see supplemental Table S1 for the primer sequences).

Results and discussion

S. nigrum is a Cd/Zn accumulator and has a high tolerance to Cd or Zn toxicity. Our previous studies analyzed the physiological and molecular mechanisms of *S. nigrum* responses to Zn/Cd toxicity and the involvement of NO in this process (Xu et al. 2009, 2010). However, the molecular mechanisms of Zn tolerance have not been elucidated, and information on Zn-responsive miRNAs in plants remains unknown. This study aimed to identify miRNAs in *S. nigrum* roots using small RNA sequencing (SRS).

Three small RNA libraries were constructed from the roots of *S. nigrum* seedlings treated with or without 200 μM $ZnCl_2$ or 100 μM NO donor SNP for 2 days. SRS was then performed to identify Zn-responsive and SNP-responsive *S. nigrum* miRNAs. The three libraries obtained more than 9.8 million total reads and approximately 9.6, 8.9, and 10.2 million unique clean reads from control, Zn, and SNP libraries, respectively (supplemental Table S2). We then obtained unannotated reads containing miRNAs by comparing the sequences of clean reads with the Silva, GtRNAdb, Rfam, and Repbase databases using the Bowtie software (Langmead et al. 2009) (supplemental Table S3). Because no *S. nigrum* genome or transcriptome database exists, these unique sRNA sequences were mapped to the *Solanum melongena* genome database (a *Solanum* species database). There are 11,205,136 total small RNAs (60.14%) representing that 887,660 (12.48%) unique small RNAs were common between Zn-treated and the control roots. Moreover, 3,195,000 total small RNAs (17.15%) representing 2,620,704 unique small RNAs (36.84%) were specific to the Zn-treated samples (Table 1, Fig. S3A), and 1,114,010 total small RNAs (13.92%) representing 12,091,195 (60.72%) small RNAs were common between SNP-treated and the control roots. In addition, 3,512,193 total small RNAs (43.87%) representing 3,956,461 unique small RNAs (19.87%) were specific to the SNP-treated root (Table 1, Fig. S3B), and 908,462 total small RNAs (12.57%) representing 11,737,440 (61.25%) unique small RNAs were common between the SNP- and Zn-treated *S. nigrum* samples. Furthermore, 3,717,741 total small RNAs (51.45%) representing 4,290,271 unique small RNAs (22.39%) were specific to the SNP-treated samples, and 2,599,902 total small RNAs (35.98%) representing 3,135,485 unique small RNAs (16.36%) were specific to the Zn-treated samples (Table 1, Fig. S3C). The majority of sRNA reads ranged from 21 to 24 nt, which is the typical miRNA distribution patterns (Fig. 1). To identify known miRNAs, all small RNA sequences were blasted against the known miRNAs in the miRNA database (http://www.mirbase.org/). After Blastn and sequence analysis, 944 conserved miRNAs in control plants, 863 conserved miRNAs in Zn-treated roots, and 956 conserved miRNAs in SNP-treated roots were identified in *S. nigrum* seedlings (Table S4).

The number of reads represents miRNA enrichment level (Yao et al. 2007; Zhao et al. 2010). In total, there are 176 differentially expressed miRNAs in Zn-treated roots (Table S5) and 475 differentially expressed miRNAs in SNP-treated roots (Table S6). Among these 176 differentially expressed miRNAs in the Zn-treated plants, 62 miRNAs were upregulated and 114 were downregulated in the Zn-treated roots (Table S5). Among these 475 differentially expressed miRNAs in the SNP-treated roots, 232 miRNAs were upregulated and 243 were downregulated in

Table 1 Summary of small RNA sequences among control, Zn-treated, and SNP-treated *S. nigrum* roots

Types	Unique sRNAS	Percentage	Total sRNAS	Percentage
Total_sRNAs	7,113,954	100.00	18,630,455	100.00
Control_&_Zn	887,660	12.48	11,205,136	60.14
Control_specific	3,605,590	50.68	4,230,319	22.71
Zn_specific	2,620,704	36.84	3,195,000	17.15
Total_sRNAs	8,005,443	100.00	19,912,937	100.00
SNP_&_Control	1,114,010	13.92	12,091,195	60.72
SNP_specific	3,512,193	43.87	3,956,461	19.87
Control_specific	3,379,240	42.21	3,865,281	19.41
Total_sRNAs	7,226,105	100.00	19,163,196	100.00
SNP_&_Zn	908,462	12.57	11,737,440	61.25
SNP_specific	3,717,741	51.45	4,290,271	22.39
Zn_specific	2,599,902	35.98	3,135,485	16.36

the Zn-treated roots (Table S6). Interestingly, we found that all of the 62 upregulated miRNAs and 114 downregulated miRNAs in the Zn-treated roots showed a similar expression pattern in SNP-treated roots (Table S7), indicating that NO may be involved in Zn toxicity-induced miRNA expression in *S. nigrum* roots. To validate the miRNA sequencing result, qRT-PCR analysis was performed to test the expression of five miRNAs (*miR6189*, *miR7502*, *miR3437*, *miR1535*, and *miR3635*). As shown in Fig. 2, these miRNAs were differentially expressed in SNP- or Zn- treated roots. The expression pattern was consistent with the small RNA sequencing result.

For further understand the roles of the *S. nigrum* miRNAs in Zn toxicity tolerance, putative targets were also predicted. For further confirmation, the expression levels of the target genes and their miRNAs were identified by qRT-PCR. The expression patterns of the miRNAs and their target genes were complementary, but not identical (Fig. 2). These data indicate a function for miRNAs in the Zn toxicity response of *S. nigrum* roots.

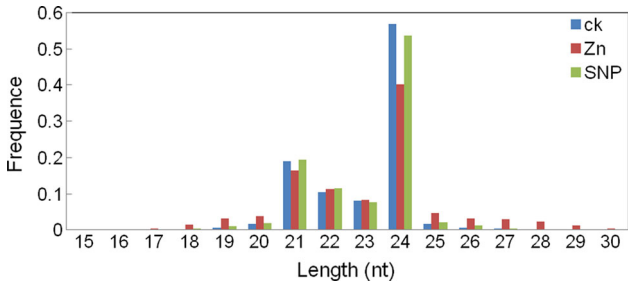

Fig. 1 Length distribution of sRNAs. Three-week-old *S. nigrum* seedlings were transferred into Hoagland solution with or without 200-μM $ZnCl_2$ or 100-μM SNP for 2 days. Total RNA was extracted from *S. nigrum* roots and the sRNAs were isolated for library preparation

Bioinformatics analysis indicated that the annotated target genes participate in plant developmental and physiological processes (Figs. S4 and S5). GO enrichment analysis indicated that the target genes are involved in the following processes in *S. nigrum* roots: modulating phenylpropanoid catabolic and metabolic processes; lignin catabolic and metabolic processes; and programmed cell death (PCD) (Fig. S5). These results indicate that Zn-induced miRNAs regulate root system development by modulating the catabolic and metabolic processes of phenylpropanoid and lignin; however, this requires further study. It is well known that heavy metal accumulation induces tolerance to pathogen infection. Mittra et al. (2004) found that pretreatment with Cd improved resistance to *Fusarium* infection in wheat. We found that the predicted target genes showed enrichment in the KEGG pathway of plant–pathogen interaction and RNA polymerase (Table S8), indicating that excess Zn modulates pathogen tolerance and the transcriptional process by miRNA pathways. However, this requires further investigation.

The predicted target gene of *miR6189* is *HMA1*, an important Zn transporter in plants. It is well known that *HMAs* mediate Zn accumulation in plants (Kramer et al. 2007). *Arabidopsis HMA1* mediated metal transport into the chloroplast. Downregulation of *miR6189* is accompanied by an accumulation of *HMA1* in excess Zn-treated *S. nigrum* roots, indicating that *miR6189* mediates the level of Zn in plants. Predicted target genes of *miR1535* include *auxin response factor 2* (*ARF2*). We found that *miR1535*

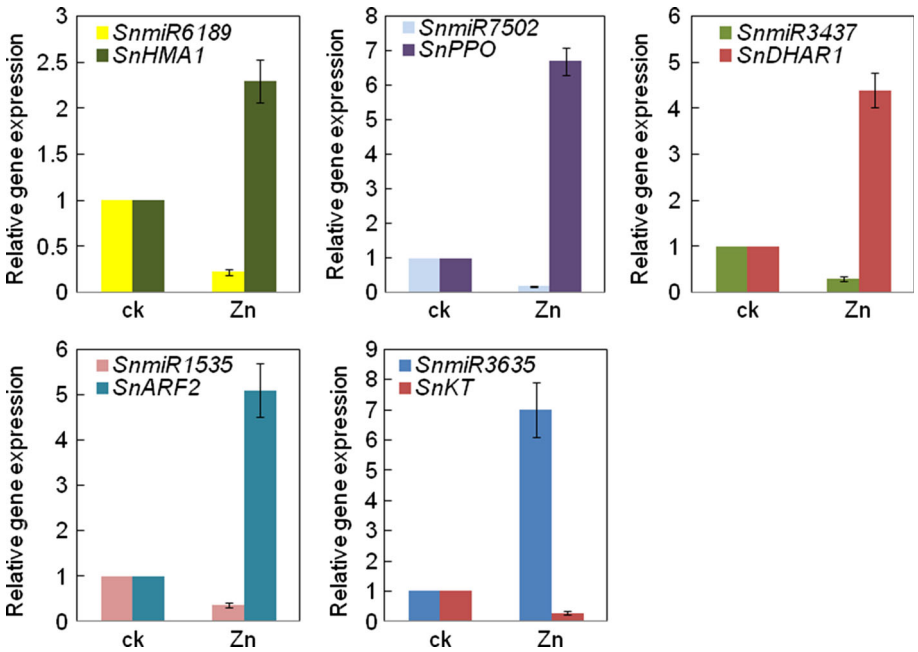

Fig. 2 Gene expression of five miRNAs and their targets in 3-week-old *S. nigrum* roots treated with or without 200-μM $ZnCl_2$ for 2 days. The expression levels of the indicated genes in the untreated roots were set to 1

was downregulated, whereas its target gene *ARF2* was upregulated in *S. nigrum* subjected to Zn toxicity. These data suggest that Zn toxicity changes the auxin signal by modulating the expression of miRNAs, such as *miR1535*. The predicted target gene of *miR7502* is polyphenol oxidase (PPO). Michael and Krishnaswamy (2011) found that excess Zn increases PPO activity in bean seedlings, suggesting that PPO plays an important role in Zn tolerance. Future study will elucidate the role of PPO in Zn tolerance and its interaction with *miR7502* in *S. nigrum*.

In this study, we found that many miRNAs are regulated in *S. nigrum* roots under excess Zn, indicating their potential role in Zn tolerance by regulating downstream target genes. Our previous study indicated that NO accumulation in roots improves Zn tolerance (Xu et al. 2010). In this study, we found that all of the differentially expressed Zn-miRNAs simultaneously respond to the exogenous NO donor SNP, indicating that NO is involved in Zn-mediated miRNA expression in plants. These data establish a foundation for a better understanding of miRNA roles in response to Zn toxicity in plants.

Author contribution statement JX conceived the study and designed the experiments. JZ, ZX, PZ, RW, and JG carried out the experiments. JZ, ZX, and PZ analyzed the data. ZX and JX wrote the manuscript.

Acknowledgements This work was supported by the China National Natural Sciences Foundation (31170228, 31272239), the National Key Research and Development Program of China (2016YFC0501901), Yunnan Province Foundation for academic leader (2014HB043), and Hebei Province National Natural Sciences Foundation for Distinguished Young Scientists (C2013503042). The authors gratefully acknowledge the Central Laboratory of the Xishuangbanna Tropical Botanical Garden for providing research facilities.

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