RESEARCH ARTICLE

Illumina sequencing revealed roles of microRNAs in different aluminum tolerance of two citrus species

Yang-Fei Zhou¹ · Yan-Yu Wang¹ · Wei-Wei Chen¹ · Li-Song Chen^{1,2} ·
Lin-Tong Yang^{1,2}

Received: 17 April 2020 / Revised: 12 August 2020 / Accepted: 11 October 2020 / Published online: 27 October 2020
© Prof. H.S. Srivastava Foundation for Science and Society 2020

Abstract Self-germinated seedlings of *Citrus sinensis* and *C. grandis* were supplied with nutrient solution with 0 mM $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ (control, $-\text{Al}$) or 1 mM $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ ($+\text{Al}$) for 18 weeks. The DW (Dry weights) of leaf, stem, shoot and the whole plant of *C. grandis* were decreased and the ratio of root DW to shoot DW in *C. grandis* were increased by Al, whereas these parameters of *C. sinensis* were not changed by Al. Al treatment dramatically decreased the sulfur (S) content in *C. grandis* roots and the phosphorus (P) content in both *C. sinensis* and *C. grandis* roots. More Al was transported to shoots and leaves in *C. grandis* than in *C. sinensis* under Al treatment. Al treatment has more adverse effects on *C. grandis* than on *C. sinensis*, as revealed by the higher production of superoxide anion (O_2^-), H_2O_2 and thiobarbituric acid reactive substance (TBARS) content in *C. grandis* roots. Via the Illumina sequencing technique, we successfully identified and quantified 12 and 16 differentially expressed miRNAs responding to Al stress in *C. sinensis* and *C. grandis* roots, respectively. The possible mechanism underlying different Al tolerance of *C. sinensis* and *C. grandis* were summarized as having following aspects: (a) enhancement of adventitious and lateral root development (miR160);

(b) up-regulation of stress and signaling transduction related genes, such as *SGT1*, *PLC* and *AAO* (miR477, miR397 and miR398); (c) enhancement of citrate secretion (miR3627); (d) more flexible control of alternative glycolysis pathway and TCA cycle (miR3627 and miR482); (e) up-regulation of S-metabolism (miR172); (f) more flexible control of miRNA metabolism. For the first time, we showed that root development (miR160) and cell wall components (cas-miR5139, csi-miR12105) may play crucial roles in Al tolerance in citrus plants. In conclusion, our study provided a comprehensive profile of differentially expressed miRNAs in response to Al stress between two citrus plants differing in Al tolerance which further enriched our understanding of the molecular mechanism underlying Al tolerance in plants.

Keywords *Citrus* · Aluminum toxicity · miRNA · Biological regulation · Antioxidant system

Introduction

In the earth crust, Aluminum (Al) is one of the most abundant metal elements. It is also considered as the main element of soil inorganic minerals. When the soil pH was higher than 5, Al exists as aluminum silicate in soil solid phase, which is non-phytotoxic form for plants. While the soil pH is less than 5, Al will be dissolved into soil solution from clay minerals and mostly exist as rhizotoxic Al^{3+} ion (Kinraide 1991; Yang et al. 2018; Zhou et al. 2017). Al toxicity has become one of constraining factors that inhibited crop growth and led to significant crop yield loss in acidic areas worldwide (Shao et al. 2015; von Uexküll and Mutert 1995). The Al-induced growth inhibition, production loss and forest degradation have been reported in

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s12298-020-00895-y>) contains supplementary material, which is available to authorized users.

✉ Lin-Tong Yang
talstoy@163.com; talstoy@fafu.edu.cn

¹ College of Resources and Environment, Fujian Agriculture and Forestry University, Fuzhou 350002, China

² Fujian Provincial Key Laboratory of Soil Environmental Health and Regulation, Fujian Agriculture and Forestry University, Fuzhou 350002, China

rice (Wang et al. 2013), soybean (Zhen et al. 2007), wheat (Oh et al. 2014), sorghum (Zhou et al. 2017), Masson pine (Larssen and Carmichael 2000), spruce (Johnson and Siccamo 1983). Along with the industrialization and improper use of chemical fertilizer, soil acidification and subsequent Al toxicity would be the challenging problems, which need to be confronted and overcome for the sake of eco-agricultural production with the characteristics of environment-friendly and sustainable development. For this purpose, understanding how the plants cope with acidic soil containing soluble Al, and unveiling the molecular mechanism underlying the Al tolerance in plants are of theoretical and practical significance.

In the past two decades, in order to discover the physiological, biochemical, and molecular responses of plants to Al stress, great efforts have been made. The secretion of malate and citrate anions from root apex, which are mediated by Al-activated malate transporter (ALMT) and multidrug and toxic compound extrusion (MATE) channels, respectively, has been the most documented exclusion mechanism in plants (Sasaki et al. 2004; Magalhaes et al. 2007). Transgenic analysis showed that over-expression of ALMT and MATE could enhance the secretion of malate and citrate anions and increased the Al tolerance of wheat and rice bean, respectively (Pereira et al. 2010; Liu et al. 2018). Besides, some plasma and vacuolar membrane-located channels, such as H^+ -ATPase, *ALS3*, *HmPAL1* and *HmVALT*, that encoded sepal plasma membrane and vacuolar Al uptake transporters, were also reported to play important roles in Al tolerance (Larsen et al. 2005; Negishi et al. 2012, 2013; Zhang et al. 2017). Furthermore, exudation of the phenolic compounds, increased flavonoid proanthocyanidin in outer cell layer, modification of cell wall compounds by pectin methylesterases, up-regulation of sulfur metabolism and antioxidant system, were also reported to be implicated in Al tolerance of plants (Guo et al. 2017a; Chang et al. 1999; Osawa et al. 2011).

MicroRNAs (miRNAs) are a class of 22–24 nt (nucleotides) long non-coding small RNAs that have the ability to repress their target gene translation or induce their degradation by base pair manner in eukaryotes. In plants, a huge number of miRNAs has been regulated during nutrient stress responses (He et al. 2014; Wang et al. 2013; Zeng et al. 2014). Since a miRNA, miR395 was reported to respond to sulfate (S) deficiency in *Arabidopsis* (Jones-Rhoades and Bartel 2004), many miRNA species have been reported to respond to nutrient stresses such as nitrogen (N) starvation, phosphorus (Pi) deficiency, copper (Cu) limitation and iron (Fe) deprivation (Zeng et al. 2014). For instance, an ubiquitin conjugase gene *PHO2*, which is responsible for Pi uptake, is mediated by miR399 in plants (Chiou and Lin 2011). The miR398 was reported to regulate Cu

homeostasis by targeting CSDs (Cu/Zn SOD isoforms), which has been involved in ROS detoxification and protection of plastocyanin in *Arabidopsis* (Naya et al. 2014). The miR827 can target NLA (nitrogen limitation adaptation) to maintain Pi homeostasis via a nitrate-dependent manner in *Arabidopsis* (Kant et al. 2011). Under N starvation, miR169 was apparently downregulated, and its target gene *NFYA* (Nuclear Factor Y, subunit A) family members were induced in *Arabidopsis* (Zhao et al. 2011). In our previous study, 52 upregulated and 82 downregulated miRNAs were successfully isolated from B-deficient roots, demonstrating remarkable metabolic flexibility of citrus roots in response to B-deficiency (Lu et al. 2014). Huang et al. (2018) reported that a total of 453 miRNAs were identified as differentially expressed miRNAs under Al stress in soybean. Of these differentially expressed miRNAs, miR166k/o, miR390g and miR396c/k mediated root elongation in Al-tolerant cultivar BX10, in contrast, miR169r triggered oxidative stress in Al-sensitive cultivar BD2. The authors proposed that these molecular processes might be important mechanisms, which conferred differential Al tolerance to BX10 and BD2. Kong et al. (2014) identified 278 differentially expressed miRNAs associated with maize root development using Solexa technology. Some expression patterns of miRNA-target pairs, including miR164-NAC1, miR156-SPL and miR172-AP2, differed significantly in different variety maize roots, and such variation probably plays important roles in maize root development and differential Al tolerance in different maize roots.

Citrus is one of important evergreen fruit trees in the world. The soil pH of many citrus orchards is acidic, which may be due to high temperature and heavy rainfall. According to our previous survey, the soil pH values for most of 319 citrus orchards investigated were less than five (Li et al. 2015). In recent years, the physiological, transcriptomic and proteomic profile of *C. sinensis* and *C. grandis*, which have different Al tolerance have been investigated and some detail mechanisms of Al tolerance in citrus plants have been unveiled (Yang et al. 2011; Guo et al. 2017b; Jiang et al. 2015). However, whether miRNAs mediated regulatory mechanisms play important roles in different Al response in citrus plants was still unclear. In order to further understand the molecular mechanism underlying Al tolerance of those two citrus plants, Illumina sequencing and other physiological methods were used to analyze and investigate the differentially expressed miRNAs and possible regulatory mechanism. Several differentially expressed miRNAs were successfully identified, which could help us to further comprehend the mechanism of different Al tolerance in citrus.

Materials and methods

Seedling culture and aluminum treatments

The uniformed seeds of ‘Xuegan’ (*C. sinensis*) and ‘Sour pummelo’ (*C. grandis*) were collected from Fujian Academy of Forestry Sciences, Fuzhou, China, and were sown in the plastic tray containing clean river sands and kept moist by spraying tap water. The seeds were cultured in green house in Fujian Agriculture and Forestry University under natural temperature and light. The seedlings of ‘Xuegan’ and ‘Sour pummelo’ were irrigated with 1/4 strength nutrient solution every other day. Eight weeks later, uniform seedlings with one sprout and two leaves were transplanted to a 6-L pottery pot (two seedlings per pot) containing clean river sands and irrigated with 1/4 nutrient solution until dipping every other day. The 1/4 strength nutrient contained the following macronutrients (in mM): KNO_3 , 1; $\text{Ca}(\text{NO}_3)_2$, 1; KH_2PO_4 , 0.1; MgSO_4 , 0.5; micronutrients (in μM): H_3BO_3 , 10; MnCl_2 , 2; ZnSO_4 , 2; CuSO_4 , 0.5; $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$, 0.065; and $\text{FeSO}_4\text{-EDTA}$, 20. Seven weeks after transplanting, each pot was supplied with nutrient solution containing 0 mM $\text{AlCl}_3\cdot 6\text{H}_2\text{O}$ (+Al) or 1 mM $\text{AlCl}_3\cdot 6\text{H}_2\text{O}$ (–Al) every other day until saturated for sixteen weeks. The pH of nutrient solution was adjusted to 4.05–4.15 with HCl or NaOH. At the end of the experiment, fully-expanded (about ten weeks old) leaves from different replicates and treatments were collected and used for all the measurements. Root apices (about 5 mm) were excised and collected from seedlings. All the samples were wrapped in aluminum foil, immersed in liquid nitrogen and stored at -80°C until extraction and measurement.

Plant dry weight (DW), contents of Al, P, S and TBARS, production of superoxide anion (O_2^-) and H_2O_2 , and root citrate secretion

Ten plants per treatment (10 replicates) from different pots were harvested and sliced into shoots and roots. The harvested tissues were fixed at 120°C for 20 min, dried at 70°C for 48 h and DW were measured. Dried roots and leaves were ground into fine powder with a pulverizer and sifted by 0.15 mm sieve. Al was assayed by the aluminium method after samples were digested in a mixture of HNO_3 : HClO_4 (5:1 v/v). Al, P and S in the solution were determined using the aluminium method (Hsu 1963), the ammonium molybdate/ascorbic acid spectrophotometric assay (Ames 1966) and the simple turbidimetric method (Long et al. 2017), respectively. The O_2^- and H_2O_2 production were measured according to Doke (1983) and Long et al. (2017), respectively. TBARS was determined

according to Hodges et al. (1999). Root citrate secretion was measured according the method described by Yang et al. (2011). There were five replicates for Al, P and S contents, and four replicates for TBARS, O_2^- and H_2O_2 production, and root citrate secretion.

Total RNA extraction and miRNA library construction

Total RNA were extracted from different samples of citrus roots using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase I (Ambion, Austin, TX, USA). The quality and concentration of extracted RNA was analyzed by Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). The minimum concentration, OD260/OD230, OD260/OD280, RIN value and 28S/18S were required to be more than 120 ng/ μL , 1.5, 1.8, 7 and 0.7, respectively. Sequencing libraries were constructed from at least 1 μg total RNA according to the instruction manual of TruSeq Small RNA Sample Prep kits (Illumina, San Diego, USA). Briefly, the sequential ligation of RNA 3' and 5' adapters to the RNA samples was performed. The ligated RNA samples were reverse transcribed followed by PCR. This procedure was used to create cDNA constructs based on the small RNA ligated with 3' and 5' adapters, which selectively enriched those fragments that have adapter molecules on both ends. The amplified cDNA fragments were purified and selected by electrophoresis in 6% TBE PAGE gel. Align the razor blade with the top of the 160 bp band of the custom ladder, then with the bottom of the 145 bp band of the custom ladder. Excise the gel fragment by connecting these cuts on the sides. Concentrate final library by ethanol precipitation. And finally, the library quality was assessed using the Agilent Bioanalyzer 2100 system.

Library sequencing, bioinformatics analysis and differentially expressed mirna identification

After cluster generation, cDNA libraries were sequenced on the Illumina HiSeq 2500 platform (Illumina Inc., San Diego, CA, USA). Illumina GA Pipeline (Version 1.6) was used to perform the original image process to sequences, base calling and quality value calculation, in which 1×50 bp single-end reads were generated.

Raw reads were subjected to an in-house program, ACGT101-miR (LC Sciences, Houston, Texas, USA) to remove adapter dimers, low quality, common RNA families (rRNA, tRNA, snRNA, snoRNA) and repeats. Subsequently, unique sequences with length in 18–25 nucleotide range were mapped to specific species precursors in miR-Base 22.0. Length variation at both 3' and 5' ends and one

mismatch inside of the sequence were allowed in the alignment. The unique sequences mapping to specific species mature miRNAs in hairpin arms were identified as known miRNAs. The unique sequences mapping to the other arm of known specific species precursor hairpin opposite to the annotated mature miRNA-containing arm were considered to be novel 5p- or 3p- derived miRNA candidates. The remaining sequences were mapped to other selected species precursors (with the exclusion of specific species) in miRBase 22.0 by BLAST. The mapped pre-miRNAs were further BLASTed against the specific species genomes to determine their genomic locations. The above two were defined as known miRNAs. The unmapped sequences were BLASTed against the specific genomes, and the hairpin RNA structures containing sequences were predicated from the flank 120 nt sequences using RNAfold software (<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>).

To predict the genes targeted by most abundant miRNAs, computational target prediction algorithms (TargetFinder, version 2.0, <http://plantgrn.noble.org/psRNATarget/seq>) were used to identify miRNA binding sites. Expectation value (score) is the penalty for the mismatches between mature miRNA and the target sequence. A higher value indicates less similarity between miRNA and the target candidate. The default penalty rule is set up by the scoring schema. Maximum expectation is the cutoff; any small RNA-target pair with an expectation more than the cutoff will be discarded in the final result. Here, we used 4.0 as the cutoff on the scoring schema. The GO terms and KEGG pathway of most abundant miRNA targets were also annotated according to the method described by Wu et al. (2019). Differential expression of miRNAs based on normalized deep-sequencing count was analyzed by selectively using ANOVA based on the experiments design. The significance threshold was set to be 0.05 in the test.

RT-qPCR analysis of miRNA and its target genes

Total RNA were extracted using TRIzol reagent, digested by DNase I, and then reverse transcribed into cDNA using Mir-X miRNA first-strand synthesis kit (Takara, Dalian, China). The miRNA special primer was designed according to the instruction of TB green advantage qPCR premix kit (Takara, Dalian, China) based on the sequence of miRNA (Table S5 (Supplementary Materials)). The 25 μ L reaction mix of miRNA RT-qPCR included 1 $\times$ TB green advantage premix, 10 μ M miRNA special primer, 10 μ M mRQ 3' primer, adequate cDNA template and ddH₂O. The experimental conditions for miRNA RT-qPCR analysis were 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 20 s. The gene special primer pairs of miRNA targets were designed using Premier Primer 5.0

software (Premier Biosoft International, Palo Alto, CA, USA) and listed in Table S6 (Supplementary Materials). RT-qPCR of target genes was carried out according to the instruction of iQ SYBR green supermix quantification kit (Bio-Rad, Hercules, CA, USA). The PCR procedure was listed as follow: 95 °C, 2 min; 40 cycles of 95 °C, 10 s, 60 °C, 30 s, 72 °C, 30 s. Relative gene expression levels were calculated using the ddCt algorithm ($2^{-\Delta\Delta Ct}$). The –Al sample was used as the control and the U6 gene (accession number: Cs7g08440.1) was used as the internal reference. There were four replicates for each gene.

Experiment design and statistic analysis

There were 20 pots for each treatment (two seedlings per pot) in a completely randomized design. There were ten replicates for plant biomass, five replicates for element measurement and four replicates for TBARS, H₂O₂ content, proline measurement and RT-qPCR analysis, respectively. Results were represented as mean $\pm$ SD for n = 3–10. Difference among treatments and cultivars were analyzed by ANOVA. Means were separated by the Duncan's new multiple range test at $P < 0.05$.

Results

Effects of Al on the plant dry weight (DW) and root/shoot ratio in *C. sinensis* and *C. grandis*

Al treatment significantly lower the DW of root, stem, shoot and whole plant in *C. grandis*, while did not change these parameters in *C. sinensis* (Fig. 1; Fig. 2A–C, E). Al did not change the root DW in both *C. sinensis* and *C. grandis* (Fig. 2D). Al significantly increased root/shoot ratio only in *C. grandis* (Fig. 2F). Al-induced alternation of plant growth was more apparent in *C. grandis* than in *C. sinensis*.

Effects of Al Treatment on the contents of Al, P and S in *C. sinensis* and *C. grandis*

Al significantly increased Al contents of root, stem and leaf of two citrus varieties (Fig. 2A–C). No difference of Al content between root samples of *C. sinensis* and *C. grandis* was observed at –Al or +Al treatment (Fig. 2A). No significant difference of Al contents of stem and leaf was observed in either *C. sinensis* or *C. grandis* under –Al condition, while the Al contents of leaf and stem were higher in *C. grandis* than in *C. sinensis* under +Al condition (Fig. 2B, C). Al significantly decreased the P contents of root, stem and leaves in *C. sinensis* and *C. grandis*. There were no significant difference of root P and leaf P

Fig. 1 Effects of Al treatment on the growth of *C. sinensis* and *C. grandis* seedlings

Fig. 2 Effects of Al treatment on dry weight (DW) of leaf (A), stem (B), shoot (stem + leaf) (C), root (D), whole plant DW (E), and the ratio of root to shoot (F) in *C. sinensis* and *C. grandis*. Bars represent mean $\pm$ SD ($n = 10$). Different letters above the bars indicate a significant difference at $P < 0.05$

under $-Al$ treatment and stem P under $+Al$ treatment between *C. sinensis* and *C. grandis*, however, the root P and leaf P under $+Al$ treatment and stem P under $-Al$ treatment were higher in *C. sinensis* than that in *C. grandis* (Fig. 3D, E). Al only decreased the root S in *C. grandis* though the root S was higher in *C. grandis* than *C. sinensis* at both $-Al$ and $+Al$ conditions. Al dramatically increased stem S and leaf S in *C. sinensis*, but did not affect S in *C. grandis* (Fig. 3H–J). Under $+Al$ condition, the contents of S in stem and leaves were higher in *C. sinensis* than *C. grandis* (Fig. 3I, J).

Effects of Al Treatment on the production of superoxide anion ($O_2^{\cdot-}$), H_2O_2 , TBARS content and citrate secretion in *C. sinensis* and *C. grandis* roots

Al significantly increased the production of $O_2^{\cdot-}$, H_2O_2 and TBARS content in *C. grandis* roots, but not in *C. sinensis* ones (Fig. 4). Under $-Al$ condition, no difference was observed in the production of $O_2^{\cdot-}$ and TBARS content between *C. grandis* and *C. sinensis* roots, while higher H_2O_2 production was observed in *C. grandis* compared

Fig. 3 Effects of Al treatment on the content of Al (A–C), P (D, E) and S (H–J) in root, stem and leaf of *C. sinensis* and *C. grandis*. Bars represent mean $\pm$ SD (n = 4). Different letters above the bars indicate a significant difference at $P < 0.05$

Fig. 4 Effects of Al treatment on the production of O_2^- (A), H_2O_2 (B), TBARS content (C) and citrate secretion (D) of root in *C. sinensis* and *C. grandis*. Bars represent mean $\pm$ SD (n = 4). Different letters above the bars indicate a significant difference at $P < 0.05$

to *C. sinensis* roots (Fig. 4A–C). Under +Al condition, the production of O_2^- and H_2O_2 was higher in *C. grandis* roots than *C. sinensis* (Fig. 4A, B). Root citrate secretion was higher in Al pre-cultured root samples (+Al) than the control (–Al) of *C. sinensis* and *C. grandis*, respectively. Meanwhile, root citrate secretion induced by Al from *C. sinensis* was higher compared to *C. grandis* (Fig. 4D).

Identification of differentially expressed miRNA induced by Al in *C. sinensis* and *C. grandis* roots

After cluster generation, different cDNA libraries were sequenced on the Illumina Hiseq 2500 platform (Illumina, CA, USA). Raw reads were subjected to an in-house program ACGT101-miR (LC Sciences, Texas, USA). After removing adapter dimers, low quality, common RNA

families (such as rRNA, tRNA, snRNA, snoRNA and other Rfam) and repeats based on Rfam database (<http://rfam.janelia.org>) (Table S1 (Supplementary Materials)), an average of 12,209,953 raw reads and 4,554,539 valid reads ranged from 18 to 25 nucleotides were obtained from nine cDNA libraries of citrus roots. Afterwards, unique sequences (length in 18–25 nucleotides) were blasted to specific species precursors in miRBase 22.0. Our data analysis showed that 31, 18, 156, 89 and 127 pre-miRNAs were clustered into group1 (gp1), gp2a, gp2b, gp3 and gp4, respectively (Table S2 (Supplementary Materials)). Further analysis revealed that 55, 19, 133, 113 and 136 unique miRNAs were clustered into gp1, gp2a, gp2b, gp3 and gp4, respectively (Table S2 (Supplementary Materials)). For the identification of differentially expressed miRNA, the unique sequences were mapped to miRBase by BLAST and BLSASTed. The remaining sequences were blasted against the citrus genome (<http://citrus.hzau.edu.cn>), and the hair-pin RNA sequences were predicated using RNAfold. Statistical significance of differentially expressed miRNAs between different treatments was analyzed using student's *t*-test based on the experimental design. The significance threshold was set to be 0.05 in the test and the fold change was set to > 2 or < 2 . Results showed that 12 and 16 differentially expressed miRNAs were identified in *C. sinensis* and *C. grandis* roots, respectively (Table S3 (Supplementary Materials)). Of these differentially expressed miRNAs, only one miRNA was identified as upregulated miRNA and 11 miRNAs were identified as downregulated miRNA in response to AI in *C. sinensis* (Table S3 (Supplementary Materials)). In contrast, five miRNAs were identified as upregulated miRNA and 11 miRNAs were identified as downregulated miRNAs in response to AI in *C. grandis* roots (Table S3 (Supplementary Materials)).

Identification of miRNA target gene and bioinformatics analysis

The miRNA target sites were screened by computational target prediction algorithms TargetFinder (Version 2.0) against citrus genome. Furthermore, the Gene Ontology terms and KEGG pathway of the targets were also annotated. A total of 166 target genes of 22 differentially expressed miRNA (8 in *C. sinensis* roots and 14 in *C. grandis* roots) with the cutoff score of miRNA-target pair ≤ 4 were listed in Table S4 (Supplementary Materials). The number of target genes of these differentially expressed miRNAs ranged from 2 to 25. GO and KEGG analysis showed that these target genes were implicated in an extensive biological processes such as DNA binding, signal transduction, stress response, post-translational

modification, cellular transport, carbohydrate and energy metabolism, etc.

RT-qPCR analysis of miRNAs and their target genes

In order to verify the accuracy and reliability of Illumina sequence on the identification of differentially expressed miRNA in citrus roots, RT-qPCR analysis of miRNA were carried out using Mir-X miRNA first-strand synthesis kit (Takara, Dalian, China) and TB green advantage qPCR premix kit (Takara, Dalian, China). Results showed that the expression patterns of selected 15 differentially expressed miRNAs were highly consistent between RT-qPCR and Illumina sequence results, revealing that Illumina sequencing was a reliable and robust technique to identify and quantify differentially expressed miRNA in citrus roots (Fig. 5). To verify whether base-pair mechanism affects the gene expression level of target mRNAs of differentially expressed miRNAs identified in this study, RT-qPCR of target genes was carried out by the instruction of iQ SYBR green quantification kit (Bio-Bad, CA, USA). A total of 22 target genes were selected to perform RT-qPCR, of which, the expression patterns of 16 genes and 6 genes showed opposite and similar patterns with their miRNA pairs, respectively, indicating that most of differentially

Fig. 5 RT-qPCR analysis of the selected differentially expressed miRNAs in *C. sinensis* and *C. grandis* roots. There were four replicates for each root sample. The heatMap was generated using TBtools (version 0.66682)

Table 1 Expression levels of miRNA target genes in *C. sinensis* and *C. grandis* roots under Al treatment

miRNAs	Target gene	Description	Relative expression level (mean ± SD)			
			<i>C. sinensis</i>		<i>C. grandis</i>	
			Control	Al-toxicity	Control	Al-toxicity
csi-miR160c-5p_R+1	Cs6g11800.3	Auxin-responsive factor AUX/IAA-related isoform 1	1 ± 0.03	2.10 ± 0.37	1 ± 0.11	0.15 ± 0.03
cas-MIR5139-p3_2ss10AG18TC	Cs5g13310.1	Alpha,alpha-trehalose-phosphate synthase [UDP-forming] 1	1 ± 0.40	2.97 ± 0.39	1 ± 0.08	0.42 ± 0.01
csi-miR477c-5p	Cs7g10070.1	DELLA protein RGL1-like	1 ± 1.59	0.05 ± 0.01	1 ± 0.28	3.05 ± 0.07
csi-miR482e-3p	Cs7g02400.1	Glycerol-3-phosphate dehydrogenase [NAD(P) ⁺]	1 ± 0.15	4.90 ± 0.77	1 ± 0.14	0.35 ± 0.06
ath-miR397a_R+1	orange1.1t03059.1	Delta-1-pyrroline-5-carboxylate synthase-like	1 ± 0.18	3.92 ± 0.19	1 ± 0.13	0.33 ± 0.02
csi-miR477a-5p	Cs1g05220.1	UDP-glycosyltransferase 76C4	1 ± 0.02	1.41 ± 0.25	1 ± 0.07	0.56 ± 0.02
mes-miR3627	Cs4g04300.2	Calcium-transporting ATPase 8, plasma membrane-type	1 ± 0.46	2.47 ± 0.17	1 ± 0.05	0.93 ± 0.11
csi-MIR398a-p5	Cs1g25770.1	Beta-galactosidase	1 ± 0.02	0.54 ± 0.07	1 ± 0.03	0.17 ± 0.08
csi-miR482f-3p_L + 1	Cs1g13410.1	LRR and NB-ARC domain-containing disease resistance protein	1 ± 0.13	0.18 ± 0.02	1 ± 0.23	1.91 ± 0.07
PC-3p-259368_11	Cs7g07770.1	Glucan endo-1,3-beta-glucosidase 5	1 ± 0.19	1.00 ± 0.04	1 ± 0.34	2.14 ± 0.45
csi-miR827_1ss21AT	Cs6g09050.3	Ubiquitin-activating enzyme E1 1	1 ± 0.45	9.75 ± 0.44	1 ± 0.27	0.20 ± 0.03
csi-miR395a	Cs3g18660.1	Low affinity sulfate transporter 3; Sulfate transporter 2.1	1 ± 0.32	4.68 ± 1.01	1 ± 0.33	0.43 ± 0.04
csi-miR477a-3p	Cs8g02700.1	E3 ubiquitin-protein ligase UPL3	1 ± 0.04	4.30 ± 0.16	1 ± 0.17	0.97 ± 0.13
csi-miR172d-5p	Cs3g20460.1	Target of MYB protein 1	1 ± 0.11	2.41 ± 0.07	1 ± 0.06	0.61 ± 0.05
csi-miR12105-3p_L-1R+1	orange1.1t04174.1	Putative F-box protein PP2-B2	1 ± 0.06	0.54 ± 0.17	1 ± 0.02	2.62 ± 0.37
cas-miR5139	Cs7g10260.1	3-oxoacyl-[acyl-carrier-protein] reductase	1 ± 0.08	2.30 ± 0.03	1 ± 0.02	3.32 ± 0.40
PC-3p-42538_155	Cs4g02520.1	Cytochrome C oxidase subunit 6b-3	1 ± 0.30	3.36 ± 0.28	1 ± 0.05	0.94 ± 0.05
csi-miR167b-5p_R+1	Cs3g02400.1	Dynamain GTPase	1 ± 0.02	1.87 ± 0.03	1 ± 0.02	0.30 ± 0.03
csi-miR482d-3p	Cs1g14030.1	Probable disease resistance protein At5g63020	1 ± 0.01	3.03 ± 0.16	1 ± 0.05	1.05 ± 0.05

expressed miRNAs responding to Al treatment in citrus roots may perform their role by down-regulating their target mRNAs (Table 1).

Analysis of gene expression patterns of enzymes involved in miRNA metabolism

The expression levels of enzymes related to miRNA biogenesis, processing, methylation, transport and degradation, such as DNA-directed RNA polymerase II (Pol II), hyponastic leaves 1 (HYL1), Argonaute 1 (AGO1), C2H2 zinc-finger protein SERRATE (SE), HASTY 1, Dicer-like 1 (DCL1), HUA enhancer 1 (HEN1) and HEN1 suppressor 1 (HESO1), were analyzed using RT-qPCR. Results indicated that the expression levels of HEN1, SE1, AGO1, HASTY1, HYL1 and HESO1 were upregulated by Al in *C. sinensis* roots, whereas the expression levels of HEN1 and

AGO1 were downregulated by Al in *C. grandis* roots (Fig. 6).

Discussion

Effects of Al on the growth, elements absorption and oxidative stress of *C. sinensis* and *C. grandis*

Similar to our previous reports, Al treatment significantly decreased the DW of leaf, stem, shoot and whole plant of *C. grandis* (Fig. 2A–C, E). Al significantly increased or did not affect the root/shoot ratio and root DW in *C. grandis*, respectively (Fig. 2D, F). In contrast, Al did not change these parameters mentioned above in *C. sinensis* (Fig. 2). Such distinct responses of different cultivars in response to Al treatment could be partially attributed to the higher secretion of organic acids (mainly malate and citrate), the

Fig. 6 RT-qPCR analysis of enzymes related to miRNA metabolism in citrus roots. *HEN1*, HUA enhancer 1 (A); *SE1*, C2H2 zinc-finger protein SERRATE (B); *DCL1*, Dicer-like 1 (C); *AGO1*, Argonaute 1 (D); *HASTY1*, the *Arabidopsis* homolog of exportin 5 (E); *HYL1*, hyponastic leaves 1 (F); *POL*, DNA-directed RNA polymerase II (G); *HES01*, HEN1 suppressor 1 (H). Bars represent mean $\pm$ SD (n = 4). Different letters above the bars indicate a significant difference at $P < 0.05$

efficient maintenance of nutrient homeostasis, the higher adaptive responses of biological processes such as cell wall, energy metabolism, signal transduction and antioxidant system in Al-tolerant cultivar, as demonstrated by our previous proteomic, transcriptomic and physiological studies (Yang et al. 2011; Guo et al. 2017b, 2018). The adverse effects of Al on plants revealed in growth inhibition and yield loss have been observed in many plants such as *Arabidopsis* (Kumari et al. 2008), *Hordeum vulgare* (Jaskowiak et al. 2018), rice bean (Wei et al. 2015), wheat (Oh et al. 2014), rice (Wang et al. 2014a), buckwheat (Wang et al. 2014b), sorghum (Zhou et al. 2017), broad bean (Chen et al. 2015) and *Citrus* plants (Yang et al. 2018; Wu et al. 2019).

We observed that Al have significantly decreased S content in *C. grandis* roots and P content in both *C.*

sinensis and *C. grandis* roots (Fig. 3D, H). This is in consistence with the result obtained by Heim et al. (2001) and Janhunen et al. (1995), who also observed that Al treatment significantly decreased the contents of calcium, potassium, P and/or Mg in Norway spruce and Scot pine roots. The nutrient imbalance caused by Al stress might be the primary physiological disorder, which could further affect a series of subsequent biological processes including signal transduction, post-translational modification (PTM), electron transport, stress response and osmotic regulation (Yang et al. 2018; Guo et al. 2017b; Janhunen et al. 1995; Yang et al. 2019). The production of reactive oxygen species (ROS), a kind of toxic byproducts of aerobic metabolism, is an inevitable event in terrestrial plants. Al stress triggered oxidative stress in plants has proved to be a decisive event for inhibition of root cell growth

(Yamamoto et al. 2003). Here, we also observed that Al treatment substantially increased the production of O_2^- , H_2O_2 and TBARS content only in *C. grandis* roots (Fig. 4A–C). This result demonstrated that *C. grandis* roots suffered more serious oxidative stress than *C. sinensis* roots and the fine-tuning oxidative balance of *C. sinensis* roots could be partially due to the upregulated activities of antioxidative enzymes and contents of antioxidants (Jiang et al. 2015; Guo et al. 2018).

Differentially expressed miRNAs in *C. sinensis* and *C. grandis* roots under Al stress

MiRNAs are a class of non-coding small RNAs ranging from 18 to 25 nucleotides (nt), which are mainly transcribed from intergenic region or intron reverse repeat region by RNA polymerase II (Pol II) (He et al. 2014; Chen 2005). The miRNAs are generated from a single-stranded RNA precursor containing a hairpin secondary structure, mediating endogenous gene silencing at the post-transcriptional level for transcript cleavage or translational repression (Zeng et al. 2014; Kuo and Chiou 2011). In the current study, we successfully identified and quantified 12 and 16 differentially expressed miRNAs responding to Al stress in *C. sinensis* and *C. grandis* roots, respectively (Table S3 (Supplementary Materials)). Furthermore, the candidate targets of these differentially expressed miRNAs were computationally predicted using TargetFinder (version 2.0) and were analyzed by RT-qPCR (Table S4 (Supplementary Materials); Fig. 6). In order to unveil the possible molecular mechanism underlying different Al-tolerance of *C. sinensis* and *C. grandis*, hereafter, we will focus on the proposed roles and biological functions of these differentially expressed miRNAs and their target genes, which have been reported in the available literatures.

The miR160 is a conserve miRNA family, which is involved in many biotic processes in plants, including seed germination, flower organ specification, leaf determination, fruit formation, regulation of pathogen associated plant innate immunity, N and S deficiency response (Chen et al. 2018; Li et al. 2010; Liang et al. 2012; Huang et al. 2010). One of the target genes of miR160, auxin response factors (ARFs), bind to TGTCTC auxin response elements in promoters of auxin response genes. ARFs work together with Aux/IAAs in auxin-mediated developmental processes (Tiware et al. 2003). Recent studies revealed that miR160-ARF10/ARF16/ARF17 module plays vital roles in root cap development, lateral root formation and primary root elongation in both *Arabidopsis* and rice (Kong et al. 2014; Gutierrez et al. 2009; Meng et al. 2009). Our Illumina sequencing result showed that the expression level of miR160 member *csi-miR160c-5p_R+1* was

downregulated by Al treatment in *C. sinensis* roots, whereas the expression level of miR167 member *csi-miR167b-5p_R+1*, which has the opposite role of miR160 in adventitious and lateral roots development in plants, was also downregulated by Al treatment in *C. grandis* roots (Table S4 (Supplementary Materials)) (Kong et al. 2014). RT-qPCR analysis showed that the expression levels of miR160 target gene ARF1 were upregulated and down-regulated by Al treatment in both citrus varieties roots, respectively (Fig. 6A). Such result may imply that the adventitious and lateral roots development would be enhanced in *C. sinensis* underground part under Al treatment, while the case is opposite in *C. grandis* (Kong et al. 2014). The altered expression levels of miR160, miR167, and miR171 were also observed in *Arabidopsis* roots, which may be crucial for root system construction under nitrogen deficiency (Liang et al. 2012).

Al treatment downregulated the expression levels of miR477 member *csi-miR477c-5p* and *csi-miR477a-5p* in *C. sinensis* roots, and *csi-miR477a-3p* and *csi-miR477c-3p* in *C. grandis* roots (Table S4 (Supplementary Materials)). The *Arabidopsis* genome encodes at least five DELLA proteins, gibberellins Insensitive (GAI), repressor of GA1-3 (RGA), RGA-like1 (RGL1), RGL2, and RGL3 (Zhao et al. 2012). One of *csi-miR477c-5p* target, DELLA protein RGL1-like (*RGL1*) was downregulated and upregulated by Al treatment in *C. sinensis* and *C. grandis* roots, respectively (Table 1). This result indicated that the expression level of *RGL1* may not be regulated by miR477 in citrus roots under Al treatment and the up-regulation of *RGL1* in *C. grandis* roots by Al indicated an altered GA and JA (jasmonic acid) signaling cascade. The down-regulation of miR477 was also found in cotton in response to drought and salinity stress (Xie et al. 2015). Yusof et al. (2016) reported that *GaSGT1* promotes cell thermotolerance and employs molecular chaperone-like activity toward temperature assaults in the yeast *Glaciozyma antarctica*. Our RT-qPCR analysis revealed that the expression levels of *csi-miR477a-5p* target SGT1-2-like protein (*SGT1*) were upregulated by Al treatment in both *C. sinensis* and *C. grandis* roots with higher expression level in *C. sinensis* roots than *C. grandis*. Such expression pattern of *SGT1* showed opposite trend with *csi-miR477a-5p* (Table 1; Fig. 5). The higher expression level of SGT1 might imply a higher tolerance of adverse stress in *C. sinensis* roots than *C. grandis*.

The target genes of miR398 and miR397 encoded Cu–Zn superoxide dismutase (CSD) and laccase, which are scavengers of ROS or involved in cell wall lignin deposition, respectively (Naya et al. 2014; Liang et al. 2012). Previous literature showed that miR398 and miR397 involved in nutrient homeostasis of plants. Huang et al. (2016) reported that miR397 played an important role in

the resilience to B toxicity in *C. sinensis*. Through its down-regulation and concomitant up-regulation of target laccase gene *LAC7*, the excess B was effectively excluded from sensitive cells by the increased deposition of lignin in the secondary cell wall. MiR398 was reported to act as a regulator in Cu homeostasis. The down-regulation of miR398 in Cu stress results in high CSDs for O_2^- detoxification, whereas high expression levels of miR398 and Fe (iron) superoxide dismutase (FSD) are observed in Cu deficient condition, which is responsible for ROS detoxification and limited Cu is transported to plastocyanin (Naya et al. 2014). Cu deprivation has also been shown to induce the expression of miR397 in *Arabidopsis* and *Brassica* (Jagadeeswaran et al. 2009). Liang et al. (2012) reported that N deficiency could downregulated the expression levels of miR397 and miR398, which may decrease P, S and Cu uptake. This is in consistency with the results of our nutrient measurement, which showed that Al apparently decreased the content of P in both *C. sinensis* and *C. grandis* roots, and the content of S in *C. grandis* roots (Fig. 3D, H). Here, we found that Al treatment significantly decreased the expression level of two members of miR397, *csi-miR397-3p_R+1* and *ath-miR397a_R+1*, whereas upregulated the expression levels of two target genes of which (Table S4 (Supplementary Materials)). Phospholipase C (PLC) is a phospholipid hydrolyzing enzyme, generating signaling messengers such as phosphatidic acid (PA) and inositol hexakisphosphate (IP6), and regulates various cellular processes in plants (Singh et al. 2015). L-ascorbate oxidase (AAO) is a copper-containing blue oxidase, catalyzing the aerobic oxidation of ascorbate to monodehydroascorbate. The expression level of AAO is related to both cell division and cell elongation, however AAO may be of more functional importance for cell elongation (Kato and Esaka 1999). The up-regulation of *PLC* and *AAO* may be regulated by the downregulated expression of miR397 and could be beneficial to the normal growth of *C. sinensis* roots under Al treatment (Table 1).

The miR3627 has been reportedly involved in abiotic stress and plant response to the nutrition status. For example, Li et al. (2017) showed that miR3627-3p (1.61-fold) and miR3627-5p (6.02-fold) were downregulated and upregulated by UVB treatment in *Prunus persica*, respectively. The target genes of miR3627-5p, related to glycolysis pathway and TCA cycle, were all downregulated, which were consistent with the up-regulation of miR3627-5p. Shen et al. (2017) reported that a MATE efflux family protein gene was regulated by a downregulated miRNA (miRNA3627) in water spinach (*Ipomoea aquatica*). Besides, the cadmium (Cd) translocation of a high-shoot-Cd cultivar T308 was enhanced due to the upregulation of MATE efflux family. And instead of sequestering Cd cation in root, the T308 tended to rise the Cd efflux by

down-regulating the expression level of miRNA3627, which could upregulate the expression of MATE efflux family, facilitating the translocation of Cd into shoot and leading to a higher Cd-accumulation in shoot. Here, we showed that Al treatment downregulated the expression level of miR3627 (*mes-miR3627*) and concomitantly upregulated its target gene membrane H^+ -ATPase 1 in *C. sinensis* roots (Table 1; Table S4 (Supplementary Materials)). The upregulated membrane H^+ -ATPase might be beneficial to the secretion of citrate from root tips of *C. sinensis* and confer higher Al-tolerance to *C. sinensis* than to *C. grandis* (Fig. 4D) (Chen et al. 2013; Yang et al. 2012).

Glycerol-3-phosphate dehydrogenase [$NAD(P)^+$] (*G3PDH*) is an enzyme that catalyzes the reversible conversion of dihydroxyacetone phosphate to glycerol 3-phosphate, linking carbohydrate metabolism and lipid metabolism. Previous studies indicated that Al treatment increased the protein abundance and gene expression level of *G3PDH* in pineapple and citrus roots (Yang et al. 2019; Chen and Lin 2010). Here, we have showed that *G3PDH* is one of target genes of *csi-miR482e-3p* in *C. sinensis* roots and of *csi-miR482d-3p* in *C. grandis* roots (Table S4 (Supplementary Materials)). The increased expression level of *G3PDH* was in consistence with the downregulated *csi-miR482e-3p* and *csi-miR482d-3p* in *C. sinensis* and *C. grandis* roots (Table 1). The altered regulation of *G3PDH* might be beneficial to switch the regular and alternative glycolysis pathway known in plants, which were catalyzed by a P-independent non-phosphorylating $NADP$ -*G3PDH* (EC 1.2.1.13) and P-dependent NAD -*G3PDH* (EC 1.2.1.12) and phosphoglycerate kinase (EC 2.7.2.3), respectively, as Al treatment significantly decreased the P contents in both *C. sinensis* and *C. grandis* roots (Fig. 3D) (Yang et al. 2012). Our previous study found that both Al and high B levels increase the protein abundance of an α -1,4-glucan-protein synthase (UDP-forming), which is responsible for the formation of cell wall polysaccharoses (Yang et al. 2018; Li et al. 2013). Here, we found that the expression level of miR5139 (*cas-miR5139-p3_2ss10AG18TC*) was induced by Al and one of its target gene α -trehalose-phosphate synthase [UDP-forming] 1 was concomitantly downregulated by Al in *C. sinensis* roots (Table 1; Table S4 (Supplementary Materials)). In contrast, a miR12105 (*csi-miR12105-3p_L-1R+1*), targeting to cellulose synthetase (UDP forming) gene, was downregulated by Al treatment in *C. grandis* roots (Table S4 (Supplementary Materials)). Such result indicated that the biosynthesis of the various cell wall components was differentially regulated by Al treatment.

Sulfur metabolism plays crucial roles in plant response to abiotic stresses. As previous studies reported that the protein abundance or gene expression level of

Fig. 7 Graphical diagram of the functions of differentially expressed miRNAs and their target genes in *C. sinensis* and *C. grandis* roots. Positive and negative regulatory actions are indicated by arrows and lines with bars, respectively

S-metabolism related enzymes, such as methionine S-methyltransferase, S-adenosylmethionine synthetase (SAMS), methionine synthase, glutathione reductase, glutathione-S-transferase (GST) and methionine adenosyltransferase, were induced by various stresses including Al (Guo et al. 2017a; Jiang et al. 2015). Here, two members of miR172, *csi-miR172b-5p_1ss3AG* and *csi-miR172d-5p*, were downregulated by Al treatment in *C. grandis* roots (Table S4 (Supplementary Materials)). Methionine S-methyltransferase-like (*MSMT*), one of the target genes of *csi-miR172b-5p_1ss3AG*, was significantly upregulated by Al in *C. grandis* roots (Table 1). Interestingly, the expression level of *MSMT* was higher in *C. sinensis* roots than *C. grandis* ones under both conditions with or without Al treatment, which demonstrated that the up-regulation of S-metabolism might be a strategic response to Al stress in plants and conferred higher Al-tolerance to *C. sinensis* than *C. grandis* under Al (Oh et al. 2014; Guo et al. 2017b; Yang et al. 2019). The lower content of S in *C. sinensis* roots and higher content of S in *C. sinensis* shoots and leaves than that of *C. grandis*, also showed that more S was transported to above ground part of *C. sinensis* than that of *C. grandis*, and *C. sinensis* roots may have a higher use-efficiency of S than *C. grandis* (Fig. 3H–J). Baculoviral IAP repeat-containing proteins (BIRCs) have multiple biological activities, including binding caspases, regulating cell cycle and modulating receptor-mediated signal transduction via protein ubiquitination (Yang and Li 2000). We have observed that *BIRC* was a target gene of *csi-miR172d-5p*. The down-regulation of *csi-miR172d-5p* might imply that ubiquitin-mediated proteolysis would be enhanced in *C. grandis* roots under Al treatment. Furthermore, the expression level of another miRNA *csi-miR12105-3p_L-1R+1* was also downregulated and its target putative F-box protein PP2-B2, which is subunit of ubiquitin protein ligase, was concomitantly upregulated by Al in *C. grandis* roots under Al treatment (Table 1; Table S4 (Supplementary Materials)). The enhanced ubiquitin mediated proteolysis was also reported in

Arabidopsis and citrus plants under P deficiency, Mg deficiency, B toxicity and/or Al stress (Guo et al. 2017b; Kumari et al. 2008; Huang et al. 2016; Jin et al. 2016; Ye et al. 2018).

In order to investigate whether the gene expression level of enzymes related to miRNA metabolism was involved in the differential expression of miRNA profile in response to Al treatment. The gene expression level of *Pol II*, *HYL1*, *AGO1*, *SE*, *HASTY1*, *DCL1*, *HEN1* and *HESO1*, were analyzed using RT-qPCR. Results showed that the expression levels of *HEN1*, *SE1*, *AGO1*, *HASTY1*, *HYL1* and *HESO1* were upregulated by Al in *C. sinensis* roots, whereas the expression levels of *AGO1* and *HESO1* were downregulated and upregulated by Al in *C. grandis* roots, respectively (Fig. 6). Such results indicated that the biological processes of miRNA biogenesis, processing, methylation, transport and degradation were more tightly controlled and flexible in *C. sinensis* roots than in *C. grandis* ones (Chen 2005; Chen et al. 2018; Dong et al. 2008).

Conclusions

Al dramatically decreased the DW of leaf, stem, shoot and the whole plant of *C. grandis*, increased root/shoot ratio in *C. grandis*, whereas did not change these parameters of *C. sinensis*. Al treatment significantly decreased the S content in *C. grandis* roots and the P content in both *C. sinensis* and *C. grandis* roots. More Al was transported to shoots and leaves in *C. grandis* than in *C. sinensis* under Al stress. Al treatment has more adverse effects on *C. grandis* than on *C. sinensis*, as revealed by the higher production of O_2^- , H_2O_2 and TBARS content in *C. grandis* roots. Via the Illumina sequencing technique, we successfully identified and quantified 12 and 16 differentially expressed miRNAs responding to Al stress in *C. sinensis* and *C. grandis* roots, respectively. The possible mechanism underlying

different Al tolerance of *C. sinensis* and *C. grandis* were summarized as following aspects (Fig. 7): (a) enhancement of adventitious and lateral root development (miR160); (b) up-regulation of stress and signaling transduction related genes, such as *SGT1*, *PLC* and *AAO* (miR477, miR397 and miR398); (c) enhancement of citrate secretion (miR3627); (d) more flexible control of glycolysis pathway and TCA cycle (miR3627 and miR482); (e) up-regulation of S-metabolism (miR172); (f) more flexible control of miRNA metabolism. In conclusion, our study provided a comprehensive profile of differentially expressed miRNAs in response to Al stress between two citrus plants differing in Al tolerance and hence, further enriched the molecular mechanism underlying Al tolerance in plants.

Funding This study was financially supported by the National Key R&D Program of China (No. 2017YFD0202000), the National Natural Science Foundation of China (No. 31301740).

Data availability The RNA-seq data was deposited in NCBI database (<https://www.ncbi.nlm.nih.gov/sra/>) with SRA accession number PRJNA600689.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

- Ames BN (1966) Assay of inorganic phosphate, total phosphate and phosphatase. *Methods Enzymol* 8:115–118
- Chang YC, Yamamoto Y, Matsumoto H (1999) Accumulation of aluminium in the cell wall pectin in cultured tobacco (*Nicotiana tabacum* L.) cells treated with a combination of aluminium and iron. *Plant Cell Environ* 22:1009–1017
- Chen X (2005) microRNA biogenesis and function in plants. *FEBS Lett* 579:5923–5931
- Chen JH, Lin YH (2010) Effect of aluminum on variations in the proteins in pineapple roots. *Soil Sci Plant Nutr* 56:438–444
- Chen Q, Guo CL, Wang P, Chen XQ, Wu KH, Li KZ, Yu YX, Chen LM (2013) Up-regulation and interaction of the plasma membrane H⁺-ATPase and the 14-3-3 protein are involved in the regulation of citrate exudation from the broad bean (*Vicia faba* L.) under Al stress. *Plant Physiol Biochem* 70:504–511
- Chen Q, Kan Q, Wang P, Yu WQ, Yu YZ, Zhao Y, Yu YX, Li KZ, Chen LM (2015) Phosphorylation and interaction with the 14-3-3 protein of the plasma membrane H⁺-ATPase are involved in the regulation of magnesium-mediated increases in aluminum-induced citrate exudation in broad bean (*Vicia faba* L.). *Plant Cell Physiol* 56:1144–1153
- Chen C, Zeng Z, Liu Z, Xia R (2018) Small RNAs, emerging regulators critical for the development of horticultural traits. *Hortic Res* 5:63
- Chiou TJ, Lin SI (2011) Signaling network in sensing phosphate availability in Plants. *Ann Rev Plant Biol* 62:185–206
- Doke N (1983) Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthora infestans* and to the hyphal wall components. *Physiol Plant Pathol* 23:345–357
- Dong Z, Han MH, Fedoroff N (2008) The RNA-binding proteins HYL1 and SE promote accurate in vitro processing of pri-miRNA by DCL1. *Proc Natl Acad Sci USA* 105:9970–9975
- Guo P, Li Q, Qi YP, Yang LT, Ye X, Chen HH, Chen LS (2017a) Sulfur-mediated-alleviation of aluminum-toxicity in *Citrus grandis* seedlings. *Int J Mol Sci* 18:2570
- Guo P, Qi YP, Yang LT, Lai NW, Ye X, Yang Y, Chen LS (2017b) Root adaptive responses to aluminum-treatment revealed by RNA-seq in two citrus species with different aluminum-tolerance. *Front Plant Sci* 8:330
- Guo P, Qi YP, Cai YT, Yang TY, Yang LT, Huang ZR, Chen LS (2018) Aluminum effects on photosynthesis, reactive oxygen species and methylglyoxal detoxification in two *Citrus* species differing in aluminum tolerance. *Tree Physiol* 38:1548–1565
- Gutierrez L, Bussell JD, Păcurar DI, Schwambach J, Păcurar M, Bellini C (2009) Phenotypic plasticity of adventitious rooting in *Arabidopsis* is controlled by complex regulation of AUXIN RESPONSE FACTOR transcripts and microRNA abundance. *Plant Cell* 21:3119–3132
- He H, He L, Gu M (2014) Role of microRNAs in aluminum stress in plants. *Plant Cell Rep* 33:831–836
- Heim A, Brunner I, Frey B, Frossard E, Luster J (2001) Root exudation, organic acids, and element distribution in roots of Norway spruce seedlings treated with aluminum in hydroponics. *J Plant Nutr Soil Sci* 164:519–526
- Hodges DM, DeLong JM, Forney CF, Prange RK (1999) Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. *Planta* 207:604–611
- Hsu PH (1963) Effect of initial pH, phosphate, and silicate on the determination of aluminum with aluminon. *Soil Sci* 96:230–238
- Huang SQ, Xiang AL, Che LL, Chen S, Li H, Song JB, Yang ZM (2010) A set of miRNAs from *Brassica napus* in response to sulphate deficiency and cadmium stress. *Plant Biotechnol J* 8:887–899
- Huang JH, Qi YP, Wen SX, Guo P, Chen XM, Chen LS (2016) Illumina microRNA profiles reveal the involvement of miR397a in *Citrus* adaptation to long-term boron toxicity via modulating secondary cell-wall biosynthesis. *Sci Rep* 6:22900
- Huang SC, Lu GH, Tang CY, Ji YJ, Tan GS, Hu DQ, Cheng J, Wang GH, Qi JL, Yang YH (2018) Identification and comparative analysis of aluminum-induced microRNAs conferring plant tolerance to aluminum stress in soybean. *Biol Plantarum* 62:97–108
- Jagadeeswaran G, Zheng Y, Li YF, Shukla LI, Matts J, Hoyt P, Macmil SL, Wiley GB, Roe BA, Zhang W, Sunkar R (2009) Cloning and characterization of small RNAs from *Medicago truncatula* reveals four novel legume-specific microRNA families. *New Phytol* 184:85–98
- Janhunen S, Palomäki V, Holopainen T (1995) Aluminium causes nutrient imbalance and structural changes in the needles of Scots pine without inducing clear root injuries. *Trees* 9:134
- Jaskowiak J, Tkaczyk O, Słota M, Kwasniewska J, Szarejko I (2018) Analysis of aluminum toxicity in *Hordeum vulgare* roots with an emphasis on DNA integrity and cell cycle. *PLoS ONE* 13:e0193156
- Jiang HX, Yang LT, Qi YP, Lu YB, Huang ZR, Chen LS (2015) Root iTRAQ protein profile analysis of two *Citrus* species differing in aluminum-tolerance in response to long-term aluminum-toxicity. *BMC Genom* 16:949
- Jin XL, Ma CL, Yang LT, Chen LS (2016) Alterations of physiology and gene expression due to long-term magnesium-deficiency

- differ between leaves and roots of *Citrus reticulata*. J Plant Physiol 198:103–115
- Johnson AH, Siccama TG (1983) Acid deposition and forest decline. Environ Sci Technol 17:294A–305A
- Jones-Rhoades MW, Bartel DP (2004) Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. Mol Cell 14:787–799
- Kant S, Peng M, Rothstein SJ (2011) Genetic regulation by NLA and microRNA827 for maintaining nitrate-dependent phosphate homeostasis in *Arabidopsis*. PLoS Genet 7:e1002021
- Kato N, Esaka M (1999) Changes in ascorbate oxidase gene expression and ascorbate levels in cell division and cell elongation in tobacco cells. Physiol Plantarum 105:321–329
- Kinraide TB (1991) Identity of the rhizotoxic aluminium species. Plant Soil 134:167–178
- Kong X, Zhang M, Xu X, Li X, Li C, Ding Z (2014) System analysis of microRNAs in the development and aluminium stress responses of the maize root system. Plant Biotechnol J 12:1108–1121
- Kumari M, Taylor GJ, Deyholos MK (2008) Transcriptomic responses to aluminum stress in roots of *Arabidopsis thaliana*. Mol Genet Genom 279:339–357
- Kuo HF, Chiou TJ (2011) The role of MicroRNAs in phosphorus deficiency signaling. Plant Physiol 156:1016–1024
- Larsen PB, Geisler MJB, Jones CA, Williams KM, Cancell JD (2005) ALS3 encodes a phloem-localized ABC transporter-like protein that is required for aluminum tolerance in *Arabidopsis*. Plant J 41:353–363
- Larssen T, Carmichael GR (2000) Acid rain and acidification in China: the importance of base cation deposition. Environ Pollut 110:89–102
- Li Y, Zhang Q, Zhang J, Wu L, Qi Y, Zhou JM (2010) Identification of microRNAs involved in pathogen-associated molecular pattern-triggered plant innate immunity. Plant Physiol 152:2222–2231
- Li XS, Bai TT, Li YF, Ruan XL, Li HP (2013) Proteomic analysis of *Fusarium oxysporum* f. sp. *cubense* tropical race 4-inoculated response to *Fusarium* wilts in the banana root cells. Proteome Sci 11:41
- Li Y, Han MQ, Lin F, Ten Y, Lin J, Zhu DH, Guo P, Weng YB, Chen LS (2015) Soil chemical properties, ‘Guanximiyou’ pummelo leaf mineral nutrient status and fruit quality in the southern region of Fujian province, China. J Soil Sci Plant Nutr 15:263–269
- Li S, Shao Z, Fu X, Xiao W, Li L, Chen M, Sun M, Li D, Gao D (2017) Identification and characterization of *Prunus persica* miRNAs in response to UVB radiation in greenhouse through high-throughput sequencing. BMC Genom 18:938
- Liang G, He H, Yu D (2012) Identification of nitrogen starvation-responsive microRNAs in *Arabidopsis thaliana*. PLoS ONE 7:e48951
- Liu MY, Lou HQ, Chen WW, Piñeros MA, Xu JM, Fan W, Kochian LV, Zheng SJ, Yang JL (2018) Two citrate transporters coordinately regulate citrate secretion from rice bean root tip under aluminum stress. Plant Cell Environ 41:809–822
- Long A, Zhang J, Yang LT, Ye X, Lai NW, Tan LL, Lin D, Chen LS (2017) Effects of low pH on photosynthesis, related physiological parameters, and nutrient profiles of citrus. Front Plant Sci 8:185
- Lu YB, Yang LT, Qi YP, Li Y, Li Z, Chen YB, Huang ZR, Chen LS (2014) Identification of boron-deficiency-responsive microRNAs in *Citrus sinensis* roots by Illumina sequencing. BMC Plant Biol 14:123
- Magalhaes JV, Liu J, Guimarães CT, Lana UGP, Alves VMC, Wang YH, Schaffert RE, Hoekenga OA, Piñeros MA, Shaff JE, Klein PE, Carneiro NP, Coelho CM, Trick HN, Kochian LV (2007) A gene in the multidrug and toxic compound extrusion (MATE) family confers aluminum tolerance in sorghum. Nat Genet 39:1156
- Meng Y, Huang F, Shi Q, Cao J, Chen D, Zhang J, Ni J, Wu P, Chen M (2009) Genome-wide survey of rice microRNAs and microRNA–target pairs in the root of a novel auxin-resistant mutant. Planta 230:883–898
- Naya L, Paul S, Valdes-Lopez O, Mendoza-Soto AB, Nova-Franco B, Sosa-Valencia G, Reyes JL, Hernandez G (2014) Regulation of copper homeostasis and biotic interactions by microRNA 398b in common bean. PLoS ONE 9:e84416
- Negishi T, Oshima K, Hattori M, Kanai M, Mano S, Nishimura M, Yoshida K (2012) Tonoplast- and plasma membrane-localized aquaporin-family transporters in blue hydrangea sepals of aluminum hyperaccumulating plant. PLoS ONE 7:e43189
- Negishi T, Oshima K, Hattori M, Yoshida K (2013) Plasma membrane-localized Al-transporter from blue hydrangea sepals is a member of the anion permease family. Genes Cells 18:341–352
- Oh MW, Roy SK, Kamal AH, Cho K, Cho SW, Park CS, Choi JS, Komatsu S, Woo SH (2014) Proteome analysis of roots of wheat seedlings under aluminum stress. Mol Biol Rep 41:671
- Osawa H, Endo I, Hara Y, Matsushima Y, Tange T (2011) Transient proliferation of proanthocyanidin-accumulating cells on the epidermal apex contributes to highly aluminum-resistant root elongation in camphor tree. Plant Physiol 155:433–446
- Pereira JF, Zhou G, Delhaize E, Richardson T, Zhou M, Ryan PR (2010) Engineering greater aluminium resistance in wheat by over-expressing *TaALMT1*. Ann Bot 106:205–214
- Sasaki T, Yamamoto Y, Ezaki B, Katsuhara M, Ahn SJ, Ryan PR, Delhaize E, Matsumoto H (2004) A wheat gene encoding an aluminum-activated malate transporter. Plant J 37:645–653
- Shao JF, Che J, Chen RF, Ma JF, Shen RF (2015) Effect of in planta phosphorus on aluminum-induced inhibition of root elongation in wheat. Plant Soil 395:307–315
- Shen C, Huang YY, He CT, Zhou Q, Chen JX, Tan X, Mubeen S, Yuan JG, Yang ZY (2017) Comparative analysis of cadmium responsive microRNAs in roots of two *Ipomoea aquatica* Forsk. cultivars with different cadmium accumulation capacities. Plant Physiol Biochem 111:329–339
- Singh A, Bhatnagar N, Pandey A, Pandey GK (2015) Plant phospholipase C family: Regulation and functional role in lipid signaling. Cell Calcium 58:139–146
- Tiwari SB, Hagen G, Guilfoyle T (2003) The roles of auxin response factor domains in auxin-responsive transcription. Plant Cell 15:533–543
- von Uexküll HR, Mutert E (1995) Global extent, development and economic impact of acid soils. Plant Soil 171:1–15
- Wang CY, Shen RF, Wang C, Wang W (2013) Root protein profile changes induced by Al exposure in two rice cultivars differing in Al tolerance. J Proteom 78:281–293
- Wang H, Chen RF, Iwashita T, Shen RF, Ma JF (2014a) Physiological characterization of aluminum tolerance and accumulation in tartary and wild buckwheat. New Phytol 205:273–279
- Wang ZQ, Xu XY, Gong QQ, Xie C, Fan W, Yang JL, Lin QS, Zheng SJ (2014b) Root proteome of rice studied by iTRAQ provides integrated insight into aluminum stress tolerance mechanisms in plants. J Proteom 98:189–205
- Wei F, He QL, Yu LG, Mei YL, Meng JC, Yu L, Jian LY, Shao JZ (2015) Characterization of an inducible C2H2-type zinc finger transcription factor VuSTOP1 in rice bean (*Vigna umbellata*) reveals differential regulation between low pH and aluminum tolerance mechanisms. New Phytol 208:456–468
- Wu YM, Wang YY, Zhou YF, Meng X, Huang ZR, Chen LS, Yang LT (2019) Analysis of interacting proteins of aluminum toxicity response factor ALS3 and CAD in *Citrus*. Int J Mol Sci 20:4846

- Xie F, Wang Q, Sun R, Zhang B (2015) Deep sequencing reveals important roles of microRNAs in response to drought and salinity stress in cotton. *J Exp Bot* 66:789–804
- Yamamoto Y, Kobayashi Y, Devi SR, Rikiishi S, Matsumoto H (2003) Oxidative stress triggered by aluminum in plant roots. *Plant Soil* 255:239–243
- Yang YL, Li XM (2000) The IAP family: endogenous caspase inhibitors with multiple biological activities. *Cell Res* 10:169–177
- Yang LT, Jiang HX, Tang N, Chen LS (2011) Mechanisms of aluminum-tolerance in two species of citrus: secretion of organic acid anions and immobilization of aluminum by phosphorus in roots. *Plant Sci* 180:521–530
- Yang LT, Jiang HX, Qi YP, Chen LS (2012) Differential expression of genes involved in alternative glycolytic pathways, phosphorus scavenging and recycling in response to aluminum and phosphorus interactions in *Citrus* roots. *Mol Biol Rep* 39:6353–6366
- Yang LT, Liu JW, Wu YM, Qi YP, Wang JL, Lai NW, Ye X, Chen LS (2018) Proteome profile analysis of boron-induced alleviation of aluminum-toxicity in *Citrus grandis* roots. *Ecotox Environ Saf* 162:488–498
- Yang LT, Zhou YF, Wang YY, Wu YM, Qian B, Wang H, Chen LS (2019) Phosphorus-mediated alleviation of aluminum toxicity revealed by the iTRAQ technique in *Citrus grandis* roots. *PLoS ONE* 14:e0223516
- Ye Q, Wang H, Su T, Wu WH, Chen YF (2018) The ubiquitin E3 ligase PRU1 regulates WRKY6 degradation to modulate phosphate homeostasis in response to low-Pi stress in *Arabidopsis*. *Plant Cell* 30:1062–1076
- Yusof NA, Hashim NHF, Beddoe T, Mahadi NM, Illias RM, Bakar FDA, Murad AMA (2016) Thermotolerance and molecular chaperone function of an SGT1-like protein from the psychrophilic yeast, *Glaciozyma antarctica*. *Cell Stress Chaperon* 21:707–715
- Zeng H, Wang G, Hu X, Wang H, Du L, Zhu Y (2014) Role of microRNAs in plant responses to nutrient stress. *Plant Soil* 374:1005–1021
- Zhang J, Wei J, Li D, Kong X, Rengel Z, Chen L, Yang Y, Cui X, Chen Q (2017) The role of the plasma membrane H⁺-ATPase in plant responses to aluminum toxicity. *Front Plant Sci* 8:1757
- Zhao M, Ding H, Zhu JK, Zhang F, Li WX (2011) Involvement of miR169 in the nitrogen-starvation responses in *Arabidopsis*. *New Phytol* 190:906–915
- Zhao M, Tai H, Sun S, Zhang F, Xu Y, Li WX (2012) Cloning and characterization of maize miRNAs involved in responses to nitrogen deficiency. *PLoS ONE* 7:e29669
- Zhen Y, Qi JL, Wang SS, Su J, Xu GH, Zhang MS, Miao L, Peng XX, Tian DC, Yang YH (2007) Comparative proteome analysis of differentially expressed proteins induced by Al toxicity in soybean. *Physiol Plantarum* 131:542–545
- Zhou D, Yang Y, Zhang J, Jiang F, Craft E, Thannhauser TW, Kochian LV, Liu J (2017) Quantitative iTRAQ proteomics revealed possible roles for antioxidant proteins in sorghum aluminum tolerance. *Front Plant Sci* 7:2043

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.