DOI: 10.1007/s10535-017-0752-5 BIOLOGIA PLANTARUM 62 (1): 97-108, 2018

Identification and comparative analysis of aluminum-induced microRNAs conferring plant tolerance to aluminum stress in soybean

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Abstract

Aluminum (Al) toxicity in acidic soils is a major factor restricting crop production. Although the molecular mechanisms of Al responses have been extensively investigated, microRNA (miRNA) mediated differential Al tolerance in different soybean genotypes remains largely unknown. In this study, two soybean [*Glycine max* (L.) Merr.] genotypes, Al-tolerant BX10 and Al-sensitive BD2, were treated with 0 and 50 μM $AlCl_3$ and then used to construct the miRNA libraries for deep sequencing. Results revealed 453 miRNAs, whose expression patterns were affected by Al stress. We also identified 32 differentially expressed miRNAs: 19 in BX10, 7 in BD2, and 6 in both genotypes. The gene ontology analysis of their putative target genes indicated that stress-responsive genes and amino-acid-metabolism-related processes preferentially existed in BX10. Comprehensive analysis demonstrated that conserved miRNAs, such as gma-miR166k/o, gma-miR390g, and gma-miR396c/k, mediated root elongation in BX10, whereas gma-miR169r triggered oxidative stress in BD2. These processes could be regarded as important mechanisms conferring differential Al tolerance in BX10 and BD2. This study provided new insights into different Al response mechanisms in various soybean genotypes.

Additional key words: abiotic stress, acidic soil, gene sequencing, *Glycine max*, target genes.

Introduction

Aluminum (Al) is an abundant metal element in the Earth's crust but is rarely involved in biological functions (Pina and Cervantes 1996, Paul *et al.* 2003). However, Al becomes released into acidic soils and exhibits phytotoxic properties (Hue *et al.* 1986). As such, Al has been considered a harmful limiting factor that reduces crop production in acidic soils, which constitute approximately 40 % of the global arable land (Matsumoto 2005, Simões *et al.* 2012). For instance, soybean, an important oil-bearing crop, is planted in acidic soils, but its yield is largely reduced by Al toxicity (Zhen *et al.* 2007, Zeng *et al.* 2012). Therefore, Al tolerance and relevant regulatory factors should be investigated to alleviate the toxic effects of Al on soybean in acidic soils. Our understanding of the Al responses has been greatly improved because of extensive studies of genes associated with Al-tolerance (Sade *et al.* 2016).

Plant responses to environmental stresses can be regulated by microRNAs (miRNAs) (Leung and Sharp 2010). A miRNA is a non-coding small RNA with a length ranging from 19 to 24 nucleotides (nt). It is important regulatory factor that leads to post-

Submitted 5 July 2016, *last revision* 15 May 2017, *accepted* 16 May 2017.
Abbreviations: DEMs - differently expressed miRNAs; GO - gene ontology; miRNA - microRNA; MFE - minimum free energy; nt - nucleotide; PCA - principle component analysis; qPCR - time quantitative PCR; RLM-5'RACE - RNA ligase-mediated rapid amplification of 5' cDNA ends; ROS - reactive oxygen species.
Acknowledgments: This research was supported by the National Key Research and Development Program of China (2016YFD0101005), the National Natural Science Foundation of China (NSFC) (grants Nos. 31271744, 31372140), the Program for Changjiang Scholars and Innovative Research Team in University (IRT_14R27), and the Important National Science & Technology Specific Project (2013ZX08011-003, 2014ZX08011-003). The first three authors contributed equally to this work.
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transcriptional gene silencing *via* targeted mRNA cleavage or translational repression (Kulcheski *et al.* 2011, Chen *et al.* 2012). Several conserved miRNAs, such as miR319, miR390, miR393, and miR398, are also involved in Al stress signalling pathways. In addition, Al-triggered miRNA expression has been investigated (Lima *et al.* 2011, Zeng *et al.* 2012). These findings help to elucidate the roles of miRNAs in plant Al stress.

The miRNA expression in wild soybean (*Glycine soja*) in response to Al stress was reported and 97 known miRNAs and 31 novel miRNAs were identified (Zeng *et al.* 2012). Of these miRNAs, 30 are differentially expressed. Some conserved miRNAs, such as miR164 and miR156, are down-regulated, whereas miR169 and miR396 are up-regulated. Some genes well characterized in stress responses, such as domain-containing disease resistance protein (NB-ARC), MYB transcription factors, and auxin response factor (ARF), are cleaved by their corresponding miRNAs under Al stress (Zeng *et al.* 2012). Chen *et al*. (2012) identified 23 Al-responsive miRNAs in *Medicago truncatula*, an important leguminous model plant, and found that most of these miRNAs are down-regulated, but their expressions display different time-response patterns. Previous studies mainly identified Al-responsive miRNAs by utilizing single genotype. The use of contrasting genotypes of one plant species might provide more comprehensive observations. Thus, miRNA-mediated differential Al tolerance in different plant genotypes may be elucidated.

Currently, 639 mature miRNAs and 573 precursor miRNAs of soybean have been registered in *miRBase* (Kozomara and Griffiths-Jones 2014). Although a series of miRNAs is involved in Al-response in legumes, the miRNA expression profiles of cultivated soybeans under Al stress have yet to be characterized. The genome-wide identification of stress-related miRNAs has been employed as an effective strategy because of its high throughput and efficiency (Nie *et al.* 2016). In our work, genome-wide analysis was conducted to identify miRNAs associated with Al response by combining tolerant and sensitive soybean genotypes. This study aimed to investigate the relationship between the miRNA expression profiles and Al tolerance and to reveal the possible mechanisms of miRNA-mediated Al responses in different soybean genotypes.

Materials and methods

Plant growth and treatments: Two soybean [*Glycine max* (L.) Merr.] genotypes, Al-tolerant BaXi10 (BX10) and Al-sensitive BenDi2 (BD2), were employed in this study (Dong *et al.* 2004, Li *et al.* 2012, Yang *et al.* 2012). Seeds were germinated at 25 °C in the dark. Four days later, the seedlings were transplanted into half-strength Hoagland nutrient solution and grown in a chamber with day/night temperatures of 26/22 °C, a 70 % relative humidity, a 16-h photoperiod, and a photon flux density of 400 mmol m^{-2} s^{-1} for one week. The uniformly grown seedlings were transferred into a vessel containing 0 (-Al) or 50 μM $AlCl_3$ (+Al) solutions and 100 μM $CaCl_2$ (pH 5.0). The seedlings (BX10-Al, BX10+Al, BD2-Al, and BD2+Al) were harvested after 48 h and root tips (approximately 1 cm) were cut and frozen in liquid nitrogen and kept at -80 °C for RNA extraction.

Isolation of RNA, library construction, and high-throughput sequencing: Total RNA was isolated by *Trizol* reagent (*Takara*, Dalian, China). Four small RNA libraries were constructed as follows: 5′ and 3′ RNA adapters were ligated to the high quality small RNA with T4 ligase and subjected to cDNA synthesis. Thereafter, PCR was performed to amplify cDNA. PCR products were separated on 15 % (m/v) polyacrylamide gel, and fragments ranging from 15 to 30 nt in length were collected. The multiplexed DNA libraries were subjected to high-throughput sequencing on an *Illumina HiSeq 2000* sequencer. This work was done in *Personalbio* (Shanghai, China).

The clean reads were obtained from raw reads by removing the contaminant sequences; they were then used for length distribution analysis. Unique sequences (15 - 30 nt) were produced from the clean reads by discarding the repeated sequences and subjected to *BLAST* pipeline against the reference genome (*Glycine max William 82, v.1.0*) for mapping information and against the *Rfam v.11.0* database for small RNA annotation, using *SOAP 2.0*.

The miRNAs were identified according to the following criteria as Meyers *et al.* (2008) reported: *1*) miRNA and miRNA* are derived from opposite stem-arms such that they form a duplex with two nucleotides, 3′ overhangs; *2*) base-pairing between the miRNA and the other arm of the hairpin, which includes the miRNA*, is extensive such that there are typically four or fewer mismatched miRNA bases; and *3*) asymmetric bulges are minimal in size (one or two bases) and frequency (typically one or less), especially within the miRNA/ miRNA* duplex.

The annotated miRNA sequences were submitted to the *miRBase* database and a plant microRNA database *PMRD* to identify miRNAs. The miRNA candidate targets were predicted using the *psRNATarget* program (Dai and Zhao 2011).

Validation of miRNA expression: Real-time quantitative PCR (qPCR) was performed to validate the miRNA expression pattern. A total of 2 μg RNA was reverse transcribed using a miRNA cDNA kit (*CoWin*

Bioscience, Beijing, China). The first-stand cDNA was used as a template and the mature miRNA sequence (Table 1 Suppl.) was used as a forward primer for real-time qPCR with miRNA real-time PCR assay kit (*CoWin Bioscience*) according to the manufacturer's instructions. Uracil-riched small nuclear RNA 6 gene (*U6*) was used as a standard to normalize each miRNA expression. The PCR reaction was done on *ABI 7500* (*Life Tech*, *Applied Biosystems,* Foster City, USA) real-time PCR system. Data were acquired using *SDS v.2.0* software (*Applied Biosystems*). We used the $2^{-\Delta\Delta Ct}$ method to calculate the relative expression (Livak and Schmittgen 2001). Two-tailed *t*-test was performed to compare the differences in expression of paired samples. Three biological replicates were used and the means were considered significantly different when $P < 0.05$.

Target gene verification: RNA ligase-mediated rapid amplification of 5′ cDNA ends (RLM-5′RACE) method (*Invitrogen*, Carlsbad, CA, USA) was applied to identify the cleavage site of the target gene. Firstly, RNA oligo was ligated to cleaved mRNA using T4 RNA ligase. Then, reverse transcription proceeded with random primers and finally, two rounds of PCR were carried out with *GeneRacer* 5′ primer/gene-specific primer and *GeneRacer* 5′ nested primer/gene-specific nested primer.

Principle component analysis (PCA) was conducted using the *Canoco for Windows v. 4.56* software. The sequencing reads were normalized using $\log_2(x+1)$ prior to being used for the PCA pipeline.

Results

We obtained 12 759 728 and 13 486 385 raw reads from BX10-Al (0 μM $AlCl_3$) and BX10+Al (50 μM $AlCl_3$) libraries, respectively; and 12 597 339 and 15 304 861 raw reads from BD2-Al and BD2+Al libraries, respectively (Table 1). After removing the junk sequences, the clean sequences ranging from 15 - 30 nt in length were collected for further analysis. Decreased abundances of miRNAs in Al-treated libraries were observed, with 5.9 and 37.3 % decline in BX10+Al and BD2+Al, respectively, compared with the controls, indicating that Al stress probably reduced the overall expression of miRNAs in both soybean genotypes, and Al-sensitive soybean genotype (BD2) imposed a larger inhibition.

We analyzed the length distributions of clean reads and found that 21 and 24 nt sequences, which are processed by dicer-like 1 (DCL1) and dicer-like 3 (DCL3) in *Arabidopsis* (Voinnet 2009), were the most abundant groups. However, after discarding the contaminant sequences, the unique sequences of small RNAs were uniformly grouped into 24 nt sequences (Fig. 1), which is consistent with the previous finding in wild soybean (Zeng *et al.* 2012).

The unique sequences were compared with known sequences in miRBase to identify known and potential new (PN) miRNAs in two soybean libraries, and allowed no more than two mismatches. A total of 453 miRNAs were annotated, including 302, 245, 309, and 300 miRNAs in BX10-Al, BX10+Al, BD2-Al, and BD2+Al, respectively (Table 1 Suppl.). Among 453 miRNA sequences, 248 belonged to known miRNAs that could match to the known sequences, whereas 205 belonged to PN miRNAs that failed to match to the known sequences. These known miRNAs included 75 conserved miRNAs from 23 families and 173 non-conserved miRNAs from 138 families. Under Al stress,

Table 1. Sequence distributions in two soybean genotypes.

Item	BX10-Al	BX10+Al	BD2-Al	BD2+Al
Raw reads	12 759 728	13 486 385	12 597 339	15 304 861
Clean reads (15 - 30 nt)	5 819 010	5 473 800	1 931 906	1 210 713

Table 2. Summary of the known and potential new miRNAs in two soybean genotypes. Potential new means those which cannot fine match to known sequences registered in *miRBase.*

Item		BX10-Al	BX10+Al	BD2-Al	BD2+Al
Known		204	165	212	197
	conserved	66	63	69	69
	non-conserved	138	102	143	128
Potential new		98	80	97	103

the total abundance of conserved miRNAs accounted for over 93 % of all the known miRNAs in each library suggesting that the conserved miRNAs probably had large contribution to the stress response in soybeans (Table 2).

To evaluate and compare the influences of genotypes and Al stresses on the expression profile of miRNAs in two soybean genotypes, principle component analysis (PCA) was conducted using miRNA-normalized reads data. Only those with combined reads in BX10 and BD2 libraries exceeding 50 were selected for PCA, following previous standards (Barrera-Figueroa *et al.* 2011). The results indicated that the first two components were extracted, accounting for 86.1 % of the total variation of miRNAs expression (Fig. 1 Suppl.). Principle component 1 (PC1), accounting for 46.5 % of the variation, separated the two treatments (-Al and +Al) of each soybean genotype; whereas PC2, explaining 39.6 % of the variation, separated two genotypes (BX10 and BD2). These results suggested that Al stress is an important factor affecting the miRNAs expression patterns in soybean.

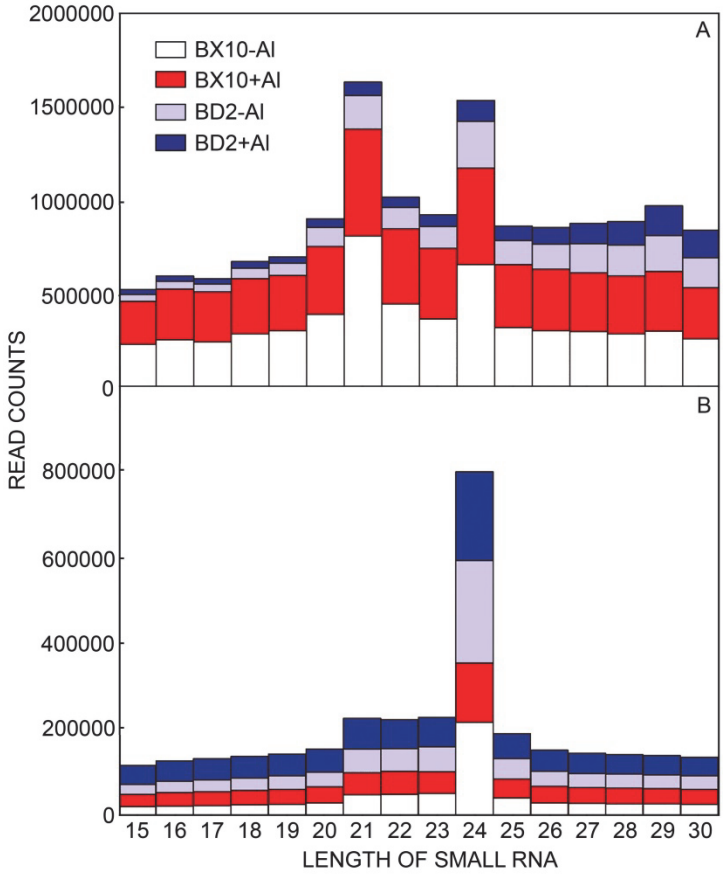

Fig. 1. Length distributions of small RNAs in two soybean genotypes. *A* - Clean reads with redundant sequences; *B* - unique sequences.

Previously, different criteria were used to identify the differentially expressed miRNAs (DEMs) (Barrera-Figueroa *et al.* 2011, Zeng *et al.* 2012, Tang 2015). In the present study, only those with combined normalized reads across four libraries ≥ 50 and $|\log_2$fold change (-Al/+Al)| ≥ 1 were regarded as differentially expressed miRNAs under Al stress. We totally detected 32 Al-responsive miRNAs in both soybean genotypes and found that most of the miRNAs were down-regulated in Al-treated plants, except one miRNA (gma-miR5786) that was up-regulated (Fig. 2). Our results revealed that 19, 7, and 6 miRNAs were differentially expressed in BX10, BD2, and in both genotypes, respectively. Of the 19 DEMs in BX10, 11 (57.9 %), *e.g.*, gma-miR159d, gma-miR162c, gma-miR166k/o/h-3p, gma-miR171b-5p, and gma-miR396c/k, were conserved and 6 (31.6 %), *e.g.*, gma-miR1507a/c-3p, gma-miR1520n, gma-miR503a, and gma-miR5678, were non-conserved. This finding implied that conserved miRNAs are probably essential for Al stress responses in BX10. Three members of the miR166 family were suppressed in BX10, among which, gma-miR166k and gma-miR166o had extremely high basal expression, suggesting that these two miRNAs might be necessary for normal growth and development in soybeans. Moreover, 15 of the 19 DEMs in BX10 showed significant differences in expression between

Al-treated samples (BX10+Al and BD2+Al) but no significant variations between Al-free samples (BX10-Al and BD2-Al), suggesting that the differential expressions of these miRNAs were probably consequential of Al stress (Fig. 2 Suppl.). By contrast, only 3 conserved miRNAs (gma-miR164k, gma-miR169r, and gma-miR482a-3p) were down-regulated in BD2. These results indicated that more conserved miRNAs were differentially expressed in BX10, an Al-tolerant soybean.

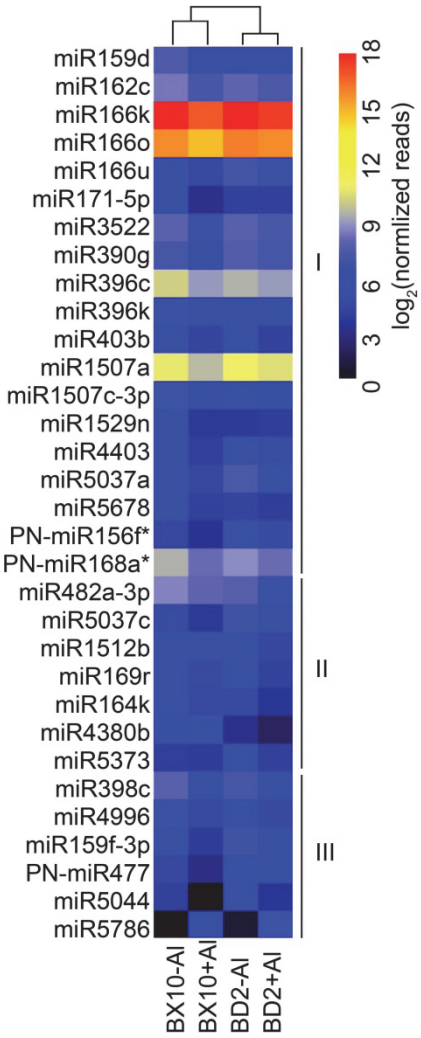

Fig. 2. Heat map of 32 differentially expressed miRNAs in two soybean genotypes made according to logarithmic transformations of normalized reads. Genes differentially expressed only in BX10 (group I), in BD2 (group II), and in common (group III) are shown.

Errors possibly occurred when detecting the abundance of miRNA sequences, especially those with low frequencies, because of high-throughput sequencing limitations. Real- time qPCR is a reliable and time-saving method to confirm the miRNA expression. We randomly validated the differential expressions of 10 miRNAs (including 6 DEMs in BX10, 2 DEMs in BD2, and 2 DEMs in common) using this method following the high-throughput sequencing results. Despite some inconsistencies, qPCR results were similar to the sequencing results (Fig. 3) as revealed by correlation analysis (Fig. 3 Suppl.).

The miRNA target gene investigation is the first step to understand their biological roles in plant development. To understand the relationship between soybean miRNAs and Al stress, we utilized the *psRNATarget* program for miRNA target prediction on the basis of position-dependent mispaired penalties and minimum free energy (MFE) ratio (Dai and Zhao 2011). A total of 442 miRNAs were predicted to target 3 180 genes with 4 381 target sites. Our results also showed that 263, 211, 267, and 252 miRNAs targeted 2 311, 1 870, 2 290, and 2 150 genes, respectively, in BX10-Al, BX10+Al, BD2-Al, and BD2+Al.

Of the 32 DEMs, 31 contained 237 putative target genes (Table 2 Suppl.). *PANTHER* (*v.10.0*) classification system was used to acquire the annotations of target genes (Mi *et al.* 2016). A total of 141 target genes were

annotated, whereas the 96 remaining genes were uncharacterized genes. Important stress-related genes, such as nucleotide binding site-leucine rich repeat (NBS-LRR), teosinte branched cycloidea proliferating cell factor (TCP), wall-associated kinase (WAK), ARF, ATP-binding cassette transporter (ABC transporter), v-myb myeloblastosis viral oncogene homolog (MYB), homeobox-leucine zipper protein (HD-ZIP), superoxide dismutase (SOD), and heat shock protein (HSP70), were predicted to be cleaved by corresponding miRNAs. Numerous predicted targets encoded DNA binding transcription factors (TFs) suggesting that these miRNAs are important in regulating gene expressions *via* targeting special TFs (Barte 2004, Hobert 2004).

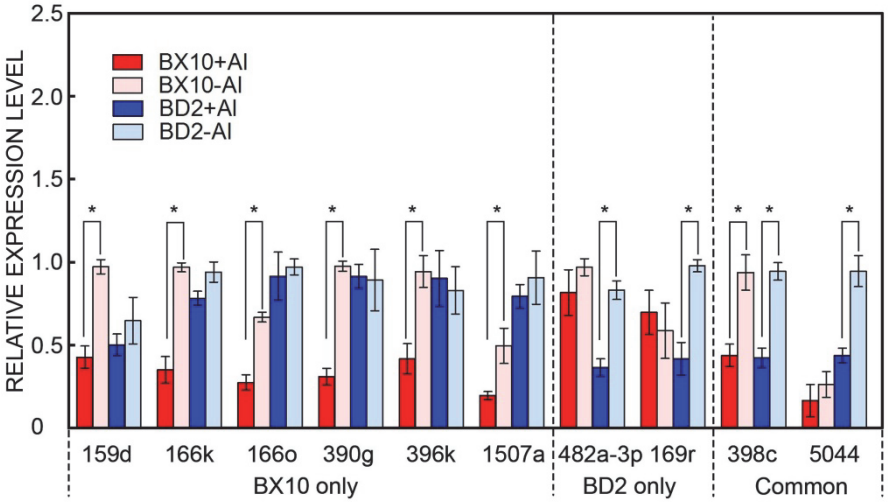

Fig. 3. Real-time qPCR validation of 10 Al-responsive miRNAs (gma-miR159d, gma-miR166k, *etc*.). The uracil-riched small nuclear RNA 6 (*U6*) gene was used as housekeeping gene. A representative sample from two biological experiments is shown. Means ± SDs, * - indicates significant difference between control and Al treatments at $P < 0.05$.

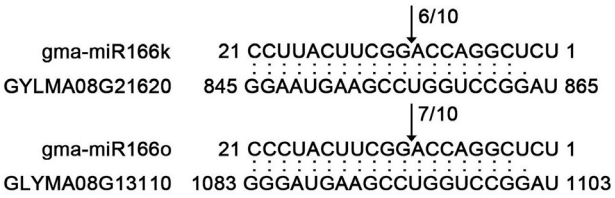

Fig. 4. Cleavage site validation of target genes. *Arrows* indicate cleavage sites. *Scores beside arrows* denote the percentage of clones with the same cleavage sites.

Because two miRNAs, gma-miR166k and gma-miR166o, had extremely high basal expression and were significantly down-regulated in BX10, their target genes were herein experimentally verified using the RLM-5′RACE method. As shown in Fig. 4, GLYMA08G21620 and GLYMA08G13110 were cleaved between the 10th and 11th bases by miR166k and miR166o, respectively. Both genes encode Class III HD-ZIP transcription factors, which are well known in transcriptional regulation in plant development and environmental response (Valdés *et al.* 2012, Xie 2015, Mandel *et al.* 2016, Yip *et al.* 2016).

Gene ontology (GO) analysis was conducted to understand the possible functions of the target genes. A total of 236 genes were mapped to reference genes of soybean (*G. max*). Genes involved in ‘stress responses’ overrepresented the GO items (expectation = 9.96, fold enrichment = 2.81, *P* value = 1.66E-03) suggesting that massive stress response-related genes were regulated by miRNAs in soybeans under Al stress. As shown in Fig. 5, in the ‘molecular function’ category, majority of the genes have catalytic, binding, and transporter activities. In the ‘biological process’ category, majority of the genes are involved in metabolic processes. In the ‘cellular component’ category, majority of the target genes exert roles in cell parts and organelles.

Specifically, 11 putative target genes were responsive to stimulus in BX10, and only 2 in BD2 (Fig. 5). Six genes involved in the apoptotic process were cleaved by three miRNAs (gma-miR159d, gma-miR159f-3p, and gma-miR162c) in BX10. Moreover, genes encoding the vesicle transport V-SNARE protein, molecular chaperone protein, and annexin, which have been previously characterized as important defense proteins (Cho and Choi 2009, Sun *et al.* 2012, Szalonek *et al.* 2015), were cleaved by DEMs in BX10. These results indicated that miRNA-mediated gene regulation was more active in BX10 than in BD2 to respond Al stress.

Metabolic pathways of the proteins encoded by putative target genes of DEMs were investigated. In BX10, the genes involved in the amino acid biosynthesis were overrepresented, whereas the genes involved in cholesterol and pantothenate biosynthesis were over-represented in BD2 (Fig. 6). These results showed that primary metabolism was enhanced in BX10, whereas secondary metabolism was strengthened in BD2.

Discussion

The role of conserved miRNAs in response to Al stress in two soybean genotypes: Recently, several studies have identified a series of miRNAs under Al stress *via* high-throughput sequencing (Chen *et al.* 2012, Zeng *et al.* 2012), expanding our knowledge on miRNA-mediated Al stress responses. In our present study, we identified 248 known miRNAs, including 75 conserved miRNAs representing 23 families and 173 non-conserved miRNAs representing 138 families. Although the number is fewer, conserved miRNAs were generally sequenced more times than non-conserved miRNAs (up to 17.5 times). A similar result was reported in two other soybean cultivars (SHB and SL10), wherein miRNAs were identified after cyst nematode infections (Zhang *et al.* 2012). In wild soybean (*G. soja*), number and expressions of conserved miRNAs were lower (Zeng *et al.* 2012). Our results supported the previous finding that conserved miRNAs are likely to be important in universal mechanism of regulation in different plant species (Song *et al.* 2011).

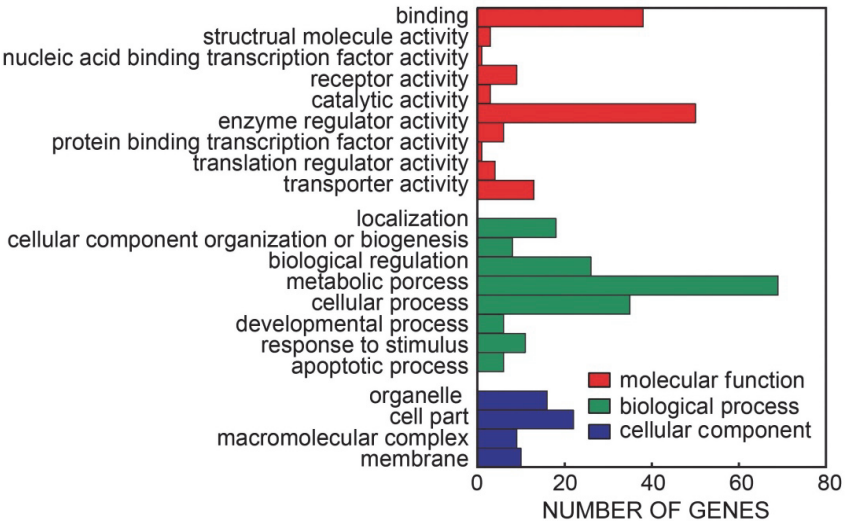

Fig. 5. Gene ontology analysis of putative target genes of differentially expressed miRNAs (DEMs) in both soybean genotypes.

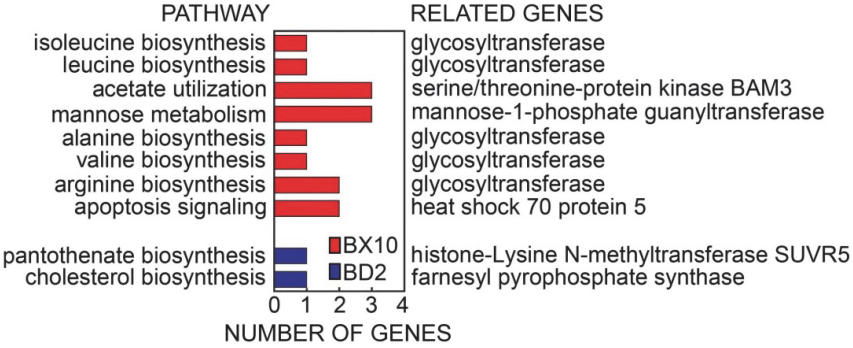

Fig. 6. Target genes involved in the metabolism pathways overexpressed in BX10 and BD2. Target genes were blasted against *PANTHER* database to acquire 'pathway' information.

However, lack of conservation does not mean lack of function (Pang *et al.* 2006). The non-conserved miRNAs are *Fabaceae* specific such as miR1507c, miR1508c, miR1509b, miR1510a, miR1512b, miR3522, miR1520, miR5037a/c, and miR5038b. Conserved miRNAs are more inclined to facilitate transcriptional regulation-related genes, whereas non-conserved miRNAs are likely to play important roles in regulating biological processes intrinsic to *Fabaceae* (Song *et al.* 2011).

Root development-related miRNAs in two soybean genotypes: The miR166 family, particularly miR166k and miR166o, were abundant in both soybean genotypes. Moreover, miR166 exhibited a relatively high expression under other stresses such as water-deficit and various heavy metals (Zhou *et al.* 2008, Ding *et al.* 2011, Li *et al.* 2011a). Therefore, miR166 is probably responsible for the cross adaptation to various stresses in plants, through the reprogramming of biological processes (Barrera-

Figueroa *et al.* 2011). In the present study, two class-III *HD-Zip* genes were cleaved by gma-miR166k and gma-miR166o, respectively. In *Arabidopsis*, miR165/166 regulate the expression of class-III *HD-Zip* genes, which are necessary to form lateral roots and vascular bundles (Floyd and Bowman 2004, Hawker and Bowman 2004). In transgenic *Arabidopsis*-overexpressing *MIR165/166* genes, *MIR165a/166a/166b* were highly expressed in roots, especially in root tips, suggesting their distinct role in root growth (Jung and Park 2007). Further evidence showed that miR166/165 overexpression could suppress the class-III *HD-Zip* genes and consequently promote root growth of *Arabidopsis* by facilitating cell division and maintenance of root apical meristem (RAM) activities (Singh *et al.* 2014). In this study, gma-miR166 had an extremely high basal expression and was down-regulated by Al in the BX10, implying that gma-miR166 (k/o) is important for both normal development and Al stress response.

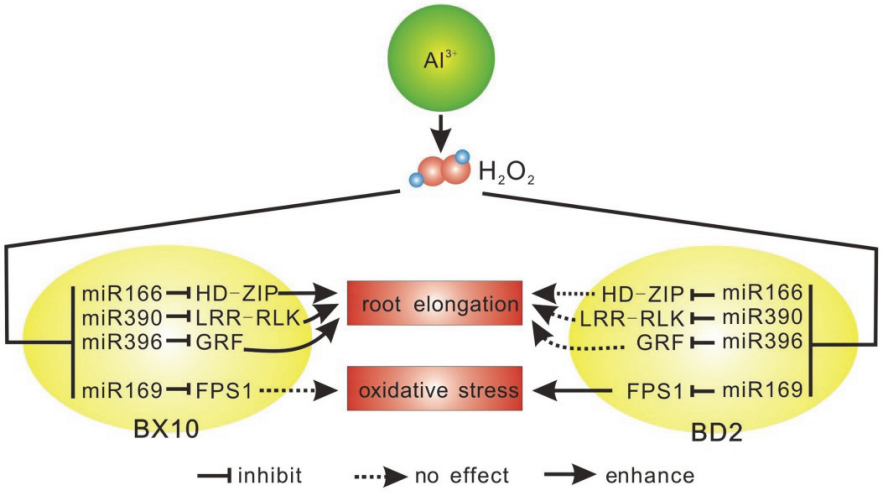

Fig. 7. A proposed mechanism for miRNA-mediated differential Al responses in two soybean genotypes. Al triggered H_2O_2 production and H_2O_2 repressed miR166, 169, miR390, and miR396 to release the suppression to the corresponding target genes. Consequently, differential root elongation and oxidative stress occurred in BX10 and BD2 in response to Al treatment.

Moreover, osa-miR390 is involved in cadmium tolerance in rice and overexpression reduced Cd tolerance and increased Cd accumulation by repressing its target gene *OsSRK*, which encodes a leucine-rich repeat receptor-like kinase (LRR-RLK) (Ding *et al.* 2016). Park *et al*. (2014) stated that *LRR-RLK* genes could be induced by salinity, osmotic stress, heat, salicylic acid, and abscisic acid, suggesting the function of *LRR-RLK* genes as receptors of external signals (Song *et al.* 2008). Another *LRR-RLK* gene in rice roots was reported previously, and this gene is involved in the specification of the outer cell layer, which protects roots from various soil stresses. Furthermore, the *Docs1* mutant-lacking LRR-RLK functions is sensitive to Al toxicity (Huang *et al.* 2012). In this study, the down-regulation of gma-miR390g, which targets the LRR-RLK gene *BAM3*, could favour Al tolerance in BX10 by the specification of the outer cell layer.

Both gma-miR396c and gma-miR396k were significantly down-regulated under Al treatment in BX10. Previous studies revealed that miR396 was down-regulated by mercury treatment in *M. truncatula* (Zhou *et al.* 2012). Moreover, overexpression of miR396c decreases salt and alkali stress tolerance in rice (Gao *et al.* 2010). Up-regulation of miR396 in transgenic *Arabidopsis* results in growth regulating factor (GRF) repression and subsequent inhibition of cell proliferation (Casadevall *et al.* 2013). Moreover, miR396 controls root cell proliferation in *Arabidopsis* by interacting with GRF (Rodriguez *et al.* 2015). In the present study, two members of the miR396 family were down-regulated in BX10, implying that root cell proliferation was likely to be favoured by the gma-miR396 and GRF gene interaction. In contrast, gma-miR396 was not significantly repressed in BD2, possibly leading to a lower GRF activity and consequently further inhibition of root growth.

The MYB transcription factors (TFs) are essential to plant growth, development, and environmental stress responses (Yang *et al.* 2014). Root epidermis differentiation and root hair formation in *Arabidopsis* are controlled by MYB genes (Koshino-Kimura *et al.* 2005, Wada *et al.* 2015), which are cleaved by miR159. Therefore, in both soybean genotypes of our study, the down-regulation of gma-miR159d was possibly required for root development under Al stress by targeting *MYB* genes.

Oxidative stress related miRNAs in two soybean genotypes: Reactive oxygen species (ROS) can severely damage cells. Al toxicity is associated with the burst of

ROS (Yamamoto *et al.* 2002). Moreover, miR398 targets two closely related Cu/Zn superoxide dismutases (CSD1 and CSD2) that can detoxify superoxide radicals (O_2^-), and down-regulation of miR398 results in the post-transcriptional *CSD1* and *CSD2* mRNA accumulation and oxidative stress tolerance (Sunkar *et al.* 2006). Our sequencing data and real-time qPCR validation demonstrated that gma-miR398c was significantly down-regulated in both soybean genotypes suggesting that miR398c may contribute to enhancement of adaptation to Al by alleviation of oxidative stress. This assumption could be confirmed by our previous results, which indicated that SOD activities are significantly enhanced in both genotypes (Zhen *et al.* 2009).

In wild soybean, miR169 is up-regulated after Al treatment (Zeng *et al.* 2012), whereas it is down-regulated in rice after Al stress (Lima *et al.* 2011). Our data revealed that gma-miR169r was significantly down-regulated in BX10 but not in BD2 after Al treatment. One of predicted target gene of miR169 encodes an *Arabidopsis* homologue farnesyl diphosphate synthase 1 (FPS1). Moreover, overexpression of the farnesyl diphosphate synthase isoform 1S gene (*FPS1S*) in tobacco increased H_2O_2 formation (Manzano *et al.* 2004) suggesting that down-regulation of miR169r might lead to the increase of H_2O_2. In BD2 soybean, inaction of gma-miR169r might increase oxidative stress, which has been proved physiologically (Zhen *et al.* 2009).

Legume-specific miRNAs in two soybean genotypes: The miR1507 is a legume-specific miRNA family member and is well conserved in soybean (Li *et al.* 2010). Two members of the miR1507 family, gma-miR1507a and gma-miR1507c-3p, were down-regulated in BX10. Their putative target genes encode a nucleotide-binding apoptotic protease-activating factor-1/R proteins and *Caenorhabditis elegans* death-4 protein (NB-ARC) domain containing disease resistance proteins. NB-ARC proteins belong to resistance proteins involved in pathogen recognition and subsequent activation of innate immune responses (Van *et al.* 2008). Moreover, overexpression of an NB-ARC protein gene (*VpCN*) from *Vitis pseudoreticulata* in *Arabidopsis* can enhance its resistance to biotic stresses (Wen *et al.* 2015). The NB-ARC protein gene RPP4 (*RECOGNITION OF PERONOSPORA PARASITICA 4*) in *Arabidopsis* is essential for defense responses and temperature-dependent cell death (Bao *et al.* 2014). Previous reports revealed that miR1507 is significantly down-regulated by Al treatment in wild soybeans (Zeng *et al.* 2012) and experienced rapid responses to Al stress in *M. truncatula* (Chen *et al.* 2012). Our data showed that two miR1507 members, gma-miR1507a and gma-miR1507c-3p, were down-regulated in BX10 implying that miR1507 might be involved in Al tolerance in soybean.

The miR5044 is a soybean-specific miRNA and has been initially reported to be down-regulated by Al treatment in wild soybeans (Zeng *et al.* 2012). One of its predicted targets encodes a serine carboxypeptidase like-1 (SCPL1). Moreover, serine carboxypeptidase (SCP) is down-regulated under heat stress in wheat (Majoul *et al.* 2003). Liu *et al*. (2008) found that OsBISCPL1 is responsible for oxidative stress adaptation in rice (Liu *et al.* 2008). In *Arabidopsis*, SCPLs function in plant metabolism, and are responsible for sinapoylmalate synthesis, a major phenylpropanoid secondary metabolite (Lehfeldt *et al.* 2000). Therefore, the down-regulation of gma-miR5044 in BX10 and BD2 could confer Al tolerance *via* the interaction with *SCPL1* gene, possibly through alleviating oxidative stress or strengthening phenylpropanoid pathway.

Overall, Al toxicity in plants is accompanied by ROS overproduction (Yamamoto *et al.* 2002, Singha and Khan 2003). Yamamoto *et al*. (2002) found that mitochondrial dysfunction and the resulting burst of ROS are a typical characteristics of Al toxicity. Several miRNAs, such as miR319, miR390, and miR398, were reported to be involved in Al-triggered H_2O_2 signalling pathway (He *et al.* 2014). Additionally, some miRNAs, such as miR159, miR166, and miR396, display different responses to H_2O_2 in rice (Li *et al.* 2011b), which suggests that Al-regulated miRNA expression is possibly achieved by the ROS (H_2O_2)-mediated signalling pathway (Fig. 7)

In conclusion, 453 miRNAs were identified in two soybean genotypes. Of these miRNAs, 54.7 % belonged to known miRNAs. Conserved miRNAs were over-whelmingly expressed in the both genotypes, especially in BX10. Furthermore, 32 miRNAs were Al responsive, and 10 of these miRNAs were validated by real-time qPCR. Target gene prediction and *GO* analysis revealed that stress response-associated genes were overrepresented in BX10 and BD2. Genes related to amino acid metabolism and secondary metabolism were targeted by miRNAs overexpressed in BX10 and BD2, respectively. Our data show that enhancing the primary metabolic pathway, alleviating oxidative stress, and promoting root cell division through miRNA regulation would facilitate the Al tolerance in BX10. This work helped to elucidate the correlation between miRNA expression patterns and differential Al tolerance in two soybean genotypes.

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