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Identification and Characterization of Known and Novel MicroRNAs in Five Tissues of Wax Gourd (*Benincasa hispida*) Based on High-Throughput Sequencing

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Abstract: MicroRNAs (miRNAs) are endogenous single-stranded non-coding small RNAs of 20–24 nucleotides and play important roles in many plant biological and metabolic processes. Wax gourd is an important vegetable of Cucurbitacea family, with great economic and medicinal value. Although miRNAs have been extensively studied in model plant species, less is known in wax gourd (*Benincasa hispida*). In this study, in order to identify miRNAs in wax groud, five independent small RNA libraries were constructed using leaf, root, stem, flower, and fruit of B227. Based on high-throughput Illumina deep sequencing. In total, 422 known and 409 novel miRNAs were identified from five libraries. Comparative analysis revealed that many miRNAs were differentially expressed among different tissues, indicating tissue-specific expression of some miRNAs. qRT-PCR verified the reliability of small RNA sequencing results. Furthermore, miRNAs with similar expression patterns among five tissues were clustered into the same profile, among which many miRNAs were found with relatively high expression in the fruit of wax gourd. MiR164-x had the highest expression in fruit than in other tissues and many NAC transcription factors were predicted as its target genes. We propose that miR164 might regulate fruit development by forming miR164-NAC module in wax gourd. Taken together, this study provides the first global miRNAs profiling of wax gourd, and lays the foundation for understanding the regulatory roles of miRNAs in the growth and development processes of wax gourd.

Keywords: wax gourd; high-throughput sequencing; microRNAs; target genes

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1. Introduction

MicroRNAs (miRNAs) are small endogenous single-stranded non-coding small RNAs of 20–24 nucleotides (nts) that negatively regulate gene expression at the post-transcriptional level [1]. The biogenesis of miRNAs has been well elaborated in plants. Firstly, the long single-stranded pri-miRNAs (primary miRNAs) are generated by RNA polymerase II from the intergenic regions [2–4]. Secondly, the pri-RNAs are processed to generate 100–200 nt pre-miRNAs (precursor miRNAs) with stem-loop structures (hairpins) catalyzed by Dicer-like I enzyme (DCL1) in the nucleus, which creates a miRNA/miRNA* complex [5,6]. Then these complexes are translocated into the cytoplasm and integrated with RNA-induced silencing complex (RISC), where the miRNA strand is incorporated with RISC and the miRNA* strand is usually degraded [7].

A large number of miRNAs have been discovered and identified from diverse plant species in the past decades. Traditional approaches including direct cloning and Sanger sequencing were widely employed for conserved miRNA identification in model plant species [8,9]. However, some miRNAs are expressed in certain tissues or developmental

stages and accumulate at relatively lower levels. The advent of next-generation high throughput sequencing technology provides a rapid and efficient opportunity for the identification of conserved miRNAs as well as low-abundance miRNAs. This powerful strategy has been applied rapidly in many plant species [10–13], and the number of miRNAs increases quickly. Up to now, the total number of registered miRNAs in miRBase increased to 48, 860 in 271 species in the current release 22 (http://www.mirbase.org/, accessed on 9 October 2021).

MiRNAs regulate gene expression by repressing the translation or cleavage of targeted mRNAs [14]. Numerous reports have demonstrated that miRNAs play important roles in a range of biological and metabolic processes, including organ formation and differentiation, and response to environmental biotic and abiotic stresses [15–23]. Some miRNAs, such as miR164 and miR166, are involved in the regulation of leaf development [24–26]. Fruit is the main consumption organ for most vegetable crops. By using miRNA sequencing, many miRNAs have been identified potentially modulating fruit development and growth. For example, 32 conserved miRNAs were differentially expressed among developmental stages in melon, and miR393 were further validated affecting melon fruit ripening [27]. Comparing miRNA abundance in three developmental stages between wild and cultivated pepper fruits, miR159 and miR172 probably were proposed possibly affecting fruit set and fruit size [28]. Based on CRISPR/Cas9-mediated knock-out of miR164 in tomato, miR164 deficient mutant developed smaller fruit than wild type fruit mainly caused by reduced pericarp cell division and expansion [29].

Wax gourd (*Benincasa hispida* (Thunb.) Cogn, 2n = 2x = 24) is an important vegetable crop of Cucurbitaceae family. The fruit size of wax gourd is diverse, ranging from 0.5 kg wild wax gourd to more than 20 kg cultivated wax gourd under normal cultivation conditions. Wax gourd could be stored over 6 months under natural conditions and has a long shelf-life, therefore it plays a significant role in ensuring the annual supply and regulating the off-season of vegetables [30]. Furthermore, it is also used for the treatment of many diseases or disorders [31,32].

Recently, the release of the reference genome of wax gourd [33] facilitated fundamental research in wax gourd. However, until now, there is no report focusing on the miRNA profile of wax gourd considering their vital biological roles. In order to obtain comprehensive knowledge of miRNAs and their target genes in wax gourd, high-throughput sequencing and bioinformatic techniques were employed to acquire the first inventory of wax gourd miRNAs population. In the present study, five small RNA libraries from root, stem, leaf, flower, and fruit were constructed and sequenced. A large number of known and novel miRNAs were identified. Besides, the expression of some miRNAs was validated. The research would lay the foundation for further studies on the regulatory roles of miRNAs in wax gourd.

2. Materials and Methods

2.1. Plant Materials

The reference genome-based wax gourd variety B227, without waxy coating on its fruit surface, was used as the experimental material source in this study. B227 was grown in the research experimental field of Vegetable Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou, China in the spring of 2017. Roots from two-week-old seedlings and stems, leaves, male flowers (indicated as flowers thereafter), and 7-days-after-pollination fruits (indicated as fruits thereafter) from each individual of the flowering stage were collected with three biological replicates. All the samples were then immediately put in liquid nitrogen and kept at −80 °C for further use.

2.2. Small RNA Library Construction and Sequencing

Each sample of tissue consisted of a pool of three biological replicates. Total RNA of the sample was extracted using the Trizol Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Subsequently, DNase I (RNase-free) (TaKaRa, Kyoto,

Japan) was used to remove DNA, and the concentration and integrity of clean RNAs were evaluated by a 2100 Bioanalyzer at 230 nm and 280 nm. A total amount of 1.5 µg RNA per sample was used as input material for sample preparations. Sequencing libraries were generated using NEBNext® UltraTM small RNA Sample Library Prep Kit for Illumina® (NEB, Ipswich, MA, USA) following the manufacturer's recommendations.

Briefly, small RNAs were separated on PAGE gel from total RNAs. After ligation of 3′ and 5′ adaptors, the small RNA fragments were used for reverse transcription and PCR amplification. Subsequently, the RNA fragments were purified by the AMPure XP system (Beckman Coulter, Beverly, MA, USA) and the library quality was assessed on the Agilent Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, CA, USA). Finally, the RNA library from each sample was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v4-cBot-HS (Illumia, San Diego, CA, USA), and deep sequenced on an Illumina Hiseq 2500 platform according to the manufacturer's instructions.

2.3. Identification of miRNAs in Wax Gourd

To obtain clean reads, low-quality reads containing more than one low-quality (Q-value $\leq$ 20) base or unknown nucleotides(N) were firstly removed. Then, reads without 3′adapters, reads containing 5′adapters, reads containing 3′ and 5′ adapters but no small RNA fragment between them, and reads containing ployA in small RNA fragment were removed. Furthermore, the sequences smaller than 15 nt or longer than 35 nt were removed. All the downstream analyses were based on high-quality clean reads. The clean tags were aligned with small RNAs from the GeneBank database (Release 209.0) and Rfam database (Release 11.0) [34] to identify and remove ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA) and small nucleolar RNA (snoRNA), and other repeats using Bowtie software [35]. All of the clean tags were also aligned to wax gourd reference genome, and those mapped to exons, introns, or repeat sequences were also removed.

Subsequently, the remaining reads were used to search against the miRBase database (miRBase 22, http://www.mirbase.org/, accessed on 9 September 2020) to detect known miRNA. In order to display the difference with the existed 5p and 3p miRNAs, the known miRNA processed from the 5′ arm or 3′ arm of the miRNA precursor is followed by an "x" or a "y". All of the unannotated tags were aligned with the wax gourd reference genome [33]. According to the criteria of novel miRNAs [36], potential novel miRNAs were predicted using Mireap software (http://sourceforge.net/projects/mireap/, accessed on 16 September 2020). Besides, the hairpin structures of novel miRNAs were predicted by Mireap_v0.2 software with default parameters setting.

2.4. Differential Expression Analysis of miRNAs

To detect the differentially expressed miRNAs, we compared the conserved and novel miRNA expression levels among root, stem, leaf, flower, and fruit by calculating TPM (tags per million reads). The TPM values were normalized based on actual miRNA count/total number of clean reads ×106. Differential expression analysis was performed using the DESeq R package (1.10.1) [37], where the miRNAs with p-value < 0.05 and $\log_2$ FC (fold expression change) greater than 1 or less than -1 were assigned as differentially expressed. The heatmap of miRNAs was drawn using pheatmap software to display miRNA expression levels in different tissues and to cluster miRNAs with similar expression patterns.

2.5. Target Gene Prediction, Functional Annotation and Pathway Analysis

Based on the sequences of known and novel miRNAs, the candidate target genes were predicted using the psRNA Target Server (http://plantgrn.noble.org/psRNATarget/, accessed on 20 September 2020) [38]. All predicted miRNA targets and differentially expressed miRNA targets were annotated based on the following databases: Nr (NCBI non-redundant protein sequences), Nt (NCBI non-redundant nucleotide sequences), Swiss-Prot (Swiss-Prot protein database), Pfam (Protein family), COG (Cluster of Orthologous

Groups database), GO (Gene Ontology database, http://www.geneontology.org/) and KEGG (Kyoto Encyclopedia of Genes and Genomes pathway database) pathway analysis (https://www.kegg.jp/kegg/pathway.html, accessed on 22 September 2020) [39]. For GO and KEGG analysis, the calculated *p*-value was processed through FDR (false discovery rate) correction, taking FDR $\leq$ 0.05 as a threshold.

2.6. Quantitative Real-Time PCR (qRT-PCR) Assay

To validate the accuracy of miRNA sequencing results and differential expression of miRNAs between five tissues of wax gourd, four known and three novel miRNAs were randomly selected for qRT-PCR analysis. qRT-PCR was performed using Mir-X miRNA qRT-PCR TB Green® Kit (TaKaRa, Kyoto, Japan) according to manufacturer's instructions on a CFX96 Real-Time PCR Detection System, with total RNA of five tissues as templates. The U6 rRNA of wax gourd was employed as the internal control for the analysis. The assay was carried out with three technical and three biological replicates. The comparison between sequencing results and qRT-PCR was analyzed using simple linear regression of correlation with 95% confidence interval in GraphPad Prism 8.3.0 software. Tissue by tissue correlation analysis was performed using Spearman's correlation test in the R package. All qRT-PCR primers used are listed in Supplementary Table S1.

3. Results

3.1. High-Throughput Sequencing and Analysis of Wax Gourd Small RNAs

To obtain a comprehensive knowledge of the small RNAs in wax gourd, five independent small RNA libraries from leaf, root, stem, flower, and fruit of wax gourd B227 were subjected for high-throughput sequencing. A total of 18,675,941, 20,161,705, 19,947,266, 23,155,945 and 16,085,205 clean reads were generated from leaf, root, stem, flower and fruit of B227, respectively (Table 1). After removing the low-quality reads, 3′ adapter, insert, 5′ adapter, polyA and reads length < 15 nt or > 35 nt, 18,284,736, 19,813,111, 19,551,447, 22,821,341 and 15,843,596 clean tags were respectively obtained from each tissue (Table 1). As shown in Figure 1, the 24 nt long small RNAs were the most abundant small RNAs in all five tissues. Except for root, the length of small RNAs is mainly distributed from 20–25 nt. In the root, the length of small RNAs presented nearly an even distribution from 16–35 nt (Figure 1), implying tissue specificity of small RNAs in wax gourd.

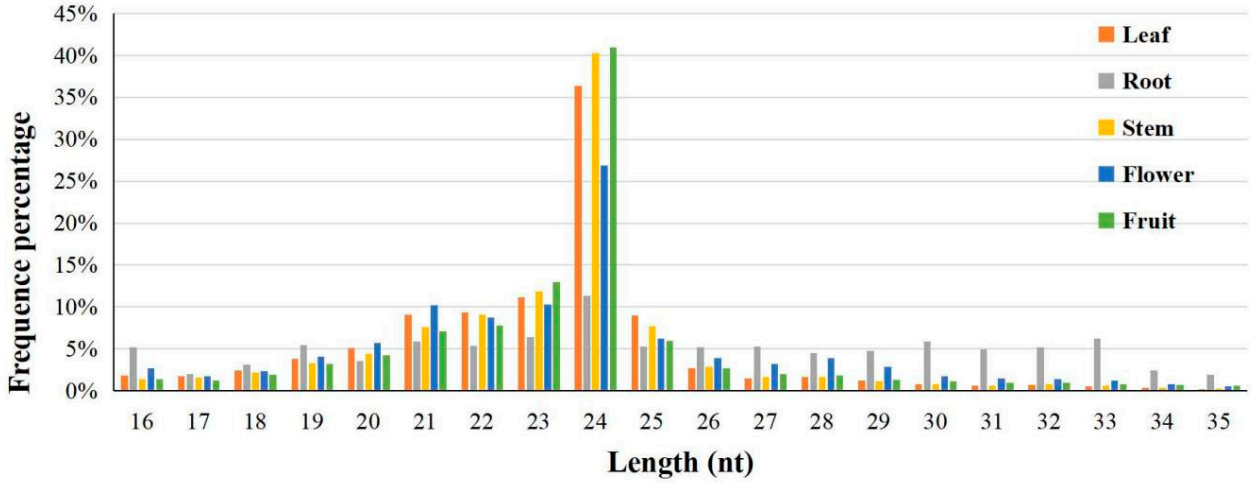

Figure 1. Length distribution of small RNAs. Y-axis represents percentage of total reads for related small RNAs. X-axis represents length of small RNAs. Five libraries are represented by different colors.

Table 1. Summary of small RNA sequencing results in five tissues of wax gourd.

Tissue	Raw Reads	Clean Reads	3′ Adapter	Insert	5′ Adapter	Poly A	Clean Tags
Leaf	19,026,019	18,675,941	264,840	71,345	14,955	1704	18,284,736
Root	20,596,286	20,161,705	244,410	37,464	22,930	699	19,813,111
Stem	20,335,677	19,947,266	270,636	68,005	14,962	1773	19,551,447
Flower	23,542,034	23,155,945	219,221	71,716	17,647	1279	22,821,341
Fruit	16,351,738	16,085,205	156,295	51,076	10,107	1253	15,843,596

3.2. Identification of Known miRNAs in Wax Gourd

In order to identify the known miRNAs in wax gourd, the generated small RNA sequences from the five libraries were aligned with the known plant miRNAs deposited in the miRBase database (release 22). Overall, the reads were matched to 422 known miRNAs for all the five libraries (Supplementary Table S2), with 255, 146, 264, 219, and 213 identified from leaf, root, stem, flower and fruit, respectively (Table 2). The expression profile of different miRNAs varied significantly. In the leaf, the TPM value of miR166-y, miR319-y, and miR167-x were all above 19,000 while some other miRNAs like miR11179-y and miR11180-y had no expression (Supplementary Table S2). The accumulation of many miRNAs in the five tissues also varied. For instance, the expression of miR319-y was 79 times more in the stem than in flower (Supplementary Table S2). The 422 known miRNAs were further mapped to wax gourd genome database [33]. Finally, 96 miRNAs were located to different chromosomes or contigs (Supplementary Table S3).

Table 2. Number of known and novel miRNAs identified in five tissues of wax gourd.

	Leaf	Root	Stem	Flower	Fruit
Known miRNAs	255	146	264	219	213
Novel miRNAs	407	381	408	408	405

3.3. Identification of Novel Potential Wax Gourd miRNAs

The characteristic hairpin structure of miRNA precursor is an indispensable feature for predicting novel miRNAs [40]. After removing known miRNAs, the remaining sequences were aligned with the wax gourd genome database [33] to discover novel and potential specific miRNAs. Overall, 409 novel miRNAs were identified from five libraries, with 407, 381, 408, 408, and 405 from leaf, root, stem, flower, and fruit, respectively (Table 2). To visualize the expression overlap between novel microRNA sets of each tissue, an UpSet plot was drawn on the Omicshare platform (https://www.omicshare.com/, accessed on 10 October 2021). As shown in Figure 2, there are nearly 20, 30, 55, 10, and 30 novel miRNAs expressed only in fruit, flower, stem, leaf, and root, respectively. More than 40 novel miRNAs were expressed in all the tissues analyzed. All novel miRNAs were successfully aligned to different chromosomes or contigs of wax gourd genome (Supplementary Table S4). The length of novel miRNA mainly ranged from 18 nt to 25 nt, with the 24 nt being the most abundant one, which counted 81.15% among all novel miRNAs (Supplementary Figure S1).

3.4. Validation of miRNAs Expression by qRT-PCR

In order to verify the reliability of small RNA sequencing results, qRT-PCR was adopted to detect the relative expression levels of five known and five novel miRNAs in five tissues of wax gourd. It could be concluded that the expression pattern among five tissues using two methods was similar (Figure 3). Thereafter, a correlation analysis was performed between qPCR data and RNA sequencing data. A moderate correlation was found between two methods (R^2 = 0.2930, $p < 0.0001$ ****). The tissue-by-tissue correlation analysis between qPCR and RNA sequencing data was also performed and it was found that the correlation between qPCR and small RNA sequencing of stem and

fruit are significant, with *p*-value 0.007547 and 0.01114, respectively (Table S7). Overall, our qPCR confirmed the reliability and availability of high throughput sequencing results.

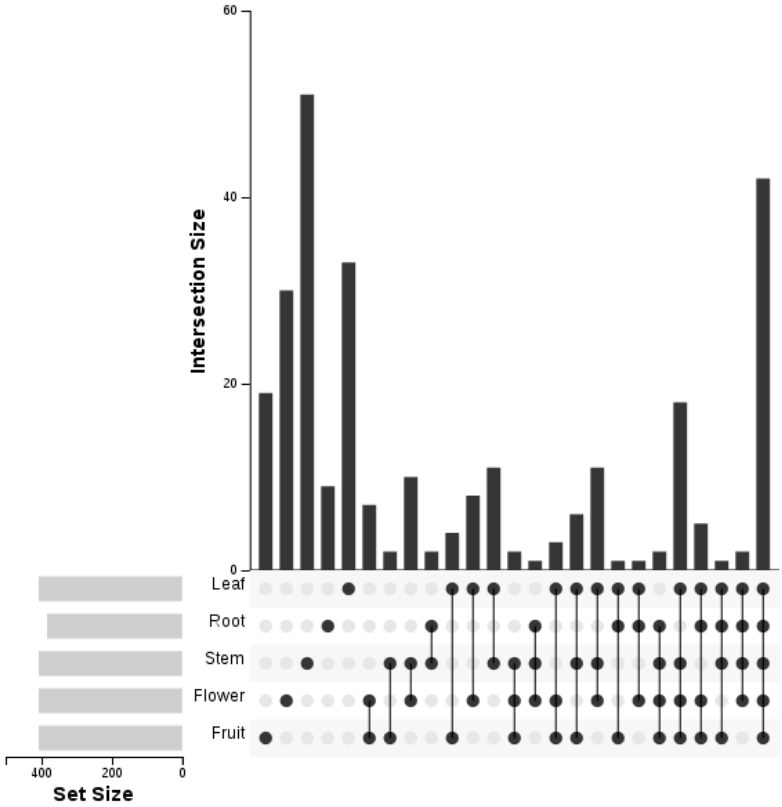

Figure 2. UpSet plot showing quantitative distribution of novel miRNAs in different tissues. The connected black dots represent different subsets of novel miRNAs according to their expressions and the bottom bar associated with each subset indicates the number of novel miRNAs exclusive to that subset.

3.5. Prediction of miRNA Targets in Wax Gourd

The psmall RNA Target Server was used to predict putative target genes of miRNAs [38]. Totally, the 831 miRNAs targeted 10440 gene sites compromised by 7536 genes. The number of target gene sites of different miRNAs differed, varying from 0 to 473 (Table S5).

Subsequently, in order to better understand the regulatory roles of miRNAs, GO analyses were performed based on their target genes. Target genes of identified miRNAs were classified into "Biological Process", "Cellular Component" and "Molecular Function", with "Biological Process" being the dominant category. "Metabolic Process", "Cellular Process" and "Single-organism Process" were the top three enriched items in the "Biological Process" category, while "Cell", "Cell part" and "Organelle" were the three most enriched items in the "Cellular Component" category (Figure 4).

3.6. Differentially Expressed miRNAs between Five Libraries

Pairwise comparison was performed among the five libraries of wax gourd to identify differentially expressed miRNA (defined by $|\log_2$ fold change$| > 1$, p-value < 0.05). The number of up- and down-regulated miRNAs between libraries is illustrated in Figure 5 (More detailed expression information see Supplementary Table S6). Eighty-two miRNAs were found up-regulated while 66 were down-regulated in root compared with that in the stem. According to the expression profile miRNAs in five tissues of wax gourd, a trend analysis was also performed to cluster those miRNAs with similar expression patterns. In total, 401 miRNAs were classified into 20 profiles (profile 0–19) (Figure 6). miRNAs in

profile 0, profile 10, profile 9, profile 12, and profile 19 had more expression in leaf, in stem, in flower, in root, and in fruit than in other tissues, respectively. miRNAs in profiles 1, 4, 6, 8, 11, 13, 15, 17, and 19 had more expression in the fruit of wax gourd. For example, MiR164-x of profile 13 and novel miRNA novel-m0037-5p of profile 6 had the highest expression in fruit than in other tissues.

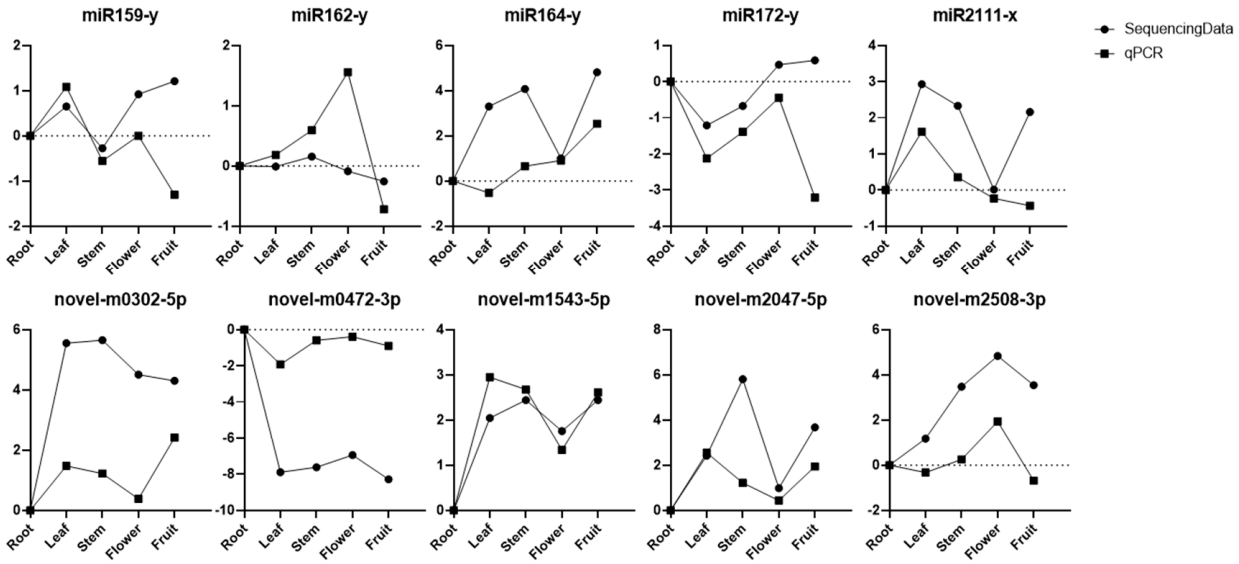

Figure 3. Expression pattern of miRNAs confirmed by qRT-PCR. The expression level of five known miRNAs and five novel miRNAs among five tissues determined by qRT-PCR was presented using $-\Delta\Delta Ct$, and that of resequencing data was presented using $\log_2$(TPMtissue/TPMroot).

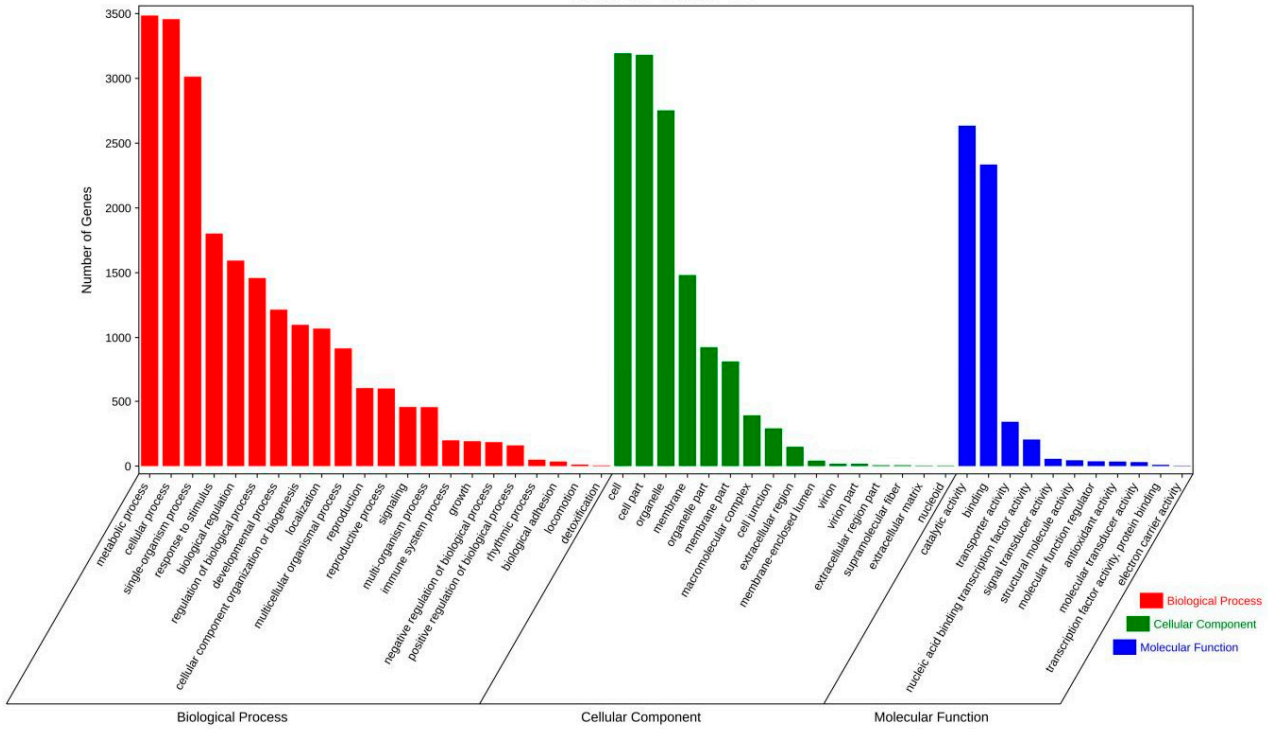

Figure 4. GO annotation of target genes of miRNAs. Y-axis represents number of genes in related GO item and X-axis represents GO items and allocated GO category.

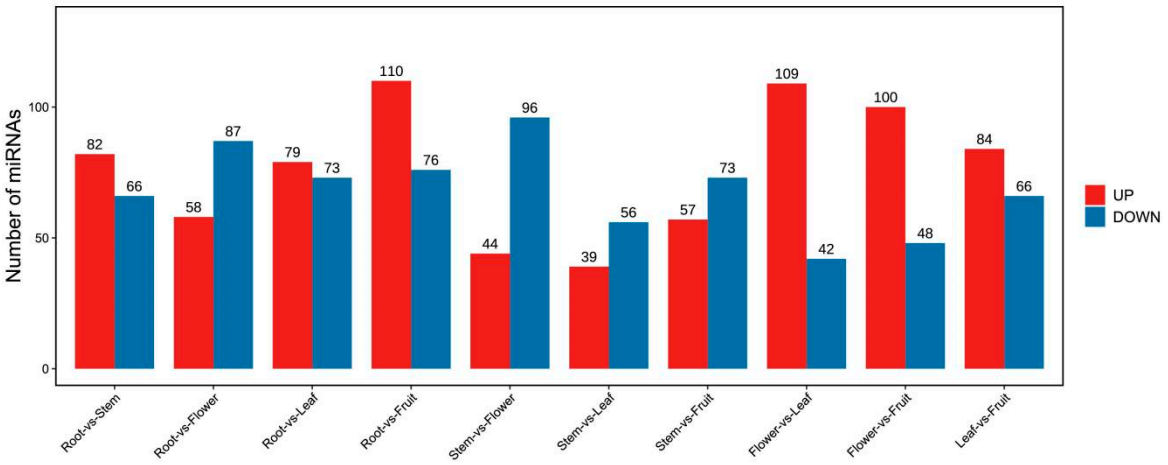

Figure 5. Number of up- and down-expressed miRNAs between tissues. Y-axis represents number of genes and X-axis represents pairwise comparison groups. Up and down expressed miRNAs are presented by red and blue color, respectively.

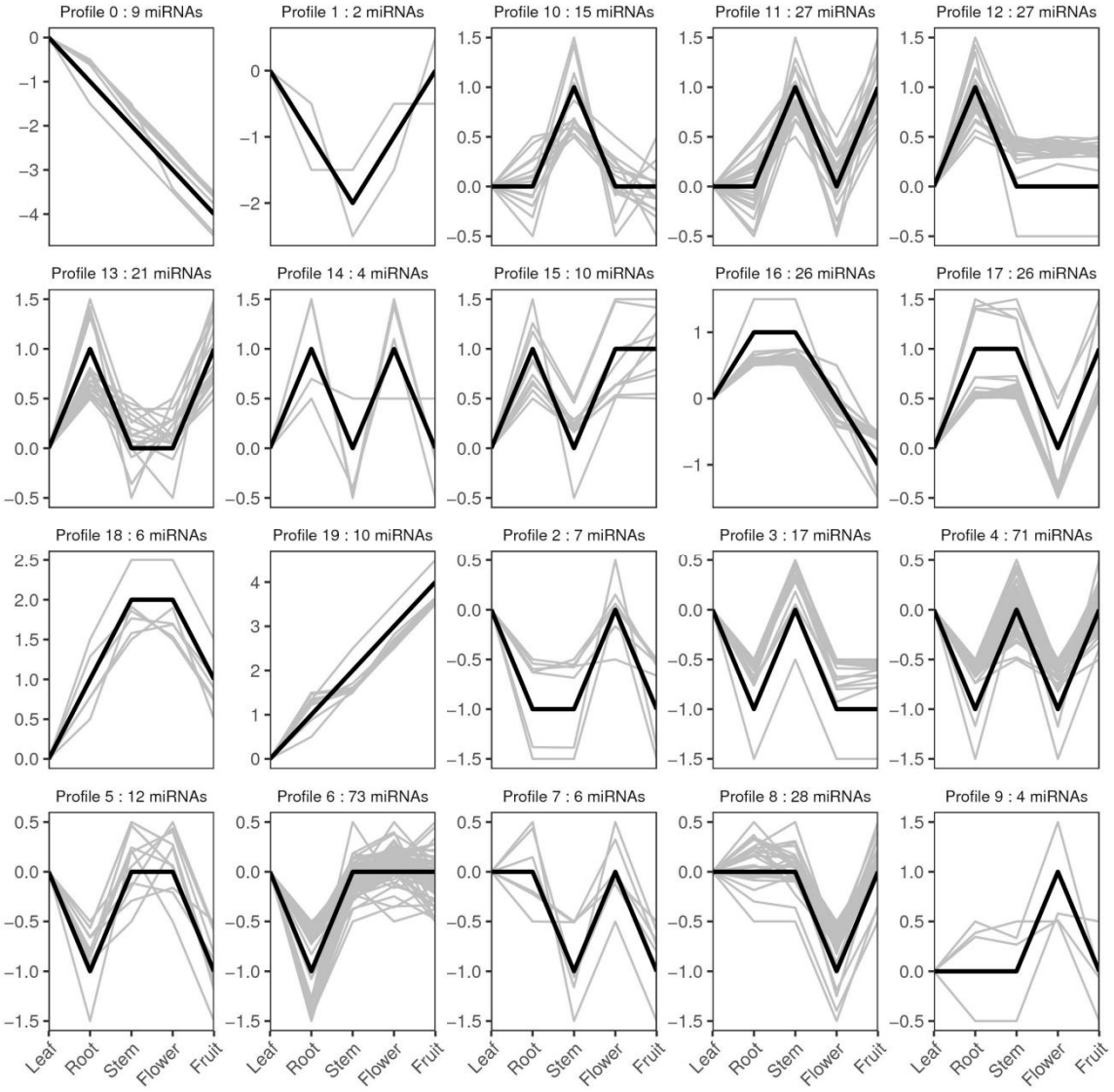

Figure 6. Trend profiles of miRNAs with similar expression pattern. In total, 20 trend profiles were generated. Y-axis represents the expression level of miRNAs among five tissues by resequencing data and X-axis represents five tissues.

3.7. Evolutionary Analysis of Wax Gourd miR164

To understand the relationship between wax gourd miR164 and miR164 from other plant species. The miR164 from *Citrus sinensis*, *Sonanum lycopersicum*, *Arabidopsis thaliana*, *Cucumis melo*, and *Oryza sativa* were downloaded from miRbase (www.mirbase.org, accessed on 19 October 2021) and the miR164-x, miR164-y, and miR164-z were also included for neighbor-joining (NJ) tree build using MEGA 11 software. It can be seen that most of miR164 among plant species are conserved and miR164-x of wax gourd was the same as that of miR164a of melon and miR164d of rice (Figure 7).

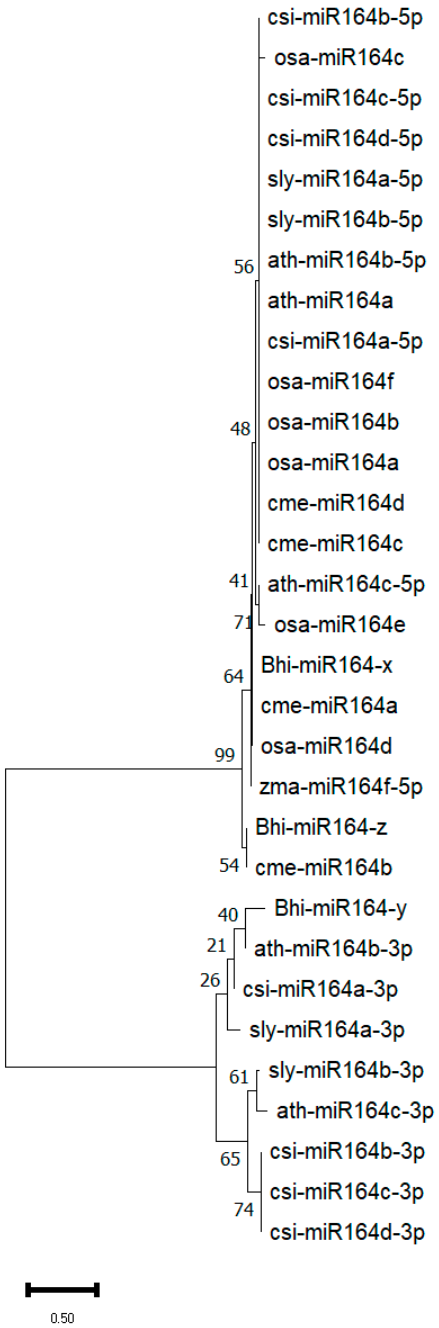

Figure 7. Phylogenetic relationships between wax gourd miR164 family members and miR164 family members of other plant species using the neighbor-joining (NJ) method with Bootstrap 1000 replicates. Bar 0.50 indicates substitutions per nucleotide position. csi: *Citrus sinensis*. sly: *Sonanum lycopersicum*. ath: *Arabidopsis thaliana*. Bhi: *Benincasa hispida*. cme: *Cucumis melo*. osa: *Oryza sativa*.

4. Discussion

miRNAs act as important regulators in transcriptional and post-transcriptional gene silencing and play essential roles in many plant physiological processes, such as plant growth and development, morphogenesis, embryogenesis, stress tolerance, and metabolism [2,41–44]. In recent years, accompanied by the rapid development of sequencing technology, a greater number of studies have focused on the identification of novel miRNAs using the next-generation sequencing technology [45–51]. Nevertheless, up to now, no information related to miRNAs has been reported in wax gourd (*Benincasa hispida*).

In this study, we firstly discovered and characterized the miRNAs of wax gourd using high-throughput sequencing from five tissues (root, stem, leaf, flower, and fruit). The major size distributions of the total small RNA reads in the five libraries ranged from 20–25 nt, in which 24 nt small RNAs were most abundant and followed by 23 nt small RNA. Similar results were also reported in other species, such as cucumber [52], persimmon [49], and Lonicera japonica [50], but different from that of radish [48], grapevine [53], Populus euphratica [54]. Furthermore, when compared with the other four libraries, the length distribution of small RNAs in the root library showed a significant difference, which implied the presence of tissue specificity of small RNAs in wax gourd. The above results suggested that the distribution of small RNAs might vary among plant species as well as tissues. It is documented that 24 nt small RNA function in heterochromatin modification, especially for genomes with a high content of repetitive sequences [55,56]. As more than 75.5% of wax gourd genome was constituted by repetitive sequences [33], it's possible that the dominant 24 nt small RNAs might play diverse functions in different processes of wax gourd.

Deep sequencing of small RNA libraries from different tissues is of great importance for comprehensively detecting the populations and roles of miRNAs [47]. Based on the sequences in the miRBase database and the reference genome of wax gourd, a total of 255 (leaf), 146 (root), 264 (stem), 219 (flower) and 213 (fruit) known miRNAs together with 407 (leaf), 381 (root), 408 (stem), 408 (flower), and 405 (fruit) novel miRNAs were identified. For both the known and novel miRNAs, the read number varied significantly. Most of the novel miRNAs existed at low copies in all five tissues (Table S2). Although presented as low levels, these miRNAs may play particular roles in specific development and cellar processes as well, such as plant development, fruit ripening, biotic and abiotic stress responses [40,47,48,54,57].

In our present study, a large number of differentially expressed miRNAs between tissues were detected by pairwise comparisons and those miRNAs with similar expression patterns were clustered into the same profile by trend analysis. miRNAs in many profiles, such as profile 13 and profile 19, had more expression in wax gourd fruit. miR164-x is a member of profile 13 and it had the highest expression in fruit than any other tissues. MiR164 was previously reported involved in fruit ripening. For instance, miR164 was highly expressed during fruit ripening of sweet orange [58]. What is more, miR164 also affects fruit development. During prickly pear cactus fruit development, miR164 expressed in all fruit development stages and homogenously expressed in fruit-related organs [59]. miR164 deficient mutant tomato slmir164aCR possessed smaller fruit than wild type indicating its role in regulating fruit development [29]. Mostly, miR164 regulates fruit development by targeting NAC transcription factors, such as *CUP-SHAPED COTYLEDON2 (CUC2)*. MiR164-CU2 module regulates fruit enlargement and therefore affects fruit size in *Arabidopsis* [60]. Evolutionary analysis revealed that most plant miR164s are relatively conserved and miR164-x of wax gourd is the same as miR164a of melon. Target gene prediction of miRNAs was performed to illuminate the probable regulatory mechanism of miRNAs. The target of miR164-x contained many NAC transcription factors, including Bhi01M001153, Bhi02M000218, Bhi02M001641, Bhi06M000313, Bhi08M000962, Bhi09M002342, Bhi09M002835 and Bhi11M002467. Therefore, miR164-x of wax gourd might also play a role in regulating fruit enlargement, which needs further validation. miRNAs in the same profile might play a similar role in regulating wax gourd biological or

metabolic processes. Nevertheless, more investigations are needed to explore the biological functions of miRNAs-mediated regulation in wax gourd.

5. Conclusions

In conclusion, we performed high-throughput small RNA sequencing to detect miRNAs in five tissues (root, leaf, stem, flower, and fruit) of wax gourd. In total, 422 known miRNAs and 409 novel miRNAs were identified and their target genes were also predicted. Differentially expressed miRNAs were clustered into 20 profiles based on the expression trend among five tissues. MiRNAs in profiles 1, 4, 6, 8, 11, 13, 15, 17, and 19 had more expression in the fruit of wax gourd. MiR164-x in profile 13 had the highest expression in fruit and was proposed to regulate fruit development by forming miR164-NAC module with its targets NAC transcription factors. To our knowledge, this is the first comprehensive report of miRNAs identification in wax gourd. Combined with bioinformatic analysis and experimental validation, these results will contribute to the functional characterization of miRNAs and understanding the regulatory roles of miRNAs in different processes of wax gourd.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app112110068/s1, Figure S1: Length distribution of novel miRNAs, Table S1: Primers used for qRT-PCR analysis, Table S2: Expression profile of all known and novel miRNAs among five tissues of wax gourd, Table S3: Genomic location and hairpin structure of reference genome mapped known miRNAs, Table S4: Genomic location and hairpin structure of novel miRNAs, Table S5: Detailed information of target genes of miRNAs, Table S6: Pairwise comparison of the expression of miRNAs among five tissues. Table S7: The Spearman's correlation test between qPCR data and RNA sequencing data tissue-by-tisse.

Author Contributions: Conceptualization B.J. and J.Y.; data curation J.Y.; formal analysis J.Y., M.W., W.L. and Q.P.; writing—original draft preparation B.J. and J.Y.; writing—reviewing and editing, B.J., J.Y. and X.H.; visualization J.Y.; resources D.X. All authors have read and agreed to the published version of the manuscript.

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