Transcriptome Analysis of Genes Involved in Aluminum Stress Responses in Peanut (*Arachis Hypogaea* L.)

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Abstract

Background

Aluminum (Al) contamination inhibits plant growth and development, however, mechanisms involved in Al stress tolerance in peanut (*Arachis hypogaea* L.) were rarely studied. The present study was comprised of four Al levels i.e., 0, 1.25, 2.5 and 5 mmol l^{-1} $AlCl_3.18H_2O$ regarded as Al0, Al1, Al2, and Al3. The respective concentrations were added in Hoagland nutrient solution and replaced every three days.

Result

Results revealed that seeding length low Al concentration (Al1) treatment had no noticeable effect on seeding lenght, while higher Al concentration (Al2 and Al3) treatment significantly inhibited seeding lenght. The differentially expressed genes (DEGs) of plant hormone metabolism pathway were significantly enriched whereas the contents of salicylic acid (SA) and abscisic acid (ABA) were up-regulated, and jasmonic acid (JA) were down-regulated to different levels. Moreover, transcription factors (TFs) and *ALMT9* and *FRDL1* genes were up-regulated at higher Al concentration and down-regulated at the lowest Al concentration (Al1).

Conclusions

Overall, Higher Al concentrations up-regulated the expression of transcription factors (TFs), and *ALMT9* and *FRDL1* genes to resist the stress of high Al concentrations whereas transcriptome analysis revealed that Al stress tolerance is closely related to endogenous hormone contents i.e., salicylic acid (SA), abscisic acid (ABA), and jasmonic acid (JA). This study preliminarily analyzed the molecular mechanism of Al tolerance in peanut and provided a theoretical rationale for developing new Al-tolerant peanut cultivars.

Background

Aluminum (Al) is the most abundant metal element, accounting for 7.45% of the total weight of the earth's crust [1]. Usually, Al exists in the earth's crust in a dormant state, however, as environmental pollution intensifies, hydrogen ions in acid rain spontaneously reacts with Al in the soil to form compounds, releasing Al^{3+}, which restricts the plant growth and development at high concentrations [2].

Peanut (*Arachis hypogaea* L.) is one of the world's four major oil crops and an essential economic crop in China [3]. However, the peanut planting areas in Southern China are mainly red and yellow soil having acidic properties. Therefore, the growth and development of peanut were impaired by Al toxicity which could lead to 20% reduction in the average peanut yield [4].

Plants can deal with the higher Al ions by external exclusion mechanism and internal compartmentalization [5]. The roots are the foremost organ that come into contact with the Al ions in growing medium [6], and high-concentration Al toxicity could cause noticeable morphological and structural changes in peanut root tip cells, manifested by inhibited root elongation and programmed cell death [7]. The aging-related *AhSAG* gene cloned in peanut was related to Al-induced cell death [8]. Moreover, Al could produce organic acids in plants, such as malic acid, citric acid, and oxalic acid which could combine with Al^{3+} to form a non-toxic complex and thus prevents Al^{3+} from entering the root tip cells [9]. There were are two significant types of transporter families related to the secretion of organic acids, which were multidrug and toxic compound extrusion (MATE) and aluminum-activated malate transporter (ALMT). The *AhFRDL1* encoding MATE family proteins had been cloned in peanut. The expression of the peanut *AhFRDL1* gene was up-regulated and increased the secretion of citric acid in the root tip, which affect the tolerance ability of the root system to Al stress [10]. In addition to the external exclusion mechanism, isolating or detoxifying the Al entering the plant is the primary mechanism of internal tolerance mechanism. Moreover, the cell wall could adsorb Al^{3+} to improve aluminum resistance in plants [11] whereas the higher pectin content in cell wall induced pectin methylesterase (PME) family genes and enhanced PME activity, thereby preventing Al^{3+} from entering the plant cells under Al stress [12]. In addition, transcription factors (TFs) STOP1, ART1 and WRKY were involved in the mechanism of plant resistance to Al toxicity [13, 14, 15], however, TFs involved in Al stress resistance in peanut were rarely investigated.

Plant hormones also play an important role in Al tolerance, for example, exogenous salicylic acid (SA) treatment could reverse the Al stress-induced pectin accumulation and PMD reduction in *Panax notoginseng* roots, thereby reducing the Al content of root pectin. Moreover, Al stress activated the endogenous SA content and NO signaling pathway of *Panax notoginseng* [16]. Exogenous indole acetic acid (IAA) could reduce the Al concentration of soybean roots and stimulate Al-induced citrate secretion and plasma membrane Hþ-ATPase activity under aluminum stress [17]. The jasmonic acid (JA) receptor COI1 mediated JA signal was involved in the regulation of *ALMT1*-mediated malic acid secretion, thereby expelling Al from the cell [18]. Exogenous abscisic acid (ABA) could enhance al-induced citrate secretion in soybean roots [19]. Overall, negative impacts of Al toxicity on the growth and yield of crop plants [20], however, as per our knowledge, molecular basis of Al stress tolerance in peanut were rarely studied. The present study used transcriptome analysis to explore the changes in the transcriptional regulation mechanism of peanut under Al stress and the effects of plant hormones on this process. The preliminary analysis of the molecular mechanism of peanut tolerance to Al stress provides a theoretical rationale for cultivating Al tolerant peanut cultivars in Al contaminated lands.

Results

Effect of different concentrations of Al on peanut seedling length

Compared with Al0, the seedling lenght of Guihua58 was not significantly affected under Al1 treatment, whilst decreased by 37.66 % and 43.77 % under Al2 and Al3, respectively.

Sequencing statistics

Sequencing data of all samples has been quality evaluated. The original bases of the sequencing data of each sample were between 5777435056 and 7275220472, the GC content of the sequence was between 45.43 % and 46.01 %, the Q20 value was above 97.39 % and the Q30 value was above 92.77 % (Table 1).

Analysis of sample relationship

The clean reads were compared with the reference genome, and the number of effective sequences that could be aligned on the reference genome for each sample sequence was between 36691674 and 45441812, and the contrast ratio was above 94.04 % and 8.46 % of multiple mapped reads on the reference genome, the unique mapped reads on the reference genome reached more than 84.8 % (Table 2). The data showed that the sequencing quality of the transcriptome data was good, the experimental process was pollution-free, and the selected reference genome information could meet the needs of subsequent analysis.

Differentially expressed genes (DEGs)

Through the comparison between samples, the results showed that Al0-vs.-Al1, a total of 633 DEGs were obtained, of which 285 were up-regulated and 348 were down-regulated. Al0-vs.-Al2, 2183 DEGs were obtained, of which 1058 were up-regulated and 1125 were down-regulated. Al0-vs.-Al3, 2294 DEGs were obtained, of which 1447 were up-regulated and 847 were down-regulated. Through the screening and analysis of DEGs between sample groups, it was found that the number of DEGs of Al0-vs.-Al3 was the largest, indicating that with the increase of Al concentration, more genes in peanut leaves participated in expression regulation in response to Al toxicity (Figure 2b). The PCA (Figure 2b) and sample-to-sample clustering analysis (Figure 2c) indicated that the same cultivar of control and Al treatment were clustered together. It showed that the library construction quality and sequencing quality results of the sequencing sample were reliable and could be used for subsequent analysis. The distance between each sample point represented the distance of the sample by PCA analysis. The closer the distance, the higher the similarity between the sample, and the better the repeatability between the samples.

KEGG pathway

The DEGs in the Al0-vs.-Al1 sample group were enriched in 12 pathways, with 123 genes annotated with KEGG. The DEGs in the Al0-vs.-Al2 sample group were enriched into 12 pathways, with 123 genes annotated with KEGG. The DEGs in the Al0-vs.-Al3 sample group were enriched in 12 pathways, with 123 genes annotated with KEGG. The KEGG pathway of DEGs was drawn by bubble chart according to the enrichment factor, and the enrichment results of the top 20 were displayed. The results showed that the significantly enriched KEGG pathways in the Al0-vs.-Al1 sample group included the plant hormone metabolism pathway, sugar metabolism pathway, and amino acid metabolism pathway. The KEGG pathways were significantly enriched in the Al0-vs.-Al2, including the plant hormone metabolism pathway, sugar metabolism pathway, and amino acid metabolism pathway. The KEGG pathways were significantly

enriched in the Al0-vs.-Al3, including the plant hormone metabolism pathway, sugar metabolism pathway, and amino acid metabolism pathway (Figure 3). According to the results of the above three sample groups, the significant difference between low Al concentration and high Al concentration lay in the metabolic pathway of plant hormones.

Effect of different Al concentrations on the SA, ABA, JA and IAA content

The contents of SA, ABA, JA and IAA in peanut leaves were determined under different Al concentrations. Compared with Al0, the SA content increased by 137 %, 368 % and 909 %, with the increase in Al concentration i.e., Al1, Al2, and Al3, respectively (Figure 4a). Compared with Al0, the ABA content was increased by 59 % and 205 % under Al2 and Al3 treatments, respectively, but found statistically similar with Al1 treatment (Figure 4b). Compared with Al0, the JA content decreased with the increase of Al treatment concentration, except for Al2 treatment (Figure 4c). Compared with Al0, the IAA contents were decreased by 23.32 %, 43.4 % and 53.06 %, respectively under Al1, Al2 and Al3 treatments, respectively (Figure 4d).

Effect of different Al concentrations on the expression of *ALMT9* and *FRDL1*

The expression levels of *ALMT9* and *FRDL1* genes were measured in peanut leaves under different Al concentrations treatment. Compared with Al0, the gene expression of *ALMT9* and *FRDL1* were significantly up-regulated under Al2 and Al3 treatments which were up-regulated by 1.3-1.5 times and 1.7-8.7 times, respectively. Compared with Al0, the *ALMT9* gene expression was significantly reduced by 77 % under Al1 treatment (Figure 5a). Compared with Al0, the expression of the *FRDL1* gene was not significantly different under the Al1 treatment (Figure 5b).

Effect of different Al concentrations on the regulation of TFs expression

The key TFs associated with Al treatments in peanut were exhibited in Figure 6. Among them, MYB (4), bHLH (9), NAC (6) and AP2 (2) were differently expressed in different treatments (Figure 6a). Ten genes from the differentially expressed transcription factor genes were randomly selected by qRT-PCR analysis (Figure 6b). The randomly selected gene expression were consistent with the results calculated by the FPKM value obtained by sequencing, indicating that the transcriptome data was reliable.

Discussion

Nowadays, with the development of transcriptome sequencing technology, transcriptome analysis is becoming an efficient and reliable research method to deeply understand plants' gene expression in a specific growth environment. Transcriptome analysis can analyze the biological processes and molecular mechanisms of plants in response to environmental stress. This study found that the plant hormone metabolic pathway was more obvious in KEGG pathway shared by the four sample groups, indicating that the plant hormonal pathway was an essential metabolic pathway in response to Al tolerance in peanut (Figure 3). Previously, Tian and Li [21] reported that Al stress inhibited the root elongation in

watermelon (*Citrullus lanatus*) whereas exogenous SA application improved the root growth in growing watermelon seedlings under Al toxic conditions. In addition, the IAA was also involved in regulating the growth and development of plant roots under Al stress [22]. Wang [23]found that the black soybean roots were inhibited by adding the IAA transport inhibitor TIBA under Al stress, indicating that IAA participated in the resistance to Al stress. Expression of JA receptor COI1 in Arabidopsis root tips was up-regulated under Al stress whereas ethylene had also been involved in regulating JA signal induced by Al in root tips, indicating that JA regulated and resisted Al stress through interaction with ethylene [24]. Al stress increased the accumulation of endogenous ABA in soybean roots and leaves and accelerated the transport of ABA, it showed that ABA might act as an Al stress response signal to regulate the Al resistance of soybeans [25]. Studies in buckwheat (*Fagopyrum Mill*) had found that high Al concentrations could promote Al detoxification by directly activating the ABA-like gene ALS3 or stimulating the increase of ABA levels [26]. This study found that with the increase of Al concentrations, the SA and ABA contents in peanut leaves were significantly up-regulated, and the content of JA was significantly down-regulated, indicating that peanut could resist Al stress by changing the content of SA, ABA and JA. There was no significant difference in IAA content, which may be due to the fact that IAA in leaves did not respond to Al toxicity (Figure 4).

Sasaki [27] isolated *TaALMT1* from wheat with different aluminum tolerance for the first time. The homologous gene *AtALMT1* isolated from *Arabidopsis thaliana* was one of the critical genes that regulated the aluminum tolerance mechanism in Arabidopsis [28]. At present, the *AhFRDL1* gene had been cloned from peanut, which had been confirmed to be a citrate transporter gene. The expression of peanut *AhFRDL1* gene was up-regulated and the secretion of citric acid from the root tip increased under Al stress [10]. The WRKY is a large family of TFs, involved in the transduction of plant defense signaling mechanism [29]. Schluttenhofe [30]found that 13 *AhWRKY* genes may be involved in SA and JA signaling pathways in cultivated peanut. In this study, the expression of *ALMT9*, *FRDL1* and *WRKY* genes were up-regulated under high concentration of Al i.e., Al2 and Al3, indicating that peanut leaves also increased the expression of organic acid secretion transporter family (*ALMT9* and *FRDL1*) and WRKY genes (Figure 5 and Figure S1). In Arabidopsis, the C2H2-type transcription factor STOP1 induced the *AtALMT1* gene to secrete malate to chelate and detoxify aluminum [31, 32, 33]. This was consistent with the results of previous studies. However, the expression of *ALMT9*, *FRDL1* and *WRKY* genes were all down-regulated under low concentration of Al (Al1) (Figure 5 and Figure S1), which may be due to low concentration of Al (Al1) promoted the absorption of other elements by the root system, thereby maintaining the growth of seeding lenght, making it offset part of the impact of Al poisoning has been eliminated [34], however further studies in this regard are still needed.

Conclusion

Al toxicity suppressed the seedling growth and root system of peanut whilst the effects were more severe at higher concentrations. Higher Al concentrations up-regulated the expression of transcription factors (TFs), and *ALMT9* and *FRDL1* genes to resist the stress of high Al concentrations whereas transcriptome analysis revealed that Al stress tolerance is closely related to endogenous hormone contents i.e., salicylic

acid (SA), abscisic acid (ABA), and jasmonic acid (JA), transcription factors (TFs) and the expression of *ALMT9* and *FRDL1* genes.

Materials And Methods

Plant materials and treatment conditions

The hydroponic experiment was carried out in the Guangzhou Key Laboratory for Research and Development of Crop Germplasm Resources, Zhongkai University of Agriculture and Engineering, Guangzhou, China (23104 N, 113281 E). The peanut cultivar 'Guihua58' was provided by Guangxi Academy of Agricultural Sciences, Nanning, China. Homogenous seeds were surface sterilized and germinated in a petri dish with wet filter paper with 20 seeds per petri dish. After three days, the seeds were transferred to a plastic culture bowl and continued to cultivate with Hoagland nutrient solution. When the seedlings grow to one leaf and one heart (one week of growth) at 27°C, the photoperiod was 12h day/12h night. The seeding lenght was measured every 24 hours the following four levels of Al were employed i.e., i.e., 0, 1.25, 2.5 and 5 mmol l^{-1} $AlCl_3.18H_2O$ regarded as Al0, Al1, Al2, and Al3, respectively. The nutrient solution was replaced once every three days.

Raw sequencing data

After RNA extraction, the magnetic beads with Oligo (dT) were used to enrich eukaryotic mRNA. A fragmentation buffer was added to interrupt mRNA randomly. mRNA was used as a template to synthesize the first cDNA strand with six-base random hexamers, then added buffer, dNTPs, RNase H and DNA polymerase I to synthesize the second cDNA strand and purified the cDNA with AMPure XP beads. The purified double-stranded cDNA was repaired, A-tailed and connected to the sequencing adapter, and then AMPure XP beads were used for fragment size selection. Finally, the cDNA library was obtained by PCR enrichment. Qubit2.0 and Agilent 2100 were used to detect the library's concentration and insert size, and the library's effective concentration was accurately quantified using the Q-PCR method to ensure the quality of the library. High-throughput sequencing was performed with NovaSeq 6000, and the sequencing read length was PE150. Raw sequencing data have been uploaded in the NCBI Gene Expression Omnibus under the accession number PRJNA754251 (http://www.ncbi.nlm.nih.gov/geo).

Enrichment Analysis

The raw data analysis was performed using BMKCloud (www.biocloud.net). The differentially expressed genes (DEGs) between the comparison groups were obtained based on certain standardized processing and screening conditions. The default parameters were FDR=0.1 and FC=2. The KEGG database was used for functional annotation, classification statistics and metabolic pathway analysis of DEGs in the comparison group.

Determination of JA, ABA, SA and IAA content

The cartridge was activated with 4 mL of methanol and 2 mL of 0.1 M aqueous ammonia solution. Fresh sample (100 mg) were homogenized with 1 mL of extraction solution (acetonitrile: water=1:1, containing sodium diethyldithiocarbamate)—ice bath for 4 h, 4 °C, 12000 rpm, 10 min. The supernatant was concentrated in vacuo, 0.1 M aqueous ammonia solution was added to the volume to 2 mL, and then passed through the MAX cartridge. The MAX cartridge was washed with 2 mL of 0.1 M ammonia solution and 2 mL of 0.1 M ammonia solution with 60% methanol and finally added 0.2 ml methanol to dissolve it. The chromatographic system used an ultra-high performance liquid system (Vanquish, Thermo, USA), and the mass spectrometry system uses a Q executive high-resolution mass spectrometry detection system (Vanquish, Thermo, USA). A liquid chromatography column was used Waters HSS T3 (50*2.1 mm, 1.8 μm). The sample volume was 2 μL. The column temperature was 40°C.

Real-time quantitative RT-PCR

RNA extraction was performed using RNAprep Pure Plant Kit (TianGen Biotech, Beijing, China). The RNA quality detection was performed using Micro-Spectrophotometer (Allsheng, Nano-300, Hangzhou, China). cDNA synthesis was used PrimeScriptRT reagent Kit with gDNA Eraser kit (TaKaRa, Beijing, China). 2×SYBR Green qPCR Mixture kit (Hlingene Corporation, Shanghai, China), Option Real-Time PCR System (Bio-Rad, CFX96, California, USA) instrument was used for real-time fluorescent quantitative PCR. cDNA was used as a template, three biological replicates were set for each sample, actin gene was used as an internal reference gene, and gene-specific primers were synthesized by Sangon Biotech, Shanghai, China.

Experimental design and statistical analyses

The experimental treatments were arranged in completely randomized design (CRD) in triplicate. Data were compiled using Microsoft Excel 2010 (Microsoft, Chicago, USA). SPSS Statistics 20.0 (IBM, Chicago, USA) was used for one-way analysis of variance, and the Tukey's test at the 5% significance level was used to separate treatment means. The R Programming Language was used for mapping.

Abbreviations

ABA: abscisic acid, Al: Aluminum, *ALMT*: aluminum-activated malate transporter, ART1: Aluminum resistance transcription factor 1, COI1: Coronatine Insensitive 1, DEGs: differentially expressed genes, *FRDL1*: Ferric reductase defective-like 1, JA:j asmonic acid, IAA: indole acetic acid, MATE: Multidrug and toxic compound extrusion, PME: Pectin methylesterases, SA: salicylic acid, SAG: Senescence associated gene, STOP1: C2H2-type zinc finger transcription factor sensitive to proton rhizotoxicity 1, TFs: transcription factors.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Raw sequencing data have been uploaded in the NCBI Gene Expression Omnibus under the accession number PRJNA754251 (http://www.ncbi.nlm.nih.gov/geo).

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

Not applicable.

Authors' contributions

YZ, GB, XW designed the experiment. GB, SL, QZ, JQ performed the experiment, data collection, lab analysis, and data analysis. SL, QZ, JQ contributed in providing chemicals, reagent, analyses, and tools. GB and SL prepare the initial draft. GB and UA finalized the initial draft. All authors read and approved the final manuscript.

Acknowledgements

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Tables

Due to technical limitations, tables 1 only available as a download in the Supplemental Files section.
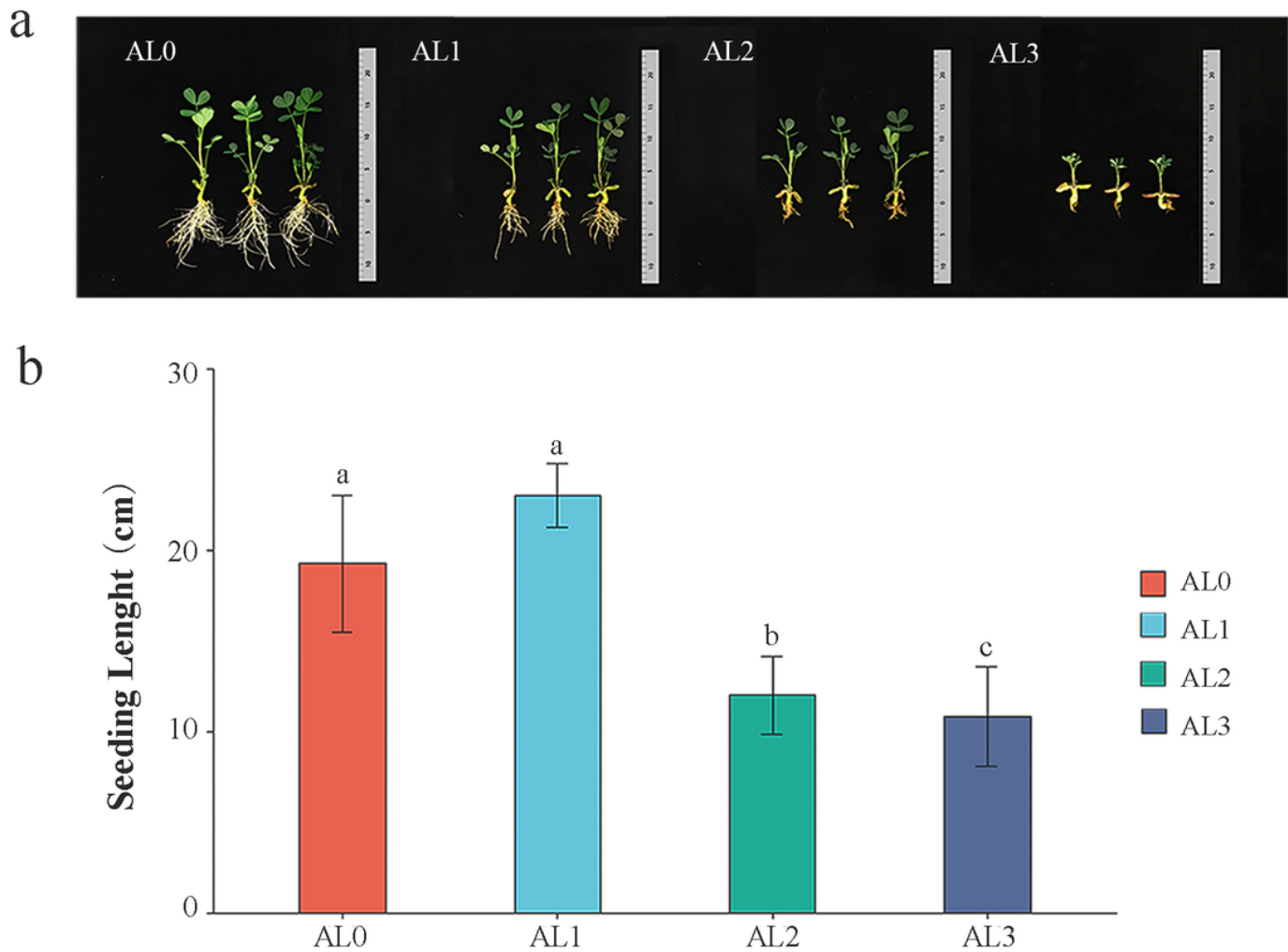
Figures

Figure 1

Seeding lenght was meatured after eleven days aluminum treatment at room temperature. Marking the same letters means P≥0.05 (LSD), there is no significant difference, the difference between different letters means P<0.05 (LSD), and the difference is significant.
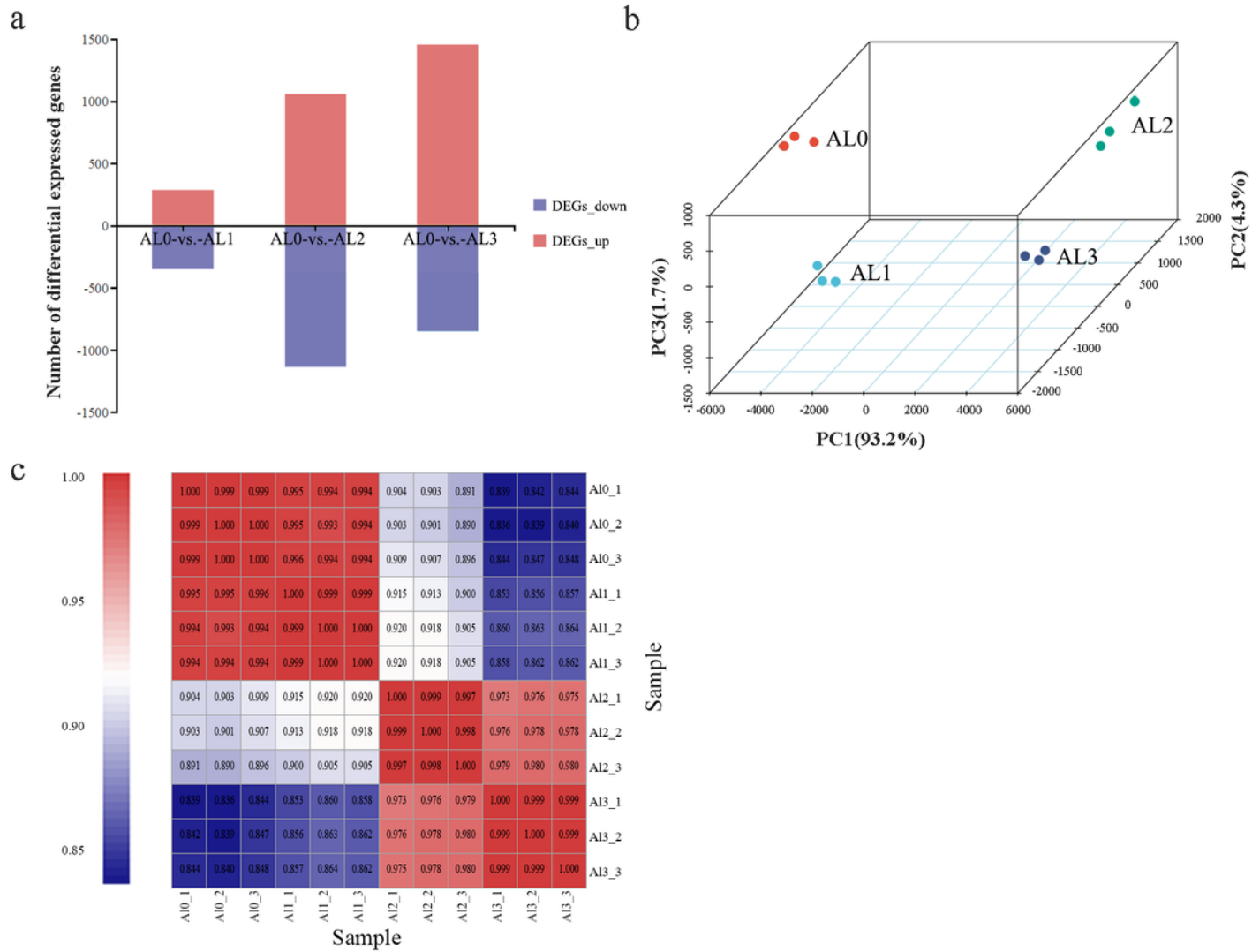
Figure 2

Summary of differentially expressed genes (DEGs) (a) DEGs. (b) Principal component analysis (PCA). (c) Sample-to-sample clustering analysis.
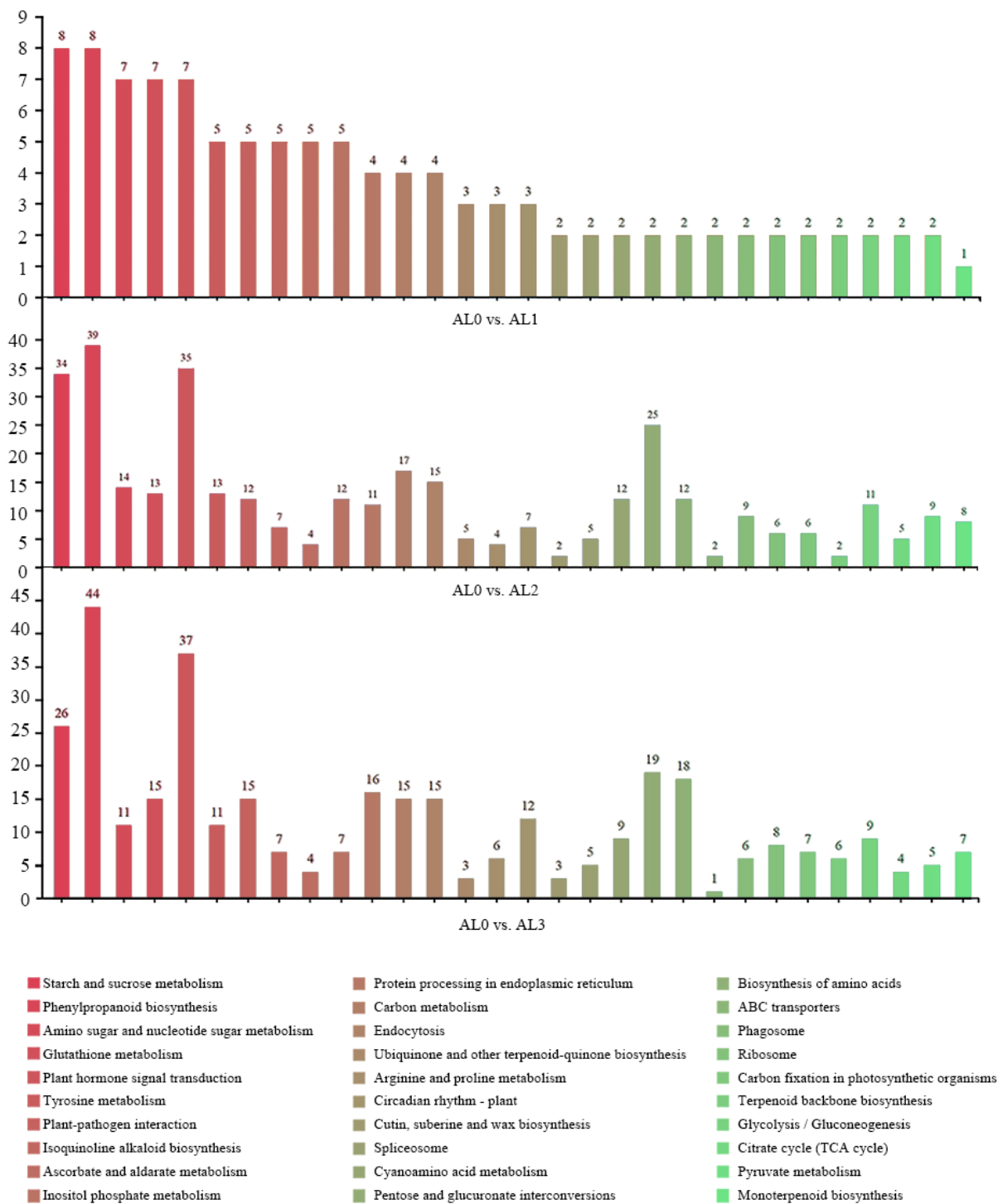
Figure 3
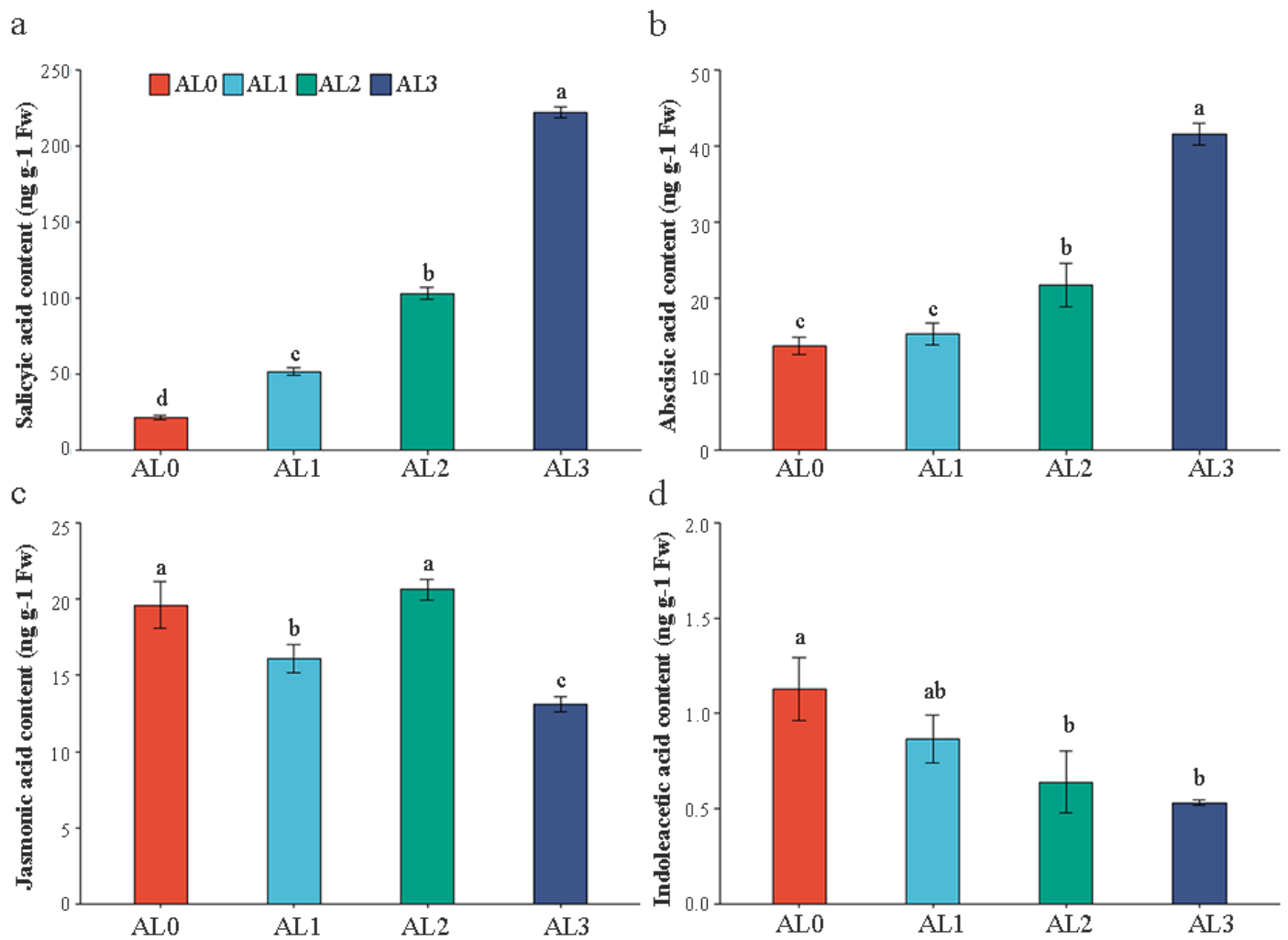
Regulations of the KEGG pathway.

Figure 4

The effect of Al treatments on (a) SA, (b) ABA, (c) IAA and (d) JA. Marking the same letters means P≥0.05 (LSD), there is no significant difference, the difference between different letters means P<0.05 (LSD), and the difference is significant.

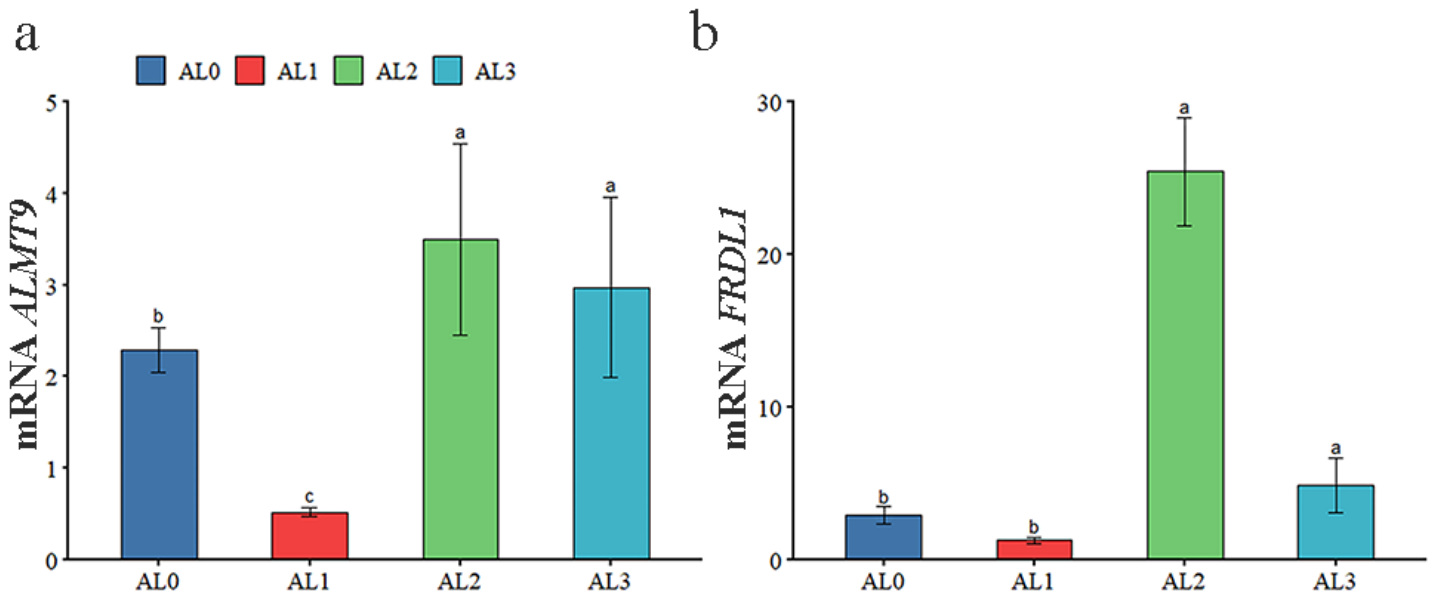**Figure 5**

Analysis of transcript levels of ALMT9 and FRDL1. Marking the same letters means P≥0.05 (LSD), there is no significant difference, the difference between different letters means P<0.05 (LSD), and the difference is significant.
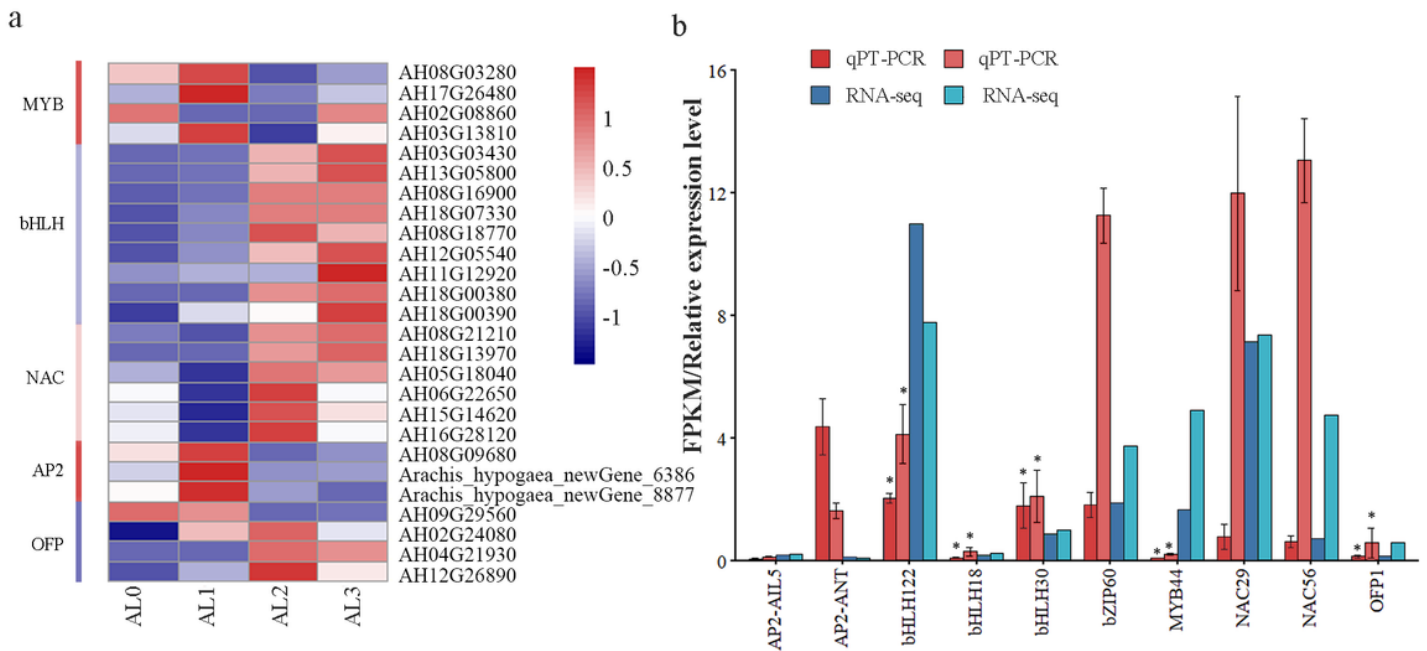

Figure 6

(a) Heatmap of known 26 TFs. Red means down-regulation, blue means up-regulation. (b) Compare the FPKM value obtained by RNA-seq analysis with the gene expression obtained by qRT-PCR analysis.

Supplementary Files

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- FigureS1.tif
- TableS1Primersequencesofgenes.xlsx
- Table1Statisticsofrawsequencingdataresults.xlsx