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Comparative analysis of miRNA expression under leaf removal conditions reveals the role of IAA in adventitious root formation in lotus (*Nelumbo nucifera* Gaertn.) seedlings

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ABSTRACT

MicroRNA regulation of targeted gene expression is involved in plant growth and development. In this study, overwater leaf removal (OLR) significantly increased the number of adventitious roots (ARs), which was inhibited upon underwater leaf removal (ULR). Additionally, indole acetic acid (IAA) content was significantly decreased in OLR and ULR after 2 days, whereas OLR IAA content was greater than the control and ULR, after 4 days. Changes in IAA content was highly correlated with the second leaf development. Therefore, miRNA expression of five libraries was analyzed using RNA-sequencing to monitor molecular regulation mechanisms of AR formation in OLR or ULR treatment. We obtained 1.2–1.4 × 10^7 reads (1.1 × 10^7 clean reads per library). The total number of miRNAs (18–30 nt) was lesser than 5% of small RNAs. Among these (known and novel), 352, 245, 376, and 434 miRNAs were differentially expressed in the A1/C0, A2/A1, B1/C0, and B2/B1 stages, respectively. Expression profiling analysis showed that these miRNAs were differentially expressed ~-4−4-fold. The associated top 20 enriched pathways were involved in plant hormone signal transduction, and starch and sucrose metabolism in A1/B1 libraries. Expression of 13 miRNAs related to AR development was monitored in the A1/B1 library, among which nine miRNAs (7 upregulated and 2 downregulated) had altered transcription levels. Nine miRNAs were selected for further expression profiling from the C0 to the A1 and B1 stages using qRT-PCR. In a total, miRNAs regulate AR formation by differential expression in OLR and ULR treatments.

Keywords

lotus
ARs
gene
leaf removal
miRNA

INTRODUCTION

Lotus is a member of dicotyledons, according to the botanical classification. It originates from India and China. Two species, *Nelumbo nucifera* and *N. lutea*, belong to the lotus family, Nelumbonaceae [1,2,3].The flowers, rhizome, and seeds of *N. nucifera* are used traditionally. Lotus is widely cultivated (more than 300 million acreages) in many provinces of China, such as Hubei (more than 200 million acreages), Jiangsu (more than 100 million acreages), Anhui, Zhejiang, Shandong, Fujian, Jiangxi, and Guangxi for cooking or processed products (e.g., leaf tea, drink, boiled, or salted lotus). Lotus is used as a popular vegetable because of its nutritional value. Additionally, it is an important component in traditional medicine to eliminate phlegm, stanch bleeding, and “clear heat” from the body. Recently, lotus farming provides considerable economic benefits to local farmers, and beautifies the environment.

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Lotus has no principal root; therefore adventitious roots (ARs) become a necessary channel for the plant to obtain water or nutrients from the soil. Additionally, ARs are also an essential aerenchyma to support plant growth and development underwater. ARs are usually located in the seedling hypocotyl and the internode of underground rhizomes of lotus. The number of ARs that form on internodes is greater than on the hypocotyl, indicating that ARs are important for rhizome (a storage organ) development. In traditional breeding of lotus, the growth of the seedlings is notably slow due to a small number of ARs. Therefore, the storage organ cannot form adequately, which delays the breeding process. Hence, elucidating the molecular regulation mechanism of ARs formation could be beneficial for lotus breeding.

AR development occurs in three stages: the inducted stage of primordium, initial formation of ARs, and the emergence of the hypocotyl [4,5]. In the first stage, the development process of normal cells in meristematic tissue changes and tends to differentiate into the primordium, which further develops into the ARs [5]. The primordium continues to develop into ARs (ARs do not emerge from hypocotyl), which is regulated by exogenous environmental conditions [6]. Finally, emergence of AR from the stem or leaf occurs in the next few days. Various factors, including internal or external conditions, widely affect every stage of AR development. The internal factors that affect AR formation include gene expression regulated by transcriptional factors or miRNAs. For the exogenous factors, several conditions, including light, sugar, hormones, mechanical damage, and flooding have a great influence on AR development. Indole-3-acetic acid (IAA) and ethylene participate in AR development. IAA is predominantly involved in primordium induction [7,8], and IAA metabolism (synthesis or transport) determines AR developmental processes [9-13]. Additionally, evidence shows that IAA and ethylene co-regulate the AR formation process. Ethylene is involved in the first stage of AR development, while IAA regulates the second and the third stage [14].

Sucrose is involved in the first stage of root formation (induced stage) and plays a similar role to hormones [15,16]. However, the relationship between sucrose and plant hormones has not been reported. Light signals, including photoperiod, light intensity, and quality, are involved in various metabolic processes during plant growth. Plant metabolic processes depend on light signals, which occur upstream of plant hormones and other photosynthetic products [17-20]. Light signals are involved in root development [21-23].

MicroRNA affects the expression of genes by degrading mature mRNAs, which directly regulates various plant metabolic processes, including AR formation [24,25]. miR393, miR164, miR160, miR1670, miR167, and miR172 regulate root formation by affecting the expression of auxin-responsive genes [26]. The change in miR172 expression profiling significantly promoted the developmental progress of ARs. Further data showed that the expression of MbARF16, MbNAC1, and MbTIR1 is regulated by miR160, miR164, and miR393, respectively, during AR development [27].

The ARs of lotus, which is a secondary organ, play an essential role in absorbing mineral nutrients and water during plant growth. Many environmental factors can affect the developmental process of the lotus plant by regulating AR formation. Recently, we found that AR formation was regulated by multiple genes, although the regulatory molecular mechanism of ARs formation is unclear. Therefore, five libraries, including inducing, initial, and developing stages of ARs, were constructed to monitor miRNA expressional changes in lotus seedlings. Based on the these changes, we aimed to establish a comprehensive regulation network for AR formation.

MATERIALS

Plant growth conditions

"Taikong 36," a species of lotus, was selected for this study. This species was bred by the Guangchang Research Base of Lotus, and has been widely cultivated in some provinces of China. "Taikong 36" was introduced from Guangxi province, and cultivated in the aquatic vegetables field of Yangzhou University, which is located in Yangzhou of Changjiang River, using a regular management method in April. For plant growth conditions, the average temperature was 25–34 °C (day) and 20–25 °C (night). The plants were kept in water for the entire growth season, with 20–40 cm in depth at an early stage and 40–80 cm at the later stage. In October, all the seeds were collected and maintained at room temperature.

Preparation of Paraffin sections

The "Taikong 36" lotus was selected for the preparation of paraffin sections. The seed was punctured and immersed in water for germination. Thereafter, the seedlings were placed in ambient light intensity at 30 °C (day), and 20 °C (night). Seedlings of complete unflattened, initial unflattened, and flattened leaves were chosen to monitor stomatal development. The first leaves to emerge after 1, 3, and 6

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days were selected and cut into 2 × 2 mm section. The leaf sections were transferred into free fatty acid fixing fluid, and vacuum conditions were simulated using a syringe for 10 seconds. Exchanging gas for 10 – 15 minutes was required, and repeated three times at room temperature overnight. Concentrations of 50%, 60%, 70%, 80%, 90%, and 100% ethanol were used to dehydrate samples for 15–20 minutes. The samples were placed into a mixed solution with a 1:1 ratio of pure xylene and absolute ethanol for a 10 minutes, thereafter pure xylene was used for approximately 30 minutes. Paraffin was used to wrap samples for 12–14 hours at room temperature, and immersed in thawed paraffin overnight before preparing paraffin blocks. Wax tape (10 μm) were made using an incisive slicer, transferred onto a glass slide, and treated with a 1:1 ratio of pure xylene and absolute ethanol, followed by absolute ethanol for 10 – 15 minutes. These samples were dried at room temperature, and an light microscope was used to observe the microstructure of the tissues.

RNA-sequencing analysis

The seed coat was punctured and immersed in water for germination. After germination, the seedlings were placed in 5 cm of water, at a temperature of 20–28 °C to continue cultivation. The seedlings were cultivated for 6 days. Next, the first overwater leaf (a leaf above the water level and at least 2 cm from the stem; the removal section was maintained above the water level for continuous growth) and underwater leaf (the leaf from the stem approximately 1 cm below the water level) were removed. The seedlings without any treatment were used as negative controls. The hypocotyls of seedlings growing for approximately 4 days were chosen to construct the CK0 library. Approximately 2 days later, the hypocotyls of the seedlings from the "above waterline" and "under waterline" treatment were used to construct the A1 and B1 libraries. After 4 days of treatment, the A2 (overwater) and B2 (underwater) libraries were also constructed to monitor miRNA expression changes. DNase I was used to purify the total RNA from each hypocotyl in the treatment and control groups after isolation, to eliminate DNA contamination. RNA (2–4 μg) was used to construct each library using sample preparation kits for gene expression (Illumina). The detailed process of library construction was illustrated by Cheng et al. (2018b) (Cheng et al. 2018b). Sequencing of miRNAs was carried out by the Beijing Institute of Genomics (BIG) using a construct.

Screening of differentially expressed miRNAs for *different types of leaf removal*

The differentially expressed miRNAs were chosen, and all the reads from each library were analyzed according to the NOISeq technique, according to [27]. The value of log2 M (fold change) was used as a threshold to assess the relative expression in each library. Based on the absolute difference value (D), a model of noise distribution was built. To judge the differential expression of miRNA among libraries, the average expression in the control (control_avg) and treatment groups (treat_avg) was computed. Next, the value of differential expression change "D" was obtained, according to the data of fold change (MA = log2 (treat_avg)/(control_avg) and the absolute value of difference (DA = |control avg – treat avg|). Finally, miRNA A was considered as differentially expressed miRNA, if MA and DA values, measured via probability, diverged significantly from the noise distribution model. In total, the fold change of expressed gene A was higher than 2 and the divergence probability was higher than 0.8, which was considered to be a differentially expressed miRNA.

Functional analysis of differentially expressed miRNAs

All differentially expressed miRNAs in each library acting as cellular components involved in molecular function and biological process, were annotated using the gene ontology (GO) tool. The function of differentially expressed miRNAs was determined by comparing the sequenced data with the National Center for Biotechnology Information (NCBI) database. The differentially expressed miRNAs involved in the above ontologies were counted when compared to the GO database, and the differentially expressed miRNAs were collected as GO terms using a hypergeometric test. For pathway analysis, the Kyoto Encyclopedia of Genes and Genomes (KEGG) tool was applied, and all differentially expressed miRNAs were grouped into various biological processes.

Analysis of expression using quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The transcriptional level of some miRNAs was identified to monitor changes in biological processes under different types of leaf removal. The treatment of seeds, types of leaf removal, and growth conditions are described above.

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The material was chosen 2 days after leaf removal (A1: overwater; B1: underwater) to document miRNA expression using qRT-PCR, as described by [28,29]. Isolation and purification of plant total RNA was performed as previously described. cDNA synthesis was performed using the First Strand cDNA Synthesis Kit (Fermentas, USA) according to the manufacturer's instructions. The mRNA level was analyzed using the Green Master Mix (Tiangen, China) on the Mx3000P machine (Stratagene) (Xiao et al. 2018) [30] in triplicates. All miRNA sequences used in this experiment were found in the NCBI database. miRNA primers were designed using primer 5.0 software (Online Resource 5). *β-actin* was used as the standard gene, and the primers were: forward primer, 5′ -AACCTCCTCCTCATCGTACT-3′, and reverse primer, 5′ -GACAGCATCAGCCATGTTCA-3′. A 25 μL reaction system was used for miRNA expression, which included 12.5 μL SYBR of Premix Ex Taq II (Tli RNaseH Plus) (2X), 10 μM forward and reverse primers, 2 μL cDNA solution, and 8.5 μL distilled water. The PCR reaction program included 30 seconds at 94 °C, 95 °C for 40 cycles, and 60 seconds at 50–61 °C. The relative expression level was determined using the $2^{-\Delta\Delta Ct}$ method described by [31].

Identification of IAA content

Broken lotus seeds were placed in 10 cm of water for germination at 28 °C for approximately 5 days (water was renewed daily). Germinated seeds were cultivated under normal growth conditions for approximately 6 days, and two types of leaf removal were carried out. After leaf removal, the hypocotyl derived from control, OLR, and ULR plants were collected at days 0, 2, 4, 6, and 8 for each treatment to identify IAA content. The hypocotyls of each sample were quenched with liquid nitrogen and ground into powder (50 mg), which were then placed in a pre-cooled centrifuge tube (2 mL). The extraction reagent (500 μL, v/v/v:isopropyl alcohol:water:concentrated hydrochloric acid = 2:1:0.002) was added, and centrifuged at 4 °C for 30 minutes at 100 rpm. Thereafter, dichloromethane (1 mL) was added, and shaken at 4 °C for 30 minutes. The solution was centrifuged at 4 °C for 5 minutes at 12,000–13,000 rpm. The supernatant (900 mL) was extracted and dried with nitrogen. Methanol (100 mL) was added into the tube to reconstitute the dried powder. After filtering, the solution (50 mL) was used for IAA identification using the C18 column for liquid chromatography.

RESULTS

Developmental process of leaves and ARs

We observed the developmental process of leaves and ARs in lotus seedlings. Leaves and AR development showed a high correlation, according to the occurrence of ARs and leaf unfolding. Lotus had no principle root, and the ARs of seedlings began to break through the hypocotyls approximately 3 days after seed germination. Many ARs emerged from the epidermis of hypocotyls 3-6 days after seedling growth (Figure 1a). We noted that the leaves of seedlings were not unfolded after 3 days of cultivation. Stomata on the overwater leaves appeared after 3 days (Figure 1b). To further observe the development of leaves, particularly aerenchyma development, the microstructure was investigated using light microscopy at days 1, 3, and 5 of seed germination. We observed that aerenchyma was undeveloped at day 1, and stomata were filled with a substance. However, aerenchyma was well developed at 5 days, and complete stomata were formed in one leaf. From growth day 3 to 6, many stomata appeared, suggesting that the aerenchyma was well developed in the leaf (Figure 1c). Hence, we considered that the formation of aerenchyma was related to the development of ARs. Therefore, different types of leaf removal was performed to elucidate the relationship between AR formation and stomatal development.

Figure 1: Growth and development ARs and leaf of lotus seedlings. a. development of ARs in the hypocotyls of lotus seedlings at 0 d, 3 d, 6 d and 9 d; b. the developmental process of leaf derived from lotus seedlings; c. The microstructure analysis of leaf vent at 0 d, 5 d and 10 d of lotus seedlings.

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Effect of different types of leaf removal on AR formation

To identify the effect of leaf removal on AR formation, OLR and ULR lines were performed under normal growth conditions. We found that the rate of AR formation on the hypocotyls was different for OLR and ULR. Development of AR was promoted when the leaf was cut from the overwater stem line. ARs occurred approximately 2–3 days earlier than in the control plant. The seedlings from ULR could not form ARs before 9 days of cultivation. Notably, we also observed that the growth of the second leaf in the OLR group was faster than that of control plants, and there was no significant change in leaf length in the ULR group (Table 1). According to the above results, we found that the exchange of oxygen is crucial for AR formation in lotus seedlings.

Identification of small RNAs in lotus seedlings with different types of leaf removal

Small RNAs regulate various biological processes by controlling gene expression. Therefore, five libraries including CK0, A1, B1, A2, and B2 stages were constructed to identify changes in metabolism through alteration of miRNA expression. After sequencing, approximately 1.2-1.4. $\times 10^7$ reads were obtained in each library. Among these, 11,035,798 (90.75%), 10,778,107 (81.07%), 11,143,980 (89.73%), 13,044,578 (93.39%), and 11,488,085 (94.6%) clean reads were found in CK0, A1, B1, A2, and B2 stages, respectively, after the low quantity reads (including adapter null, insert null, adapter null, smaller than 18 nt, and PolyA) were removed (Table 2). Small RNA analysis showed that some uniq-sRNAs (65.69% and 26.86%) and total-sRNAs (17.55% and 8.71%) were specifically expressed in the A1 and B1 libraries, respectively (Online Resource 1). Among them, less than 10% of miRNAs were identified in these libraries, and the majority of small RNAs were identified as un-annotated(Figure 2a). Additionally, most sRNA lengths obtained in each library were between 18~30 nt, (24 nt being the mode) (Figure 2b). We observed that “U” and “G” were the main nucleotides among the 23 cleavage sites in the CK0, A1, B1, A2, and B2 libraries (Figure 2c).

Analysis of differentially expressed miRNAs in these five libraries

The change in gene expression can reflect an alteration of metabolism in certain environments. In this study, some miRNAs were found to change the transcriptional level under different types of leaf removal conditions (Online Resource 4). It was shown that 346 miRNAs, including 322 upregulated (94 known, 228 novel) and 54 downregulated miRNAs (14 known, 40 novel) were altered from C0 to B1. From the C0 to the A1 stage, a total of 352 miRNAs changed expression, among which 265, including 122 known and 143 novel miRNAs, were upregulated, and 137 miRNAs (26 known and 101 novel) were downregulated

Table 1: The effect of different types of Leaf removal on adventitious root rates and the growth of second leaf length.

	1 d	3 d	5 d	7 d	9 d	11 d
CK (%)	0 b	0 b	21 b	38 b	53 b	75 b
Second leaf length (cm)	0.58±0.032a	0.61±0.019b	0.75±0.029b	0.95±0.086b	4.15±0.027b	5.46±0.059b
Underwater (%)	0 b	0 b	0 c	0 c	22 c	45 c
Second leaf length (cm)	0.62±0.042a	0.65±0.022b	0.68±0.014b	0.71±0.029c	1.29±0.018c	2.15±0.035c
Overwater (%)	26 a	67 a	93 a	100 a	100 a	100 a
Second leaf length (cm)	0.59±0.052a	1.05±0.018a	2.98±0.031a	5.68±0.085a	8.75±0.047a	12.87±0.15a

Table 2: The detail information of reads sequenced by RNA-seq technique.

Catalogue	C0 stage	B1 stage	A1 stage	B2 stage	A2 stage
Total reads	12338713	13494300	12576760	14223100	12308050
High quality	12161210 (100%)	13295260(100%)	12419282(100%)	13968299 (100%)	12143844(100%)
Clean_reads	11035798 (90.75%)	10778107 (81.07%)	11143980(89.73%)	13044578(93.39%)	11488085(94.6%)
3′adaptor null	296404 (2.44%)	184643 (1.39%)	245490(1.98%)	192126(1.38%)	174361(1.44%)
Insert null	31859 (0.26%)	97322 (0.73%)	80385(0.65%)	59622 (0.43%)	26116(0.22%)
5'adapter null	8701 (0.07%)	25283 (0.19%)	27675(0.22%)	17992 (0.13%)	11420 (0.09%)
Smaller than 18nt	788436 (6.48%)	2209877(16.62%)	921277(7.42%)	653732(4.68%)	443760(3.65%)
PolyA	12	28	475	28	102

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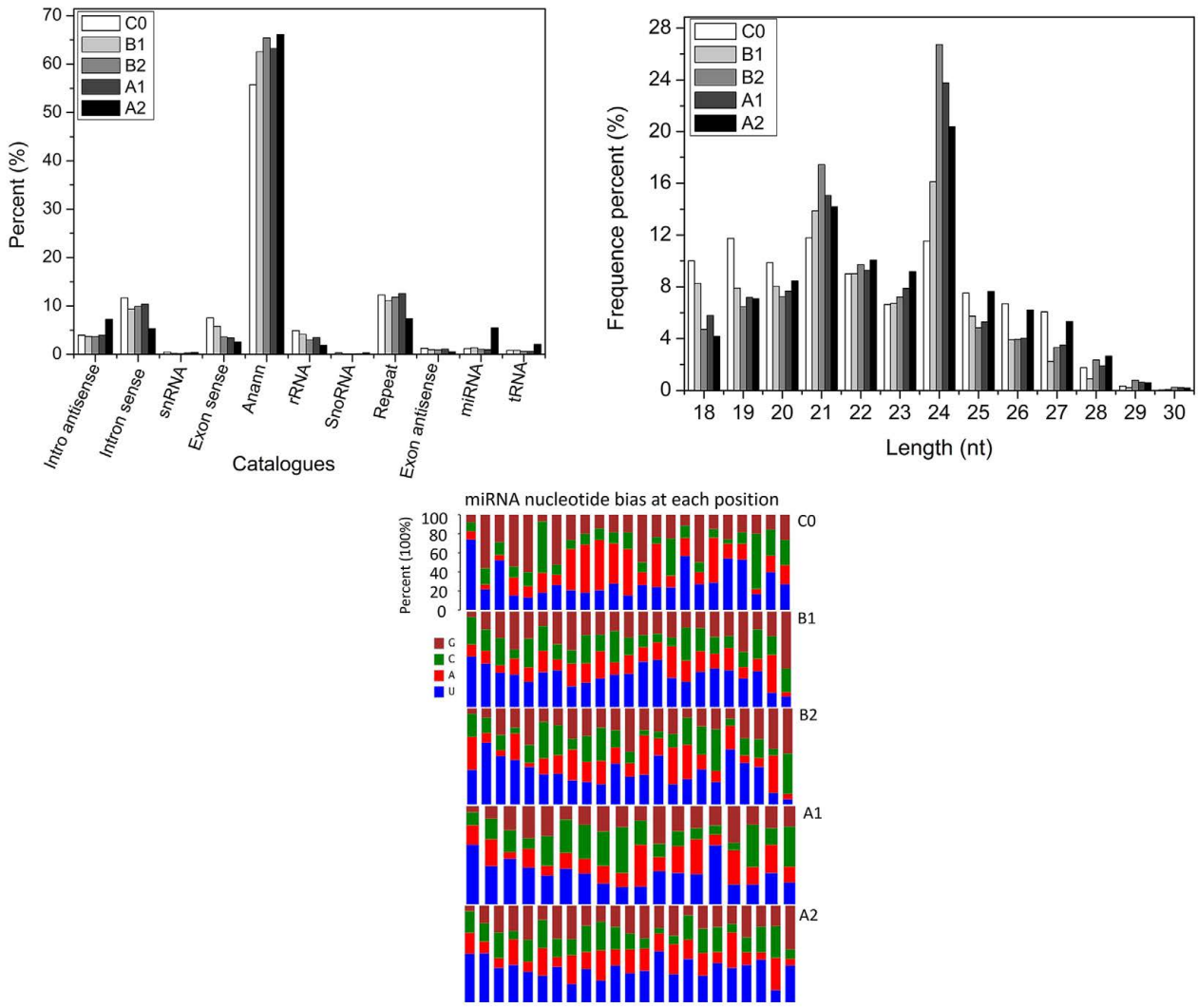

Figure 2: Analysis of miRNAs sequenced by RNA-seq technique in five libraries. a. The categories of these small RNA in C0, B1, B2, A1 and A2 libraries. b. Tags length sequenced by RNA-seq technique in C0, B1, B2, A1 and A2 libraries. c. miRNA Nucleotide bias at each position in C0, B1, B2, A1 and A2 libraries.

Table 3: The number of differentially expressed miRNAs in B1/C0, B2/B1, A1/C0 and A2/A1 libraries.

Libraries	Categuries	Expression profiling	Number	Total Number
C0 Vs B1	Known	Up-regulated	94	108
		Down-regulated	14	
	Novel	Up-regulated	228	268
		Down-regulated	40	
B1Vs B2	Known	Up-regulated	116	175
		Down-regulated	59	
	Novel	Up-regulated	129	239
		Down-regulated	110	
C0 VsA1	Known	Up-regulated	122	148
		Down-regulated	26	
	Novel	Up-regulated	143	204
		Down-regulated	101	
A1 VsA2	Known	Up-regulated	32	71
		Down-regulated	71	
	Novel	Up-regulated	80	174
		Down-regulated	94	

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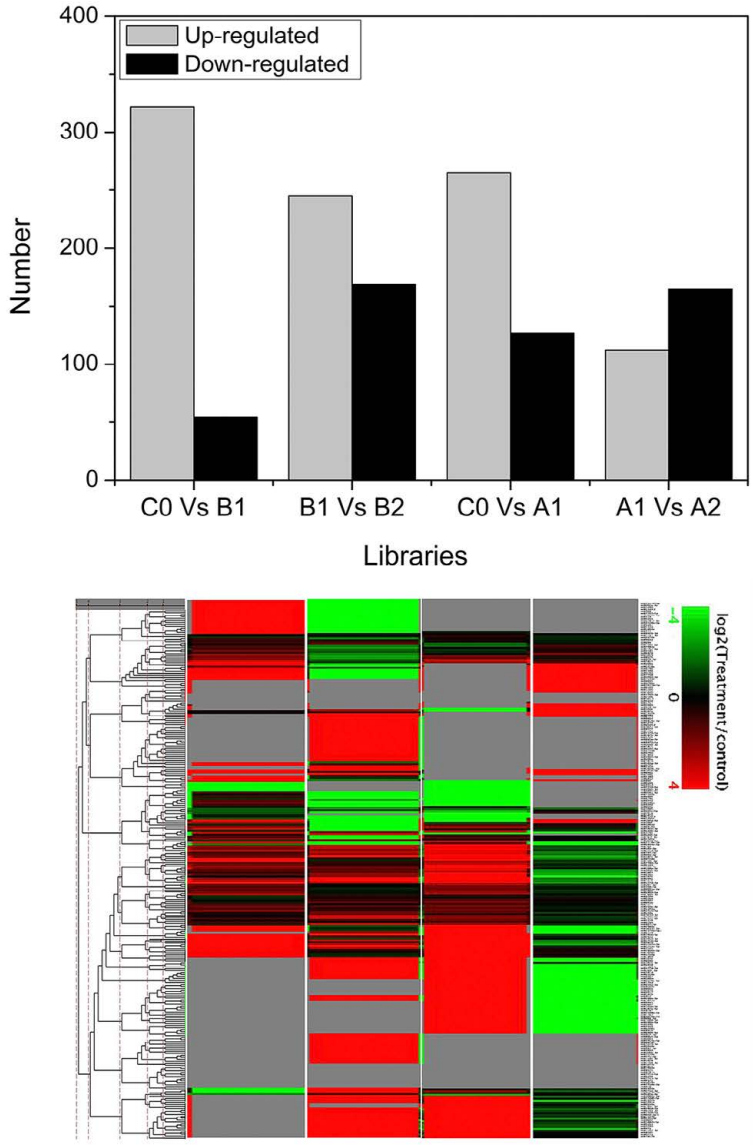

Figure 3: Expression analysis of miRNAs in C0, B1, B2, A1 and A2 libraries. a. screen of differentially expressed miRNAs in B1/C0 libraries, B2/B1 libraries, A1/C0 libraries and A2/A1 libraries. b. Fold change of miRNA expression n B1/C0 libraries, B2/B1 libraries, A1/C0 libraries and A2/A1 libraries.

at the transcriptional level. Many miRNAs were also found to change expression in B2/B1 and A2/A1 libraries, suggesting that miRNAs are involved in the entire process of AR formation in lotus seedlings (Figure 3a, Table 3). Simultaneously, we observed that most miRNAs changed expression between -4–4-fold in the A1/C0, B1/C0, A2/A1, and B2/B1 libraries (Figure 3b).

Functional analysis of the differentially expressed miRNAs

KEGG and GO tools were used to analyze differentially expressed miRNAs in five libraries during AR formation. Significant differences in metabolism were found with different types of leaf removal, particularly in the A1/C0 and B1/C0 libraries (A/B libraries). Therefore, the top 20 statistics of pathway enrichment were analyzed in A1/B1 libraries. The most common differentially expressed known miRNAs were involved in plant hormone signal transduction, followed by necrosis factor kappa beta and toll-like receptor signaling pathways (Figure 4a). We observed that the largest number of differentially expressed novel miRNAs participated in starch and sucrose metabolism, followed by porphyrin and chlorophyll II metabolism (Figure 4b). According to KEGG analysis, three different

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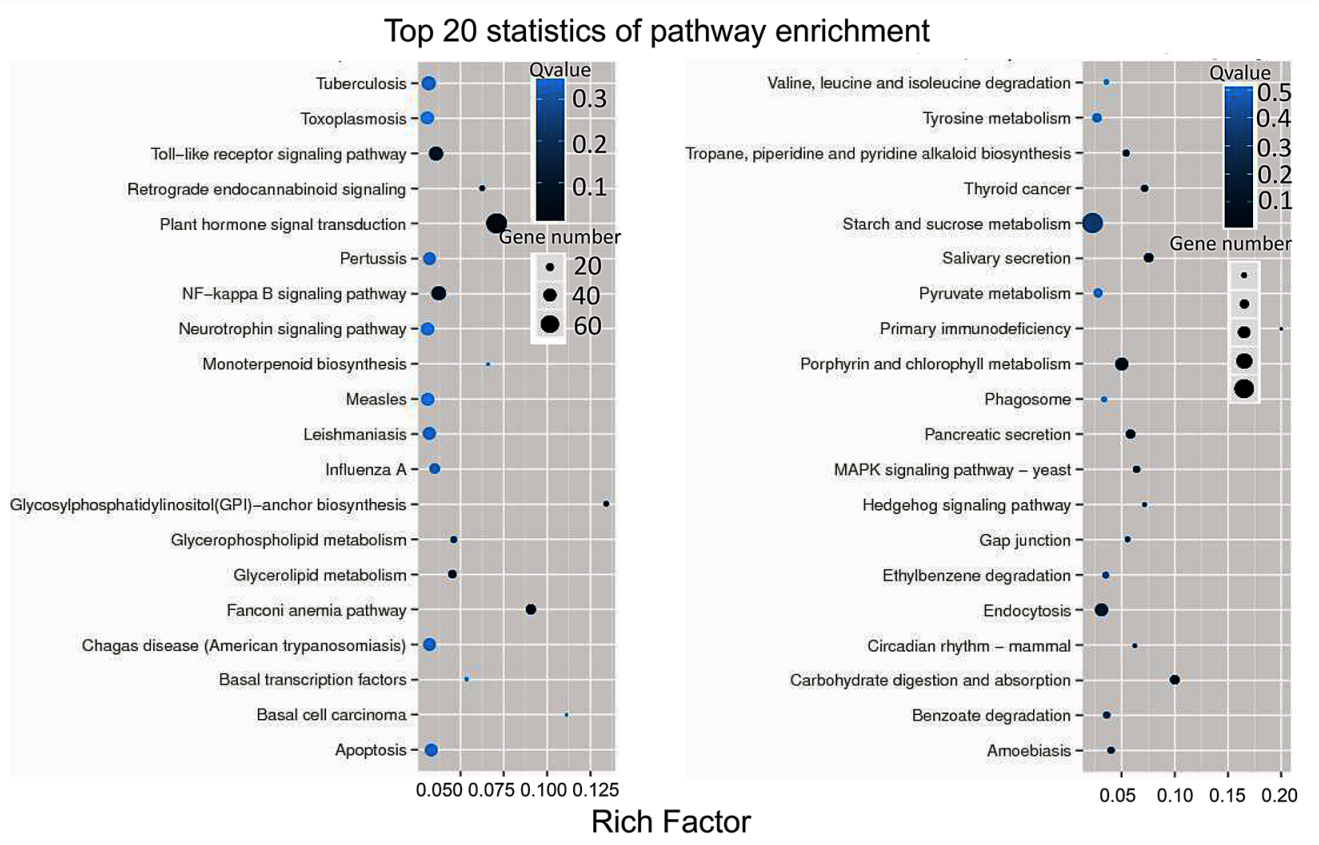

Figure 4: Differentially expressed miRNAs was annotated by KEGG tool in A1/B1 libraries. a. Top 20 statistics of pathway enrichment of known miRNAs in A1/B1 libraries. b. Top 20 statistics of pathway enrichment of novel miRNAs in A1/B1 libraries.

functions were found for differentially expressed known and novel miRNAs when comparing A1/C0 with B1/C0 libraries (A1/B1 libraries). The most common known and novel miRNAs were involved in the metabolism process, cellular part, and catalytic activity for biological processes, cellular components, and molecular functions, respectively (Online Resources 2 and 3). Hence, miRNAs associated with plant hormone metabolism were further analyzed in detail. We found that 28 miRNAs (18 known and 10 novel) participated in auxin, gibberellin, abscisic acid, brassinosteroid (BR), and ethylene metabolism or signal transduction. Among these, 14 miRNAs were upregulated (12 known, 2 novel) and 14 miRNAs (7 known and 7 novel) were downregulated. Further analysis showed that miRNAs involved in auxin metabolism showed the most change in expression, suggesting that auxin is crucial for AR formation in lotus seedlings (Table 4).

miRNAs relevant to AR formation

We compared the miRNAs with known relation to AR formation in A1/B1 libraries. It was seen that a total of 13 miRNAs (6 known miRNAs: miRNA171d-5p, miRNA395a, miR396a-3p, miR396b-5p, miR160a, and miR162-3p; 7 novel mir: novel mir407, novel mir141, novel mir24, novel mir340, novel mir499, novel mir611, and novel mir133) were found in A1/C0 and B1/C0 libraries. Among these, miR396a-3p, novel mir407, novel mir340, and miR162-3p did not alter the transcriptional level in A1/B1 libraries. Expression levels of mRNA171d-5p, miR396b-5p, novel mir141, novel mir24, miR160a, miR162-3p, and novel mir133 were significantly increased, and miRNA395a and novel mir499 were markedly decreased in the A1/B1 libraries. miR396b-5p and novel mir499 were the most differentially expressed miRNAs (Figure 5a). Additionally, the secondary structure of some important miRNAs (the most changed miRNAs, mRNAs involved in AR formation, or miRNAs related to key signal transduction) were also analyzed (Figure 5b). Simultaneously, miR162-3P, miR160f-5p, miR952b, miR1683, 171b-3p, miR168a, miR8558b, miR482d, and miR397a were selected to monitor expression using qRT-PCR. We found that the expression tendency derived from qRT-PCR was similar to that in the RNAs-seq technique (Figure 6).

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Table 4: miRNAs involved in plant hormone signal transduction in A1/B1 libraries.

microRNAs involved in plant hormone signal transduction				
Gene ID	Log_2(A1/B1 ibraries)	P-Value	Function annotation	Mature sequence
miR5568c-5p	18.52	0	Glucuronokinase	TCGTCCGAATTGTAGTCG
miR8181	18.15	0	Two-component response regulator ARR-B family	TGGTGGTGGCGGGTGACG
miR8181	18.15	0	Protein brassinosteroid insensitive 2	TGGTGGTGGCGGGTGACG
miR7817b	14.78	0	ATP-dependent RNA helicase DDX47/RRP3	TCTTTTCTGTTTAACGGT
miR160a-5p	14.40	0	Auxin response factor	TGCCTGGCTCCCTGAATGCT
miR6466-5p	13.99	0	Serine/threonine-protein kinase CTR1	GTGGTAGAGCATTTGACTGA
novel_mir_46	7.16	0.00002	Ethylene-insensitive protein 3	TTTGGTCTTTTCTTGTTTGGT
miR171b-3p	2.96	6.31E-77	DELLA protein	TTGAGCCGTGCCAATATCACG
miR393b-5p	2.32	1.09E-77	Transport inhibitor response 1	TCCAAAGGGATCGCATTGATT
novel_mir_208	2.06	1.77E-07	BR-signaling kinase	TGTGGCACTCTTGTAATTTTTG
miR162-3p	1.91	0	SAUR family protein	TCGATAAACCTCTGCATCCAG
miR390a-5p	1.63	0.00000003	Interleukin-1 receptor-associated kinase 1	AAGCTCAGGAGGGATAGCGCC
miR171d-5p	1.33	1.39E-208	Auxin responsive GH3 gene family	TATTGGTCCGGTTCAATTAG
miR157a-5p	1.19	0	Protein brassinosteroid insensitive 1	TTGACAGAAGATAGAGAGC
novel_mir_551	-6.67	0.00003	Transcription factor MYC2	TGGGCTTGCCTCGAGGGACGTG
novel_mir_948	-6.91	0	Transcription factor MYC2	TTCCCAGTCTGAACGTGCCTC
novel_mir_798	-7.30	0	DELLA protein	TTGGAGAGGGAGAGAGTGAGC
novel_mir_970	-7.60	3.29E-07	Arabidopsis histidine kinase	TCTCGAACTGGATTGGATGCC
novel_mir_787	-7.79	3.91E-08	Two-component response regulator ARR-B family	TGGGCTTGCCTCGAGGGACGT
novel_mir_1025	-7.91	0	Interleukin-1 receptor-associated kinase 1	TGGGCTTGCCTCGAGGGACGTG
novel_mir_475	-9.07	0	Auxin-responsive protein IAA	CAATCTCAGATCTGTAAATTC
miR6196	-9.90	1.71E-32	DELLA protein	AGGAGAGGAGTGGAGAGGAGC
novel_mir_743	-10.93	1.91E-45	Gibberellin receptor GID1	TGTTAAGTCGTCGCCGGGTGG
miR160f-5p	-11.54	1.04E-99	Auxin response factor	TGCCTGGCTCCCTGTATACC
miR1149	-12.91	0	ABA responsive element binding factor	TGTTGTCTGGCTCGAGGTC
miR9748	-14.16	0	Brassinosteroid resistant	GAGGAAGGTGGGGATGAC
miR9748	-14.16	0	Protein brassinosteroid insensitive 1	GAGGAAGGTGGGGATGAC
miR6195	-16.3	0	Ethylene-responsive transcription factor 1	AATAGTAGGATGGCACGTGAG

miRNAs related to IAA, BR, and ethylene metabolism

Plant hormone metabolism and signal transduction pathways play critical roles in the formation of ARs in lotus seedlings. In this study, we found that a large number of miRNAs involved in IAA, ETH, and BR signaling pathways were altered at the transcriptional level. A total of four miRNAs, including miR393b-5p, miR160a-5p, miR9748, and miR171d-5p, were involved in IAA metabolism. Four genes, *TIR1*, *ARF*, *GH3,* and *SAUR* were regulated by miR393b-5p, miR160a-5p, miR9748, and miR171d-5p, respectively. Concurrently, it was found that five miRNAs regulated *BRI1* (miR157A-5P, miR9748, and miR390A-5P) and *BIN2* (miR8181), and *BZR1/2* (miR9748), which are related to BR metabolism or signal transduction pathways, and changed their transcriptional levels with different types of leaf removal during AR formation. Aside from two important hormones, we also found that two miRNAs (CTR1: miR6466-5p and ERF1/2: miR6195) relevant to ethylene metabolism, changed expression (Figure 7).

Identification of IAA content with different types of leaf removal

The seedlings, approximately 8–10 cm in length, were cut at the first overwater and underwater leaves, and the IAA content was analyzed at days 0, 2, 4, 6, and 8 of treatment. No significant difference in IAA content was found at 0 days. The IAA content in the control plant was higher than that of the experimental plant at day 2, and there was no difference between OLR and ULR groups. After 4 days of treatment, we found that IAA content in plants with OLR was notably higher than that in control and ULR groups. ULR plants maintained the lowest level of IAA content for the duration of the investigation, suggesting that poor ventilation influenced IAA content, which directly affected the development of ARs in lotus seedlings (Figure 8).

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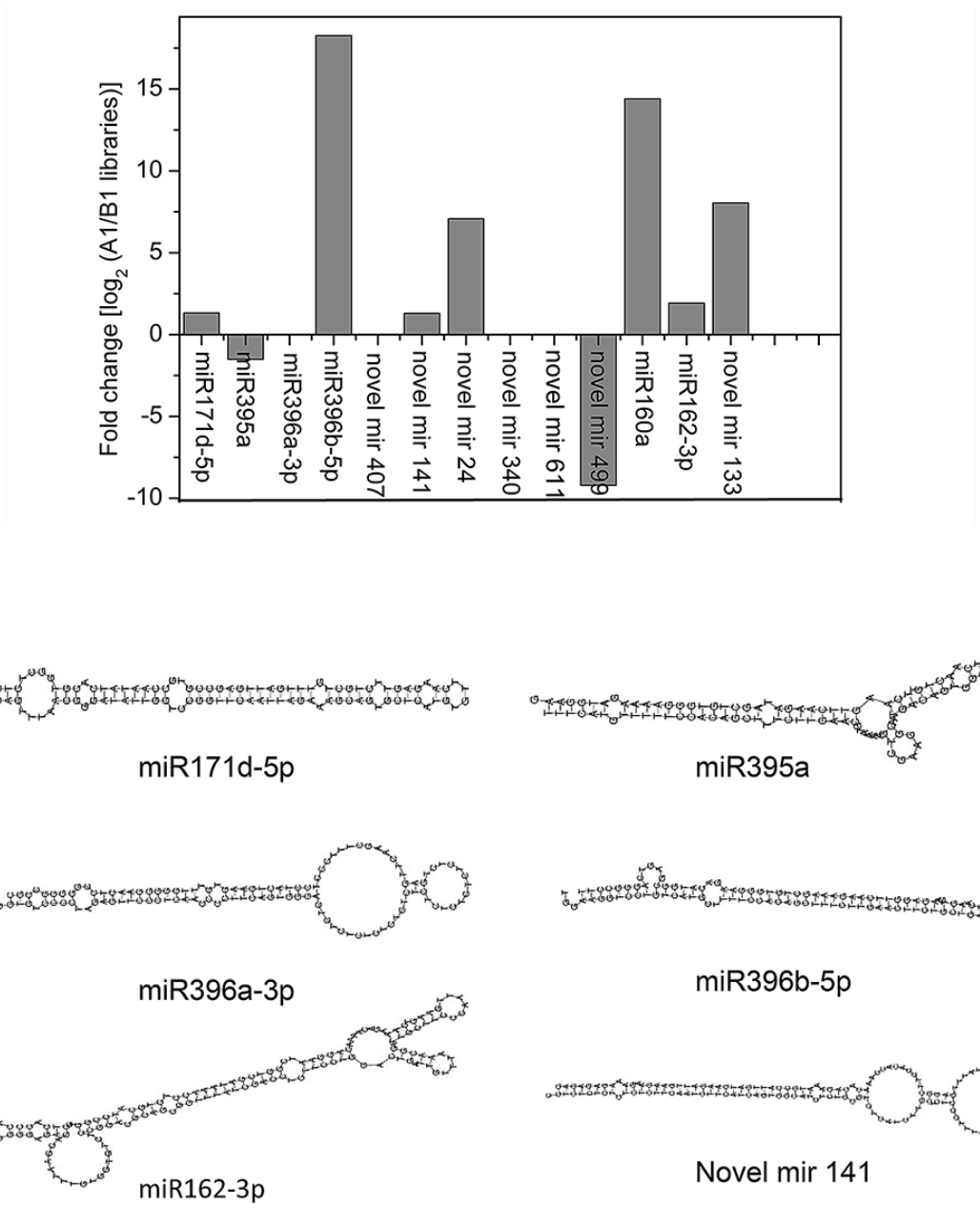

Figure 5: miRNAs related with ARs development in A1/B1 libraries. a. the transcriptional level of known or novel miRNAs involved in ARs formation. b. The secondary structure of known or novel miRNAs related with ARs development.

DISCUSSION

The effect of ARs formation in plants

Vegetative propagation through the regulation of AR development is popular in horticultural crops [32]. ARs are required for plant survival under various conditions, particularly submergence, and plants attempt to maintain normal growth by forming a large number of ARs [33-36]. Therefore, oxygen plays a critical role during plant growth and development. AR development is regulated by multiple factors, including plant hormones, light intensity, cutting, nutrition, and temperature, which have a profound influence on AR formation [37-39]. In plant propagation, leaf removal is a common method for producing ARs within a short time. In lotus plants, we found that OLR and ULR have different effects on AR formation (Table 1), which might be derived from oxygen supply. This was further confirmed by the microstructure of the leaf, whereby the process of

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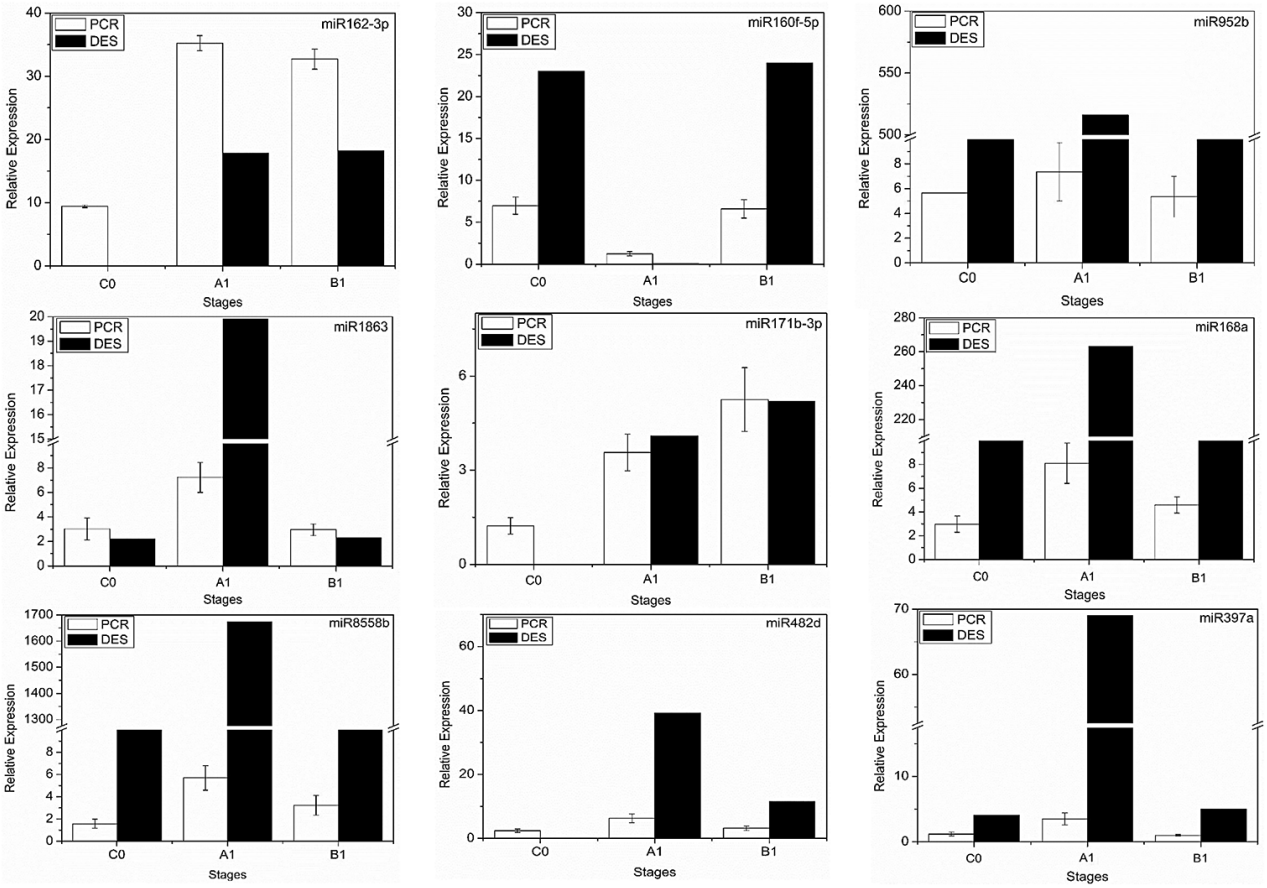

Figure 6: Expression analysis of nine miRNAs in C0 stage, B1 stage and A1 stage using qRT-PCR method and RNA-seq technique.

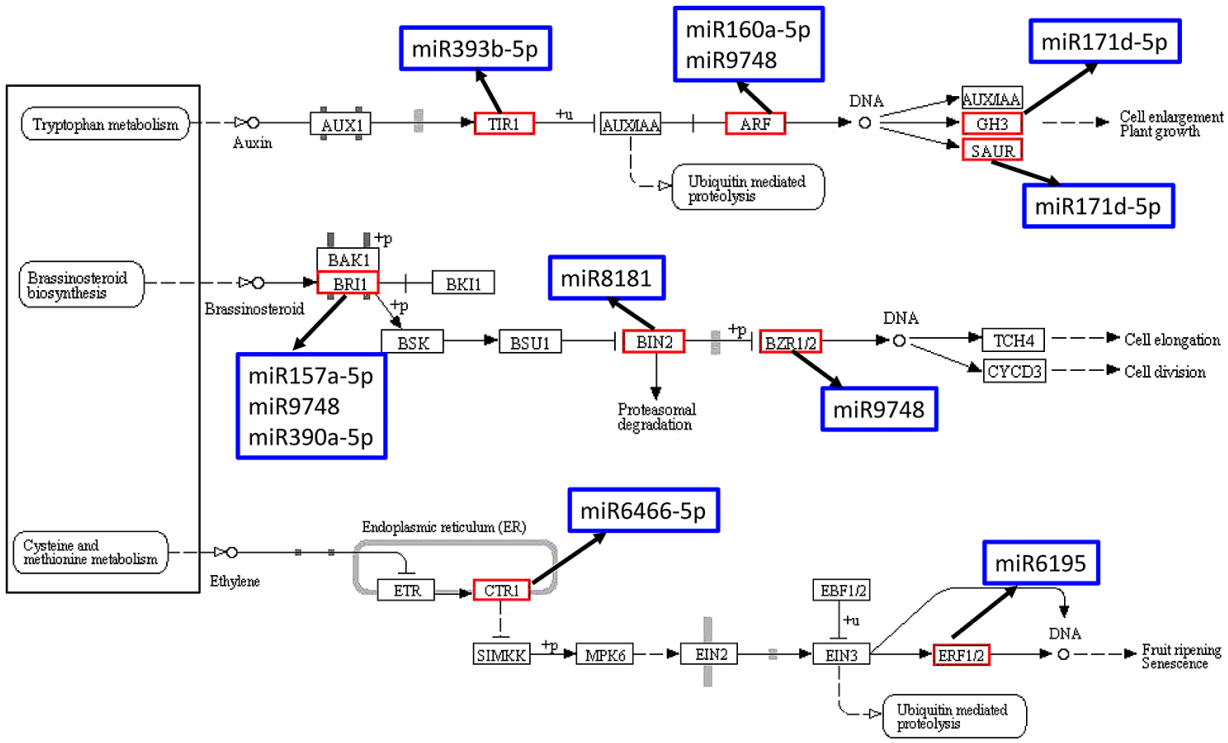

Figure 7: All the important miRNAs involved in IAA, brassinosteroid and ethylene signal transduction pathway. All the up-regulated miRNAs was marked by red box, and the miRNAs in involved in each pathway was listed in green box.

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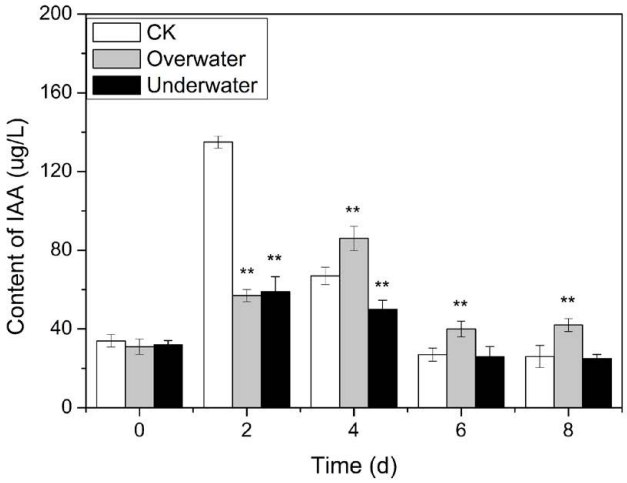

Figure 8: Identification of IAA content at 0 d, 2 d, 4 d, 6 d and 8 d in the hypocotyls of seedlings treated with different leaf removals (leaf removal under waterline, leaf removal over waterline).

aerenchyma formation was highly correlated with AR development (Figure 1). Additionally, we also found that the second leaf treated with OLR had a higher growth rate than control plants and ULR(Table 1), which might lead to a higher IAA content or other substances important for plant growth.

Identification of miRNAs in plants

ARs play an important role in plant growth, particularly where the principal root is not well-developed. In lotus plants, three obvious developmental processes: induction, initial primordium formation, and expressed stages, were found in seedlings [40]. In this study, miRNAs of OLR and ULR were identified using Solexa technology, which has been widely applied in plants [41]. We obtained 1.0–1.3 × 10^7 clean reads in five libraries, including CK0, A1, B1, A2, and B2 libraries (Table 2). Among these, we identified less than 10% of miRNAs (Figure 2a), including 346, 352, 414, and 245 miRNAs in B1/C0, A1/C0, B2/B1, and A2/A1 libraries, respectively (Figure 3, Table 3). In previous years, many plant metabolic processes were elucidated based on changes in genes or protein expression [42], although RNA-seq techniques are required to explore key genes or proteins.

IAA, BR, *and* ethylene metabolism or responding during AR formation

The incidence of ARs plays a critical role in plant growth and metabolism, yield, fruit quality, and stress adaptation, particularly in plants with underdeveloped or no principal roots. The development of ARs is strictly regulated by plant hormones and other factors [43]. IAA is an essential plant hormone involved in various biological processes such as plant growth [44,45]. This study found that IAA plays a critical role in every stage of root formation. Moreover, IAA regulates the functional conversion between xylogenic metabolism and AR formation [46]. IAA synthesis and transport is involved in the development of the primary root by inducing accumulation of functional cells related to AR formation [47,48]. In this study, we found that miR171d-5p, which is responsible for IAA synthesis, was increased in the A1/C0 and B1/C0 libraries, suggesting that the accumulation of endogenous IAA may be different between control and experimental plants. This was further confirmed by the notable decrease in IAA content 2 days after treatment, which indicated that the removal of the first leaf affected the synthesis of IAA, which is regulated by miR171d-5p (Figure 4, Online Resource 4). However, in the A1/B1 stages, we found that miR171d-5p expression was enhanced, and IAA content was not significantly different between the ULR and OLR groups (Table 4). This result implies that another synthetic pathway in plants.. IAA mediates many plant biological processes, including AR formation, by regulating the expression of auxin-responsive genes. Evidence suggests that *miR160* can negatively regulate *ARF17*, and *miR167* positively regulates *ARF6* and *ARF8* to affect AR formation [49]. Additionally, the transport of IAA is important for AR formation [50]. In this study, it was shown that miR160a-5p, novel_mir_475, and miR160f-5p were differentially expressed in the OLR and ULR groups during AR formation (Table 4), suggesting that different forms of ARs might be derived from the expression of these miRNAs. Concurrently, another miRNA (miR162-3p) responsive to SAUR proteins (IAA transport carries) was also involved in AR development in A1/B1 libraries (Figure 5). This phenomenon was noted by Kong et al. (2014), whereby the change in SAUR1 expression regulated by miR10515 significantly enhanced IAA content, which directly promotes AR [51]. Therefore, it was concluded that any factor that affected IAA synthesis, transport, or signal transduction could change the biological process of AR formation.

We also found that some miRNAs involved in ethylene and BR pathways were also differentially expressed (Table 4, Figure 7), which indicated that ethylene participated in the formation of ARs in lotus seedlings. Enhancement of endogenous ethylene can significantly promote AR formation [52]. Further analysis showed ethylene regulation in the induction and growth stages of AR [53,54]. Therefore,

exogenous treatment of 1-aminocyclopropane-1-carboxylic acid (ACC; an ethylene synthesis precursor) improves the number of ARs in tomato by affecting the accumulation of endogenous [55]. BRs are involved in AR formation by regulating cell differentiation [56]. Plant transforming with *OsCKI1*, a gene involved in BR metabolism, has adverse effects on AR development [57]. Further analysis showed that the interaction between BRs and auxin regulate AR formation [58]. It was elucidated that several miRNAs, including miR157a-5p, miR390a-5p, miR8181, and miR9748 showed different expression in A1/B1 stages, suggesting that BRs exert a negative role on AR formation in lotus seedlings (Table 4, Figure 7).

CONCLUSION

To monitor the different effects of two types of leaf removal at the molecular level, five libraries were constructed at different growth stages of lotus AR formation. A large number of clean reads (1.0–1.3 × 10^7) were obtained with approximately 346, 352, 414, and 245 differentially expressed miRNAs in the B1/C0, A1/C0, B2/B1, and A2/A1 libraries, respectively. Majority of these miRNAs were involved in plant hormone metabolism and signal transduction, particularly IAA metabolism. The change in endogenous IAA accumulation is the predominant mechanism behind AR development in OLR and ULR groups of lotus seedlings.

DECLARATIONS

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Conflicts of interest/Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

Not applicable.

Consent to participate

Not applicable

Consent for publication

Not applicable.

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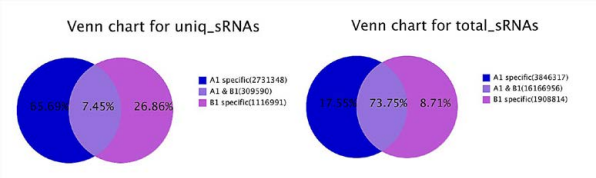
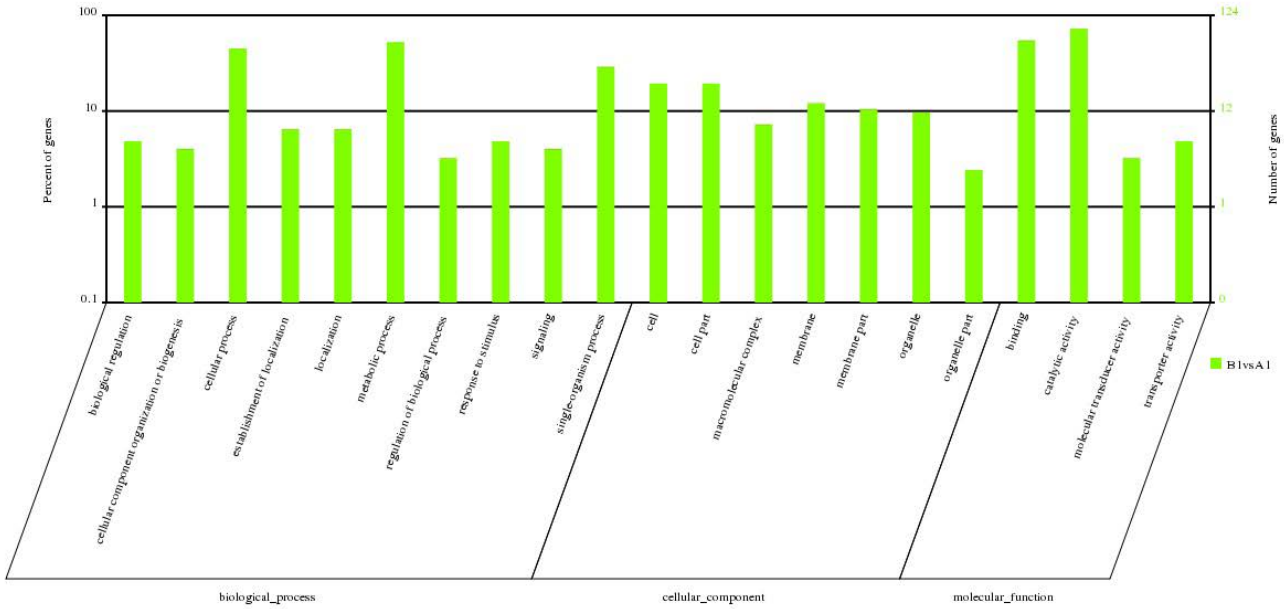
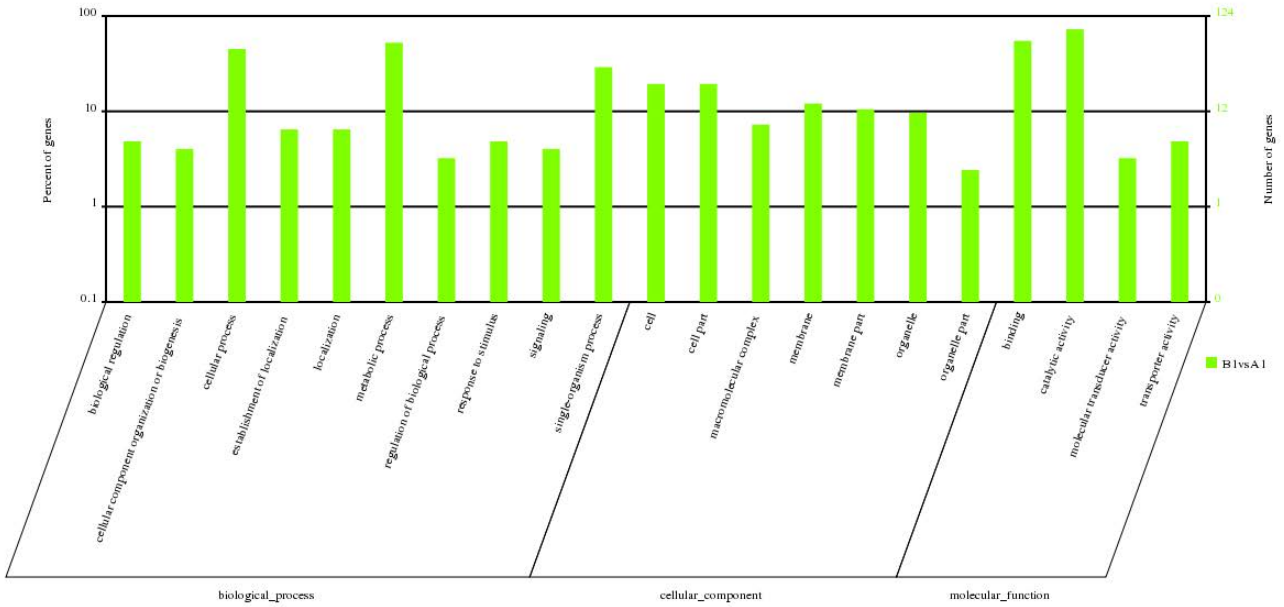
Research Article



Online Resource 1: (Additional Table S1) Information of uniq-sRNAs and total-sRNAs involved in A1 and B1 libraries



Online Resource 2: (Additional Figure S1) The biological processes of known miRNAs by GO tool involved in A1/B1 libraries.



Online Resource 3: (Additional Figure S2) the biological processes of novel miRNAs by GO tool involved in A1/B1 libraries.

Table S1: Primers used for qPCR identification

miRNA	Primer (5'-3')
miR397a	gcgcgTCATTGAGTGCAGCGTTGA
miR482d	gcgTTTCCGATCCCACCCATACCC
miR8558b	cgcgTTTCCGATCCCACCCATACCT
miR168a-5p	cgTCGCTTGGTGCAGGTCGGGAA
miR1863	cgcgcgTCTAGCTCTGATACCATGTTGAGT
miR952b	cgcgcgAACGAGGATCCATTGGAG
miR157a-5p	ccggcgcgTTGACAGAAGATAGAGAGC
miR171b-3p	cgcgTTGAGCCGTGCCAATATCACG
miR162-3p	cgcgcgTCGATAAACCTCTGCATCC
miR160f-5p	cTGCCTGGCTCCCTGAATGCC