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ORIGINAL ARTICLE

Identification of aluminum-responsive microRNAs in *Medicago truncatula* by genome-wide high-throughput sequencing

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Abstract MicroRNAs (miRNAs) play important roles in response of plants to biotic and abiotic stresses. Aluminum (Al) toxicity is a major factor limiting plant growth in acidic soils. However, there has been limited report on the involvement of miRNAs in response of plants to toxic Al^{3+}. To identify Al^{3+}-responsive miRNAs at whole-genome level, high-throughput sequencing technology was used to sequence libraries constructed from root apices of the model legume plant *Medicago truncatula* treated with and without Al^{3+}. High-throughput sequencing of the control and two Al^{3+}-treated libraries led to generation of 17.1, 14.1 and 17.4 M primary reads, respectively. We identified 326 known miRNAs and 21 new miRNAs. Among the miRNAs, expression of 23 miRNAs was responsive to Al^{3+}, and the majority of Al^{3+}-responsive mRNAs was down-regulated. We further classified the Al^{3+}-responsive miRNAs into three groups based on their expression patterns: rapid-responsive, late-responsive and sustained-responsive miRNAs. The majority of Al^{3+}-responsive miRNAs belonged to the 'rapid-responsive' category, i.e. they were responsive to short-term, but not long-term Al^{3+} treatment. The Al^{3+}-responsive miRNAs were also verified by quantitative real-time PCR. The potential targets of the 21 new miRNAs were predicted to be involved in diverse cellular processes in plants, and their potential roles in Al^{3+}-induced inhibition of root growth were discussed. These findings provide valuable information for functional characterization of miRNAs in Al^{3+} toxicity and tolerance.

Keywords Aluminum toxicity · High-throughput sequencing · Medicago · MicroRNA

Abbreviations

miRNA	MicroRNA
pre-miRNA	MicroRNA precursor
pri-miRNA	MicroRNA primary transcript
miRNA*	MicroRNA star
qRT-PCR	Quantitative real-time PCR

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Introduction

MicroRNA (miRNA) is one type of endogenous non-coding small RNAs with approximate length of 21 nt, and is a large family of small RNA (sRNA). miRNAs have been found in animals (Lee and Ambros 2001) and plants (Llave et al. 2002). miRNA-like small interfering RNAs (siRNAs) were also reported in fungi (Lee et al. 2010). Similar to siRNAs, miRNAs play an important role in regulation of gene expression. The major difference between miRNA and siRNA lies in their way of generation (Jones-Rhoades et al. 2006). siRNAs are processed from long, double-strand RNAs; whereas miRNAs are generated from single-strand RNAs with stem-loop structure, called pre-miRNAs (Bartel 2004). The generation of miRNA in plants differs

from animals. In plants, the primary transcripts of miRNAs (pri-miRNAs) which are translated by RNA polymerase II are digested by DCL1, a dicer like enzyme, leading to the generation of pre-miRNAs, thereafter pre-miRNAs were digested by DCL1 to produce miRNA/miRNA* duplexes (Kurihara and Watanabe 2004). Mature miRNAs which depart from miRNA/miRNA* are loaded into AGO1, an argonaute protein, to assemble RNA-induced silence complex (RISC) (Jones-Rhoades et al. 2006). Multiple proteins participate in this process, including HYPONASTIC LEAVESI (HYL1) (Lu and Fedoroff 2000; Wu et al. 2007), SERRATE (SE) (Lobbes et al. 2006), nuclear cap binding complex (CBC) (Fang and Spector 2007), HUA ENHANCER1 (HEN1) (Park et al. 2002), and DAWDLE (Yu et al. 2008). miRNAs direct RISC to their target mRNAs, and repress the expression of their target genes by cleavage or inhibition of translation (Jones-Rhoades et al. 2006). In a recent study, Wu et al. (2010) reported that miRNAs can also regulate expression of target genes by DNA methylation.

It has been widely reported that miRNAs are involved in the regulation of numerous physiological processes in plants, including seed germination (Reyes and Chua 2007), flower development (Chen 2004), root development (Gutierrez et al. 2009). There is also a growing body of evidence showing that miRNAs play a role in transduction of hormonal signals in plants, such as auxin (Guo et al. 2005; Meng et al. 2009), abscisic acid (Reyes and Chua 2007) and gibberellins (Achard et al. 2004). In addition, the involvement of miRNAs in responses of plants to biotic and abiotic stresses has been reported (for example, Sunkar and Zhu 2004). These include cold (Jian et al. 2010), drought (Li et al. 2008a; Trindade et al. 2009; Wang et al. 2011), salinity (Ding et al. 2009; Liu et al. 2008), nutrition deficiency (Bari et al. 2006; Liang et al. 2010; Pant et al. 2008), heavy metals stress (Zhou et al. 2008) and oxidative stress (Sunkar et al. 2006).

Aluminum (Al) is the most abundant metal in the Earth's crust and usually occurs in non-phytotoxic forms of aluminosilicate under most conditions. However, it is solubilized to phytotoxic Al^{3+} species in acidic soils, and becomes a major factor limiting crop production and yield in the acid soils. Inhibition of root elongation is one of the earliest and most distinct symptoms of Al^{3+} phytotoxicity (Ryan et al. 1993). The root apex in general and the root transition zone in particular have been identified as critical sites for sensing Al^{3+} toxicity and expressing tolerance to Al^{3+} (Ryan et al. 1993; Sivaguru and Horst 1998). Many physiological processes have been identified to be associated with Al^{3+} toxicity and tolerance (Rengel and Zhang 2003; Ryan et al. 2011). However, the primary mechanisms underlying the Al^{3+} phytotoxicity remain largely unknown and elusive. The involvements of miRNAs in mineral stress have been reported (see review Sunkar et al. 2007), but there has been no report on systemic identification of Al^{3+}-responsive miRNAs and their targets at the global genome level by high-throughput sequencing. *Medicago truncatula* is an annual legume species distinguished by its small diploid genome and easy transformation, and has been used as a model plant to study functional genomics of legume plants (Trinh et al. 1998). Identification of new miRNAs in plants on a genome-wide scale is one of the essential steps for functional characterization of miRNAs. To understand the role of miRNAs in response of plants to Al^{3+} toxicity, we identified a number of conserved and non-conserved miRNAs that were responsive to toxic Al^{3+} by high-throughput sequencing, and their potential role in mediation of Al^{3+}-induced inhibition of root growth and development was discussed.

Materials and methods

Plant materials and growth conditions

Seeds of *Medicago truncatula* (cv. Jemalong A17, kindly provided by Dr. Carroll Vance, USDA-ARS, Plant Science Research, St. Paul, MN, USA), were soaked in concentrated, anhydrous sulfuric acid for about 5 min to scarify seed coat, and then washed thoroughly with water. After kept at 4°C in 0.8% agar plates for 3 days, the seeds were germinated at 25°C in dark for 2 days. Seedlings were grown hydroponically in aerated nutrient solution. The nutrient solution contained 1 mM NH_4NO_3, 2.5 mM KNO_3, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 0.25 mM K_2SO_4, 0.25 mM $CaCl_2$, 100 μM FeNaEDTA, 30 μM H_3PO_3, 5 μM $MnSO_4$, 1 μM $ZnSO_4$, 1 μM $CuSO_4$, 0.7 μM Na_2MoO_4, 1 mM NH_4NO_3, 2.5 mM KNO_3 and 50 μM KCl at pH 5.8. Seedlings were grown in a growth chamber under conditions of a 16/8 h light/dark cycle at 25°C for 1 week. During seedling growth, the nutrient solution was changed every 2 days.

Aluminum treatment

Seven-day-old *M. truncatula* seedlings were exposed to solution containing 0.5 mM $CaCl_2$ with 10 μM $AlCl_3$ (pH 4.5) for varying periods to determine the effect of Al^{3+} on root growth, while *M. truncatula* seedlings of the same age incubated in 0.5 mM $CaCl_2$ solution (pH 4.5) were used as controls. Root tips ($\approx$1.5 cm in length) were collected to isolate total RNA for construction of sRNA libraries after exposure to $AlCl_3$ solution for 4 and 24 h, respectively. The corresponding root tips grown in the control solution were used to construct library of sRNA for control.

Construction and sequencing of sRNA libraries

Total RNA was isolated from root tips of the following three groups: control group (CK), and groups treated with Al for 4 h (Al4) and 24 h (Al24), respectively. sRNA libraries were constructed by the methods described previously (Hafner et al. 2008; Wang et al. 2011). Briefly, sRNA with the length of 18–30 nt was separated and purified on a 15% TBE-urea denaturing PAGE gel. The 5′- and 3′-RNA adapters were ligated to sRNA with T4 RNA ligase (TaKaRa). Thereafter, the adapter-ligated sRNAs were transcribed to single-stranded cDNA with superscript II reverse transcriptase (Invitrogen). PCR was conducted using the primer designed according to the adapter sequence to amplify single-stranded cDNA template to double-stranded cDNA. PCR products were sequenced on a Solexa sequencer (Illumina) at the Beijing Genomics Institute (BGI), Shenzhen, China.

Analysis of high-throughput sequencing data

The raw reads obtained from the Solexa sequencer were cleaned by removing contaminant reads including those reads with 5′-primer contaminants, reads without 3′-primer, reads with poly A, and reads with length less than 18 nt. Clean reads were then used to analyze length distribution. Thereafter, clean reads were mapped to *Medicago truncatula* genome Mt3.5.1 (http://www.medicagohapmap.org/downloads_genome/Mt3.5/Mt3.5.1_pseudomolecules.tar.gz) using SOAP (Li et al. 2008b), with no mismatches being allowed.

All clean reads were annotated using different databases. The known miRNAs were annotated by comparing to miRBase 16 (ftp://mirbase.org/pub/mirbase/CURRENT/miRNA.dat.gz). rRNAs, scRNAs, snoRNAs, snRNAs and tRNAs were annotated by BLASTn to NCBI Genbank database and Rfam database ($e = 0.01$). Small interfering RNA (siRNA) is a double-strand RNA with 22–24 nt in length, each strand is 2 nt longer than the other on the 3′ end. According to this structural feature, we aligned tags from clean reads to each other to find those sRNAs that meet this criterion. These tags may be potential siRNA candidates and were removed from new miRNA analysis. Reads that were not annotated were used to predict new miRNA.

Pre-miRNA candidates were predicted using MIREAP (http://sourceforge.net/projects/mireap/). Parameters were set as follows: minimal miRNA sequence length of 18 nt; maximal miRNA sequence length of 25 nt; minimal miRNA reference sequence length of 20 nt; and maximal miRNA reference sequence length of 23 nt. Maximal copy number of miRNAs on reference was set to be 20 nt, and maximal free energy allowed for a miRNA precursor was −18 kcal/mol. Maximal space between miRNA and miRNA* was set to be 300 nt. Minimal base pairs of miRNA and miRNA* were taken as 16; maximal bulge of miRNA and miRNA* was 4. Maximal asymmetry of miRNA/miRNA* duplex was 4 nt. Flank sequence length of miRNA precursors was 20 nt. New miRNAs' pre-miRNA stem-loop structure was constructed by m-fold (Zuker 2003).

Target predicting was carried out following the rules proposed by Allen et al. (2005): (1) no more than four mismatches between sRNA and target (G–U bases count as 0.5 mismatches); (2) no more than two adjacent mismatches in the miRNA/target duplex; (3) no adjacent mismatches in positions 2–12 of the miRNA/target duplex (5′ of miRNA); (4) no mismatches in positions 10–11 of miRNA/target duplex; (5) no more than 2.5 mismatches in positions 1–12 of the miRNA/target duplex (5′ of miRNA); and (6) minimum free energy (MFE) of the miRNA/target duplex should be >75% of the MFE of the miRNA bound to its perfect complement.

In miRNA expression analysis, *P* value was calculated as:

$$P(x|y) = \left(\frac{N_2}{N_1}\right)^y \frac{(x+y)!}{x!y!\left(1+\frac{N_2}{N_1}\right)^{(x+y+1)}}$$

$$C(y \le y_{\min}|x) = \sum_{y=0}^{y \le y_{\min}} p(y|x)$$

$$D(y \ge y_{\max}|x) = \sum_{y \ge y_{\max}}^{\infty} p(y|x)$$

Normalized read count was calculated as:

$$\text{Normalized read count} = \text{actual miRNA count}/\text{total count of clean reads}) \times 1{,}000{,}000$$

If one miRNA has no read in a library, the normalized read count of this miRNA in the library was arbitrarily set to be 0.001 for further calculation.

Quantitative real-time PCR

Total RNA was isolated from *M. truncatula* root tips exposed to different solutions identical to those described above using Trizol (Invitrogen) according to the manufacturer's protocols. For determination of miRNA expression, RNAs were reverse-transcribed by One Step PrimeScript® miRNA cDNA Synthesis Kit (TaKaRa), which added a ploy (A) tail to the 3′-end of miRNA and with transcription leading by a known oligo-dT ligate. SYBR Premix Ex Tag II (TaKaRa) was used for qRT-PCR. Small nuclear RNA U6 was used as an internal reference. All primers used in this paper were given in Supplemental Table S1. qRT-PCR experiments were performed on

Mx3000P™ PCR system (Agilent-Stratagene, USA). PCR program was set as: (1) 95°C 30 s; (2) 95°C, 5 s; thereafter 60°C, 30 s, 40 cycles.

Results

Deep-sequencing results of *M. truncatula* sRNA

To identify miRNAs that were responsive to toxic Al^{3+}, three sRNA libraries were constructed from *M. truncatula* root tips exposed to solution without Al^{3+} (control, CK) and solution containing 10 μM ACl_3 for 4 h (Al4) and 24 h (Al24), respectively. A high-throughput sequencing technology, Solexa, was employed to sequence these libraries. This led to the generation of 17.1, 14.1 and 17.4 M raw reads from libraries of CK, Al4 and Al24, respectively (Table 1). After removal of contaminant reads, clean reads with length in the range between 18 and 30 nt were obtained from the raw reads. Most clean reads were those with a length of 19–26 nt (Fig. 1). Reads with length of 24 nt were the most abundant, followed by the reads with length of 21 nt (Fig. 1).

About 11.8 M clean reads from CK (2,869,846 unique reads), 9.7 M clean reads from Al4 (1,598,588 unique reads) and 11.7 M clean reads (2,967,233 unique reads) from Al24 were mapped to *M. truncatula* genome sequence (Mt3.5.1, released in November 2010) with SOAP (Li et al. 2008b). miRNA, tRNA, siRNA, snRNA, snoRNA, rRNA, repeat regions, exon and intron RNA reads were annotated, respectively. Reads that were used for prediction of new miRNAs for CK, Al4 and Al24 were 6,179,361, 3,556,311 and 6,589,467, respectively (Table 1).

Table 1 Statistical analysis of sequencing reads for the three libraries

	Total reads	Unique reads
CK		
Raw reads	17,194,661	
Clean reads	15,863,178	5,038,244
Mapped to genomic	11,845,080	2,869,846
miRNA	926,887	4,001
Without annotation	6,179,361	3,301,112
Al4		
Raw reads	14,125,168	
Clean reads	12,272,519	2,883,437
Mapped to genomic	9,768,712	1,598,588
miRNA	795,748	2,504
Without annotation	3,556,311	1,876,252
Al24		
Raw reads	17,438,054	
Clean reads	16,248,638	5,410,314
Mapped to genomic	11,732,713	2,967,233
miRNA	976,809	4,259
Without annotation	6,589,467	3,588,917

Library for CK was constructed from *M. truncatula* roots exposed to solution without Al (pH 4.5), libraries for Al4 and Al24 were constructed from *M. truncatula* roots exposed to solution containing 10 μM Al (pH 4.5) for 4 and 24 h, respectively

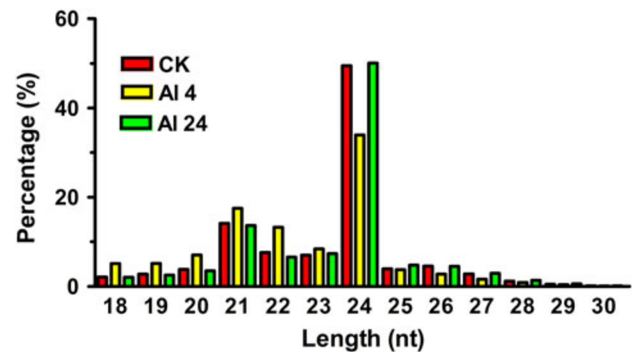


Fig. 1 The length distribution of sRNAs in the libraries of control (CK), treatment with Al for 4 h (Al4) and 24 h (Al24)

Identification of known miRNA in *M. truncatula*

To identify known miRNAs from the three libraries constructed in this work, clean reads were used to compare known *M. truncatula* miRNA precursors or mature miRNA sequence using miRBase 16.0 (Griffiths-Jones et al. 2008). There were 291, 262 and 282 miRNAs that were matched to the known *M. truncatula* miRNA from the libraries of CK, Al4 and Al24, respectively (Table 1; Supplemental Table S2). A total of 326 known miRNAs was identified in the three libraries. Some of the miRNAs exhibited extremely low expression levels in the three libraries (normalized count less than 10), i.e. mtr-miR171g and mtr-miR2588a. As the use of low expression miRNAs is prone to cause false results in express analysis, these miRNAs were not used for further analysis. After removing these miRNAs, a total of 67 known miRNAs belonging to 24 families was used for further analysis (Supplemental Table S3). The read counts differed among the 67 known miRNAs. For instance, mtr-miR166 was sequenced 391, 456 and 494K times in the libraries of CK, Al4 and Al24, respectively. This was the highest frequency among the miRNAs detected, and the frequency of mtr-miR156g was the second in terms of its sequenced frequency (Fig. 2).

Eight known miRNAs*, including mtr-miR1507*, mtr-miR1509*, mtr-miR1510a*, mtr-miR1510b*, mtr-miR2086*, mtr-miR2087*, mtr-miR2088a* and mtr-miR2089* were sequenced in the three libraries. Interestingly, the sequences of mtr-miR1510a* and mtr-miR2088a* occurred more

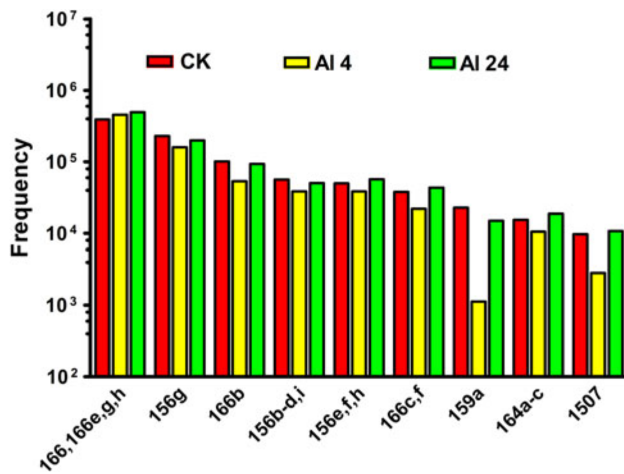


Fig. 2 Frequency distribution for those miRNAs that had the most reads from the three libraries (CK, Al4 and Al24)

frequently than those of their corresponding mature miRNAs, while the frequency of mtr-miR2089* sequence was comparable to its corresponding mtr-miR2089 (Table 2). A similar situation has been reported (Zhang et al. 2009). miRNAs are thought to be more stable than miRNA* in vivo due to their combination with RISC (Jones-Rhoades et al. 2006). There is a hypothesis that the amounts of miRNA are ten times more than those of miRNA* (Rajagopalan et al. 2006). Accordingly, our results indicate that mtr-miR1510a*, mtr-miR2088a* and mtr-miR2089* are likely to be true mature miRNAs.

Table 2 Frequency of eight known miRNA*s and their miRNA in the libraries of CK, Al4 and Al24

Name	Sequence frequency		
	CK	Al4	Al24
mtr-miR1507	9,708	2,835	10,731
mtr-miR1507*	415	117	240
mtr-miR1509	1,320	691	914
mtr-miR1509*	38	6	41
mtr-miR1510a	776	300	393
mtr-miR1510a*	1,271	1,012	924
mtr-miR1510b	3,777	2,052	3,923
mtr-miR1510b*	306	93	222
mtr-miR2086	5,490	2,961	4,493
mtr-miR2086*	272	164	342
mtr-miR2087	105	72	137
mtr-miR2087*	19	5	17
mtr-miR2088a	306	105	201
mtr-miR2088a*	653	279	506
mtr-miR2089	828	262	824
mtr-miR2089*	874	396	786

Identification of novel miRNAs in *M. truncatula*

Based on the criteria for annotation of novel miRNA (Ambros et al. 2003; Meyers et al. 2008), a stem-loop precursor is a prerequisite for the annotation of new miRNA. The secondary structures of potential miRNA precursors were obtained using m-fold (Zuker 2003). There were 252 potential pre-miRNAs that met the requirements for new miRNAs (Supplemental Table S4). miRNA* has been used as strong evidence to identify miRNA (Meyers et al. 2008). We identified 21 candidates that had complementary miRNAs* (Table 3), suggesting that these candidates may be new miRNAs. The lengths of the 21 new miRNAs ranged from 21 to 23 nt with 85.7% being 21 nt in length, a classical length of miRNA. Among the 21 new miRNAs, 14 started with a 5′-uridine, which is also a hallmark of miRNA (Yao et al. 2007). The stem-loop structures of 21 new miRNA precursors are shown in Fig. 3 and Supplementary Figure S1. Several new miRNAs potentially expressed from multiple miRNA precursors, i.e. pmiR-007 and pmiR-017 (Table 3). Similarly to the known miRNAs, new miRNAs with normalized read-count less than ten in the three libraries were removed from the expression analysis, leading to nine miRNAs that were further analyzed.

Identification of Al^{3+}-responsive miRNAs

Depending on their read counts, results obtained from the high-throughput sequencing can also be used to compare the difference in expression of miRNAs among different treatments. For comparison among the inter-libraries, the reads of the three libraries had to be normalized. In the libraries of Al4 and Al24, miRNAs were designated as 'up-regulated' if their normalized read counts were greater than those in the control library, the fold-changes were greater than 2 and $P < 0.05$. Similarly, miRNAs were designated as 'down-regulated' if their normalized read counts in Al4 and Al24 libraries were lower than those in the control library, the fold-changes were less than 0.5 and $P < 0.05$. We identified 20 miRNAs belonging to 15 families as down-regulated and 3 miRNAs belonging to 3 families as up-regulated in Al4 library by these criteria (Fig. 4; Supplemental Table S5). There were four down-regulated miRNAs belonging to four families, and one up-regulated in the Al24 library (Fig. 4; Supplemental Table S5). These results indicate that the number of Al^{3+}-induced down-regulated miRNAs is greater than that of up-regulated miRNAs.

Samples were collected after exposure of *M. truncatula* seedlings to solutions containing toxic Al^{3+} for 4 and 24 h, and referred to the two treatments as 'short-term' and 'long-term' Al^{3+} treatment, respectively. Among the

Table 3 Summary of new miRNAs

Name	*L*	Sequence	Loci	miRNA* count	Normalized read count		
					CK	Al4	Al24
pmiR-001	21	GGCAUGGGAUAGUAGGGAAGA	AC235678:65782:65900:+	24,114	1,158.03	1,651.98	716.74
pmiR-002	22	UAUCUGUGAUCUUGGACACCAA	Chr1:16010420:16010512:+	1	0.63	0.001	0.001
pmiR-003	22	UACGUGUGUCUUCACCUCUGAA	Chr2:18000278:18000408:+	60	519.44	162.48	496.90
pmiR-004	21	UGUGAAUGAUGCGGGAGCUAA	Chr3:19927118:19927243:+	86	70.98	44.82	41.85
pmiR-005	22	UAAAUGUGUUGGAAUUAAGGUU	Chr3:22394764:22394883:+	6	23.83	17.763	18.28
pmiR-006	21	UACAGUGUUUGAACAAAGGCC	Chr3:6675930:6676018:−	2	0.95	0.001	0.001
pmiR-007 (loci A)	21	UCACUAAUGGAUUGGGGGUCC	Chr3:23406587:23406685:−	1	0.63	0.001	0.001
pmiR-007 (loci B)	21	UCACUAAUGGAUUGGGGGUCC	Chr3:31917926:31918023:−	1	0.63	0.001	0.001
pmiR-007 (loci C)	21	UCACUAAUGGAUUGGGGGUCC	Chr7:8968911:8969010:−	1	0.630	0.001	0.001
pmiR-007 (loci D)	21	UCACUAAUGGAUUGGGGGUCC	Chr8:28460673:28460772:+	3	0.63	0.001	0.001
pmiR-008	21	UUCGACUCUAACUGGAUGGUC	Chr4:3266845:3266927:+	7	272.01	0.001	220.26
pmiR-009	21	AAUAUGACGGAGUGUAAAUGC	Chr4:12634035:12634131:+	1	0.76	0.90	0.001
pmiR-010	21	UUAAUCUGAACCAUACAUUUA	Chr4:31077256:31077452:+	1	0.76	0.001	0.001
pmiR-011	21	UGAUAUCAGGCAACUCGGUCC	Chr4:42479761:42479861:+	1	5.23	0.001	1.726
pmiR-012	21	UGCGUAUACUGUGGCGGUAAG	Chr5:16231190:16231317:−	25	18.22	0.001	0.001
pmiR-013	22	AUAUUCCAAAAUGAUCUUUGAA	Chr5:24320293:24320501:−	4	1.20	0.001	0.62
pmiR-014	21	UCGCAGGAGUGAUGGGACCGG	Chr6:2484496:2484624:−	178	17.52	28.27	148.38
pmiR-015	21	UUGAAGAUGAGUUGUUGUCCA	Chr6:12560450:12560556:−	4	0.44	0.41	0.74
pmiR-016	21	UUUAAUUUAUAUACAUCGUCA	Chr7:26377742:26377870:+	1	0.88	1.06	0.74
pmiR-017 (loci A)	21	AAAUAGGACCAGAGGGAGUAU	Chr7:8204238:8204390:−	3	0.630	0.001	0.001
pmiR-017 (loci B)	21	AAAUAGGACCAGAGGGAGUAU	Chr7:8230002:8230154:−	3	0.630	0.001	0.001
pmiR-018	21	CAUUUGGAGAGACAUAGACAA	Chr7:33922012:33922100:−	6	12.86	9.45	13.91
pmiR-019	21	GAUGGUAUUAGUUUGGGACUG	Chr8:9356240:9356352:+	1	1.70	1.22	0.001
pmiR-020	21	AAUUAGGAGAUCUCAAGGCAA	Chr8:166171:166372:−	1	0.69	0.001	0.68
pmiR-021	21	UCGGGCGACGGUGGUGACAAU	Chr1:11384648:11384759:+	53	0.001	26.56	0.001

Normalized read count, (actual miRNA count/total count of clean reads) × 1,000,000

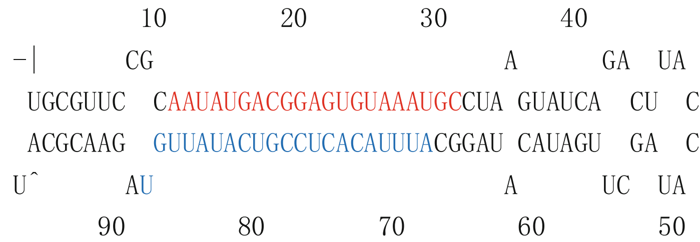
No. candidate number of new miRNAs, *Name* interim name for new miRNAs, *L* length of miRNA, *miRNA* count* miRNA* count in three libraries

identified Al^{3+}-responsive miRNAs, we further classified them into three groups based on their expression patterns. The changes in miRNA expression in response to treatment with 4 or 24-h Al treatment exclusively were referred to as ‘rapid responsive’ and ‘late responsive’ miRNAs, respectively, while the changes in miRNA expression in response to both 4 and 24-h Al treatment were referred to as ‘sustained responsive’ miRNAs. Among the Al^{3+}-responsive miRNAs, 18 miRNAs belonging to 13 families were classified as ‘rapid responsive’, 4 miRNAs belonging to 4 families were categorized as ‘sustained responsive’ (Table 4). In contrast to the ‘rapid’ and ‘sustained responsive’ miRNAs, only one miRNA was found to be ‘late responsive’ miRNA (Table 4). These results reveal that the majority of Al^{3+}-responsive miRNAs is likely to be associated with rapid changes in physiological processes in response to Al^{3+}. miR390 was the only late responsive miRNA (Table 5). Recent reports demonstrated that miR390 regulates lateral root development (Marin et al. 2010; Yoon et al. 2010).

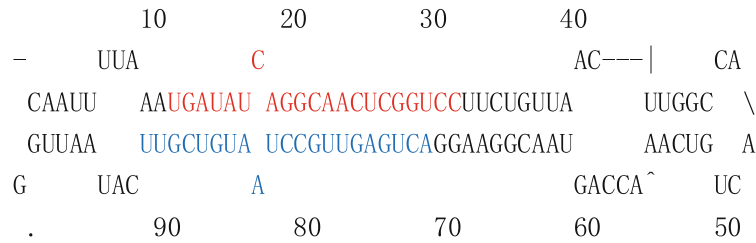
Verification of high-throughput sequencing data by quantitative real-time PCR

To verify the data obtained from the high-throughput sequencing, we studied the responses of the Al^{3+}-responsive mRNAs obtained by the high-throughput sequencing to Al^{3+} treatment by qRT-PCR (Table 6). Only one member of each Al^{3+}-responsive miRNA family was selected for qRT-PCR analysis because miRNAs within the same family have similar mature miRNA sequences and qRT-PCR can hardly distinguish them. In general, the changes in Al^{3+}-responsive miRNAs obtained by qRT-PCR were comparable to those obtained by the deep-sequencing. The time-course of many miRNAs examined by qRT-PCR in response to Al^{3+} showed a rapid decrease

pmiR-009



pmiR-011



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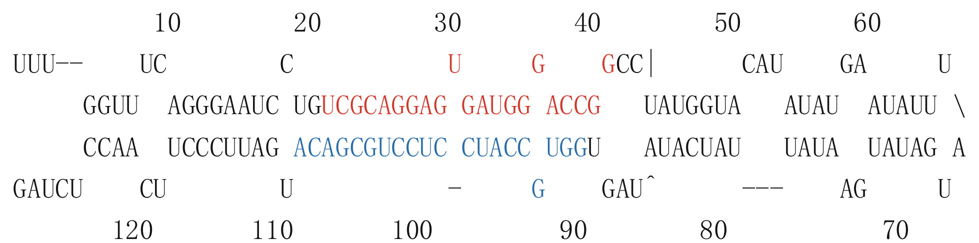

pmiR-016

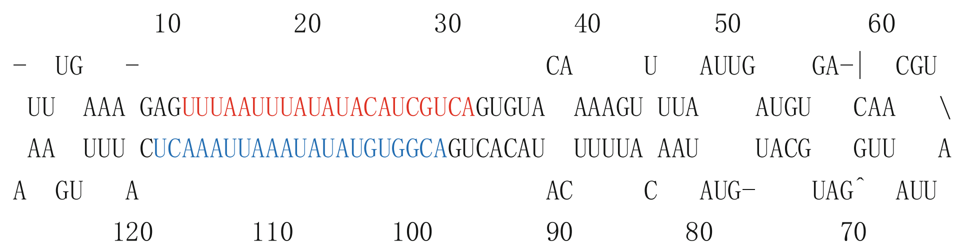

Fig. 3 Stem-loop structures for six novel miRNA precursors. Mature miRNAs of the remaining stem-loop structures were given in Supplemental Fig. S1. Mature miRNAs were marked by *red* and miRNA stars were marked by *blue*. The *numbers* indicate the nucleotide sites form 5′ of miRNA precursors

with short-term Al^{3+} treatment (1–12 h) and recovered after long-term (12–48 h) exposure to Al^{3+}, i.e. mtr-miR159a, mtr-miR162, mtr-miR396a, mtr-miR1507, mtr-miR2088a, mtr-miR2089, mtr-miR2597, mtr-miR2668, pmiR-003 and pmiR-008. These results were correlated with the miRNAs that exhibited rapid response to Al^{3+} measured by the high-throughput sequencing. Furthermore, expression of sustained Al^{3+}-responsive miRNAs such as mtr-miR1510a, mtr-miR2199, pmiR-012 pmiR-014 and pmiR-021 showed steady changes when treated with Al^{3+}. In addition, the only late Al^{3+}-responsive miRNA, mtr-miR390, displayed slight down-regulation by treatment with short-term Al^{3+} and significant up-regulation after long-term expose to Al^{3+}.

Expression of several miRNAs in response to Al^{3+} obtained by the high-throughput sequencing differed from that obtained by the qRT-PCR. For example, deep-sequencing results showed that there was a significant change in expression of miR160 in response to Al^{3+} treatment, while their expression was little responsive to Al^{3+} treatment when determined by the qRT-PCR (Table 6). Note that no read-counts were found for pmiR-021 in the control library, leading to very high values for the fold changes in Al4 and Al24 libraries. However, the

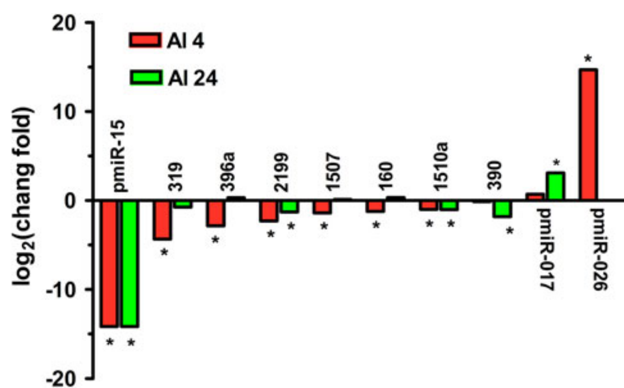

Fig. 4 Comparison of miRNA expression among the three libraries (CK, Al4 and Al24). To identify Al^{3+}-responsive miRNAs, we compared the normalized expression of miRNAs in the three libraries (CK, Al4 and Al24). The relative change in response to Al treatment was expressed as $\log_2$ (Al4/CK) and $\log_2$ (Al24/CK). A positive value indicates a higher miRNA expression in the Al4 and Al24 than in the CK library, while a negative value means a lower miRNA expression. *Star marks* mean that the $|\log_2$ (fold change in Al4) $|$ or $|\log_2$ (fold change in Al24)$| >1$, and $P < 0.05$

Table 4 Classification of Al^{3+}-responsive miRNA

Categories	Name of miRNAs
Rapid responsive miRNAs	mtr-miR159a, mtr-miR160, mtr-miR160b, mtr-miR160d, mtr-miR160e, mtr-miR162, mtr-miR319, mtr-miR319b, mtr-miR396a, mtr-miR396b, mtr-miR1507, mtr-miR2088, mtr-miR2089, mtr-miR2597, mtr-miR2668, pmiR-004, pmiR-010, pmiR-026
Late responsive miRNA	mtr-miR390
Sustained responsive miRNAs	mtr-miR1510a, mtr-miR2199, pmiR-015, pmiR-017

results from the qRT-PCR revealed that the Al^{3+}-induced changes in expression were much lower than those from the deep-sequencing results. We speculate that these differences may result from the higher sensitivity and less specificity of qRT-PCR than those of the deep-sequencing technology. Despite of the differences in response of miRNAs to Al^{3+} treatment, the majority of miRNAs displayed comparable expression patterns in response to Al^{3+} by the qRT-PCR and the high-throughput sequencing.

Prediction of target for new miRNAs

Plant miRNAs are highly complementary to their targets (Jones-Rhoades and Bartel 2004), and this feature has been used to predict targets of miRNAs in plants (Rhoades et al. 2002). The targets for the new miRNAs identified in the present study were predicted using Mt3.5.1 according to the rules set by Allen et al. (2005). The predicted targets for the new miRNAs were involved in many plant physiological processes, including plant development, defense, ion transport (Supplemental Table S6). For example, the predicted target for pmiR-003 was a TIR-NBS-LRR resistance protein which is involved in plant defense (Noutoshi et al. 2005; Mestre and Baulcombe 2006; Yang et al. 2008).

Discussion

Al^{3+} phytotoxicity is a major factor limiting plant growth in acidic soils worldwide (Kochian et al. 2005). Although extensive studies on identification of genes responsible for Al phytotoxicity and tolerance have been conducted (see review Ryan et al. 2011), there has been little information on the roles of miRNAs in Al^{3+} phytotoxicity, especially at whole genome level. In the present study, we constructed three libraries: control, without Al^{3+} treatment (CK); treatment with Al^{3+} for 4 h (Al4); and 24 h (Al24) from *M. truncatula* root tips. Identification of miRNAs in *M. truncatula* by the high-throughput sequencing technology

Table 5 Al^{3+}-responsive miRNAs with known functions

Name	Responsive in Al4	Responsive in Al24	Targets	Function	Reference
miR159	Down-regulated	NR	MYB	Seed germination	(Alonso-Peral et al. 2010; Reyes and Chua 2007)
miR160	Down-regulated	NR	Auxin response factor	Root cap formation, seed germination, embryo development	(Liu et al. 2007; Liu et al. 2010; Wang et al. 2005)
miR319	Down-regulated	NR	TCP	Flowering development, leaf development, cold response, drought response, jasmonate signal	(Schommer et al. 2008; Sunkar and Zhu 2004; Zhou et al. 2010)
miR390	NR	Down-regulated	Auxin response factor	Lateral root development	(Marin et al. 2010; Yoon et al. 2010)
miR396	Down-regulated	NR	GRF	Cell proliferation, leaf development	(Liu et al. 2009; Rodriguez et al. 2010)

"NR" means that miRNAs were not responsive to Al^{3+}

Table 6 Time-dependent changes in expression of miRNAs in response to Al^{3+} treatment measured by qRT-PCR

Name	1 h	4 h	12 h	24 h	48 h
mtr-miR159a	0.396 ± 0.07171	0.35 ± 0.07573	0.7283 ± 0.03027	0.8876 ± 0.08609	0.9011 ± 0.05876
mtr-miR160	0.6918 ± 0.06200	1.072 ± 0.1464	1.134 ± 0.1116	1.236 ± 0.1253	1.036 ± 0.06049
mtr-miR162	0.2179 ± 0.006824	0.4592 ± 0.08314	0.6467 ± 0.01788	1.06 ± 0.1467	0.8237 ± 0.1314
mtr-miR319	0.03554 ± 0.002166	0.2418 ± 0.01456	0.03114 ± 0.03114	0.5890 ± 0.04939	0.01274 ± 0.001023
mtr-miR390	0.77 ± 0.07101	0.8189 ± 0.1237	0.4969 ± 0.08250	0.3236 ± 0.06208	0.3513 ± 0.1246
mtr-miR396a	0.5409 ± 0.003312	0.5863 ± 0.08689	0.7481 ± 0.06705	0.8221 ± 0.1166	1.028 ± 0.1625
mtr-miR1507	0.3174 ± 0.04144	0.5355 ± 0.1205	0.5189 ± 0.04408	1.015 ± 0.2424	1.204 ± 0.02760
mtr-miR1510a	0.4368 ± 0.02610	0.4706 ± 0.01993	0.3889 ± 0.05160	0.4909 ± 0.04759	0.4566 ± 0.02877
mtr-miR2088a	0.4933 ± 0.02588	0.5267 ± 0.07036	0.8661 ± 0.1401	0.9748 ± 0.1523	0.9249 ± 0.1612
mtr-miR2089	0.4131 ± 0.05806	0.5153 ± 0.04429	0.8585 ± 0.1418	0.9663 ± 0.2309	1.211 ± 0.04769
mtr-miR2199	0.4357 ± 0.04298	0.3595 ± 0.07111	0.621 ± 0.1641	0.5548 ± 0.02692	0.4398 ± 0.07098
mtr-miR2597	0.1405 ± 0.1065	0.4284 ± 0.1595	0.3509 ± 0.1604	0.7161 ± 0.08551	0.6946 ± 0.2832
mtr-miR2668	0.4846 ± 0.02810	0.5562 ± 0.1653	0.6643 ± 0.09781	0.7765 ± 0.06983	0.7784 ± 0.1368
pmiR-004	0.1534 ± 0.01419	0.5039 ± 0.05112	0.6508 ± 0.1172	1.258 ± 0.1951	1.145 ± 0.1312
pmiR-010	0.06428 ± 0.003356	0.1617 ± 0.03180	0.4505 ± 0.1674	0.9239 ± 0.1020	1.083 ± 0.2651
pmiR-015	0.08578 ± 0.02146	0.1502 ± 0.02686	0.06878 ± 0.03593	0.2244 ± 0.06808	0.222 ± 0.06967
pmiR-017	2.520 ± 0.6657	2.416 ± 0.6372	10.85 ± 1.317	17.80 ± 0.2044	16.29 ± 3.505
pmiR-026	2.519 ± 0.6421	3.02 ± 0.6828	7.275 ± 1.286	2.961 ± 0.1345	1.076 ± 0.2663

Data are mean ± SD of three independent experiments. Seedlings of *M. truncatula* were exposed to solution containing 10 μM $AlCl_3$ (pH 4.5) for 1, 4, 12, 24 and 48 h, respectively

such as Solexa or Roche 454 has been reported in several recent studies (Szittya et al. 2008; Jagadeeswaran et al. 2009; Lelandais-Briere et al. 2009). 375 miRNAs in *M. truncatula* have been identified using the database of miRBase (Griffiths-Jones et al. 2008). In the present study, we sequenced *M. truncatula* root tips at the whole-genome level by Solexa technology. The three libraries with greater than 45 M reads in total were the most abundant for the identification of miRNAs in *M. truncatula*. The obtained reads allowed us to analyze the miRNAs with low abundance. In addition, the use of latest *M. truncatula* genomic database (Mt3.5.1) in the present study also facilitates identification of miRNAs as the database contains more sequence information for *M. truncatula.* In contrast to other studies of identification of miRNAs in *M. truncatula*, roots particularly root tips, were used to isolate miRNAs.

In the present study, a total of 326 known miRNA and 33 new miRNA was identified; among them, 23 miRNAs were identified to be responsive to Al^{3+}. Zhou et al. (2008) identified a number of Al^{3+}-responsive miRNAs by bioinformatic approach. However, our findings differed from those reported by Zhou et al. (2008). For instance, Zhou et al. (2008) reported that miR171, miR319, miR393, and miR519 are up-regulated, and that miR166 and miR398 are down-regulated in response to Al^{3+} treatment. In contrast, we found that expression of miR393 and miR398 were too low to be used for further expression analysis in the three libraries. Furthermore, we failed to sequence miR519, and found that miR519 was not recorded in the miRBase database. In the present study, we observed that miR166 and miR171 were not responsive to Al^{3+}. Moreover, we also found that miR319 was a rapid responsive miRNA such that it was down-regulated after treatment with Al^{3+} for 4 h, while its expression was not responsive to the treatment with Al^{3+} for 24 h. Several possible explanations may account for the differences between our findings and those reported by Zhou et al. (2008). The miRNAs were predicted with a bioinformatic approach by Zhou et al. (2008). This approach has been suggested to be insufficient for the identification of miRNAs (Meyers et al. 2008). The application of high-throughput sequencing technology to identify miRNAs in the present study can overcome some problems associated with bioinformatic approach. In addition, since the root tip is the critical site for sensing toxic Al^{3+} (Ryan et al. 1993), we used root tips to construct the sRNA library, while leaves rather than root tips were used in studies on the effect of Al^{3+} on the expression of miRNAs by Zhou et al. (2008).

In addition to Al toxicity, plants grown in acidic soil also suffer from Mn toxicity (Taylor et al. 1998). There are reports demonstrating the cross-talk between Al^{3+} and Mn toxicity. For instance, Yang et al. (2009) found that excess Mn can increase the accumulation of Al^{3+} in soybean roots, and that high Al^{3+} concentration alleviates Mn

toxicity. More recently, Valdes-Lopez et al. (2010) reported that the expression of miR319, and miR159 is up-regulated in roots and nodules upon exposure of *Phaseolus vulgaris* to toxic level of Mn. Our results showed that the Mn-responsive miRNAs were also responsive to Al^{3+} such that mtr-miR319 and mtr-miR159a were rapid responsive, down-regulated miRNAs. These findings indicate that the metal toxicity associated with Al^{3+} and Mn may affect plant by targeting the same miRNAs.

Recent studies revealed that Al^{3+}-induced inhibition of root elongation may result from disruption of auxin distribution in roots by targeting auxin polar transporters PIN2 and AUX1 (Kollmeier et al. 2000; Doncheva et al. 2005; Sun et al. 2010). The Al^{3+}-responsive miR160 and miR390 have been shown to modulate root growth by regulating auxin response factor (ARF) (Wang et al. 2005; Marin et al. 2010; Yoon et al. 2010). We found that mtr-miR160 family was rapidly, down-regulated by Al^{3+}. It has been reported that targets of miR160 are ARF10 and ARF16 that regulate the development of root cap (Wang et al. 2005). Therefore, the rapid down-regulation of miR160 in response to Al^{3+} treatment may be involved in inhibition of root elongation by up-regulating ARF.

miR390 was the only late responsive miRNA such that its expression was down-regulated in the Al24 library. miR390 has been reported to control the generation of tasiRNA (TAS3-derived trans-acting short-interfering RNA), which regulates lateral root emergence by targeting transcription factors such as ARF2, ARF3 and ARF4 (Marin et al. 2010; Yoon et al. 2010). As root elongation in *M. truncatula* can be rapidly suppressed by Al^{3+} (Sun et al. 2007), the down-regulation of miR390 after 24 h of exposure to Al^{3+} may be an indirect effect of Al^{3+}.

We identified five new Al^{3+}-responsive miRNAs in *M. truncatula*. Two of them, pmiR-003 and pmiR-008 were predicted to target mRNA of the TIR-NBS-LRR resistance protein. NBS-LRR proteins are considered to be involved in resistance to many types of pathogens (Hammond-Kosack and Parker 2003). Furthermore, the latest research revealed that this family plays an important role in the symbiotic process in legume species (Colebatch et al. 2004). We found that pmiR-003 and pmiR-008 were rapidly responsive miRNAs, and that both were down-regulated in Al4. These results may suggest that pathogens resistance protein may also be involved in Al^{3+} toxicity.

In summary, we identified a group of new miRNAs in the legume model plant of *M. truncatula* by a deep-sequencing method, and discovered a number of miRNAs that were responsive to Al^{3+}. These findings provide valuable information for functional characterization of miRNAs in response of legume plants to Al^{3+} phytotoxicity.

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