

Identification and analysis of key miRNA for sex differentiation in hermaphroditic *Juglans mandshurica* Maxim.

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Abstract

Background: *Juglans mandshurica* Maxim. is a monoecious heteromorphic plant. The mechanism of miRNA-mediated sex differentiation and dioecious heterozygosity is unclear.

Results In this study, 310 known and 278 unknown miRNA were identified and 129 differentially expressed miRNA were screened using high-throughput sequencing technology for microRNA sequencing analysis of male and female flower buds of the protogyny and protandry of *Juglans mandshurica*. 638 miRNA target genes predicted using PSRobots and these target genes are significantly enriched in reproductive, floral development and phytohormone signaling pathways. Therefore, it is hypothesized that miR159c-3p, miR156d-3p, miR172d, miR171b, miR164a, miR167a, miR319a, miR399a, and miR396t et al. may be the key miRNA affecting sex differentiation in *Juglans mandshurica*, among which miR164a, miR166c, and miR171c-5p may be the key miRNA for heterosexualism differentiation in *Juglans mandshurica*. 9 randomly selected miRNAs with significant differences were subjected to real-time fluorescence quantitative PCR to verify the accuracy of high-throughput sequencing. Combined with transcriptome sequencing results for co-analysis, a differential miRNA-differential mRNA primary regulatory network was established.

Conclusion It was further confirmed that these miRNA play an important role in the sex differentiation of *Juglans mandshurica*. This study lays the research foundation for the eventual discovery of the molecular mechanism of sex differentiation and heterosexualism in *Juglans mandshurica*.

1. Introduction

Heterosexualism is an intermediate transition form from monoecious to dioecious plants[1–5]. According to the sequence of flower sex differentiation time can be divided into two types of female flower first mature individuals and male flower first mature individuals, and formed two kinds of mating groups, namely protogyny and protandry[2, 3, 6–8]. Among flowering plants, dioecious heterozygosity is uncommon and is currently found in only 13 families and 20 genera, and most of them are angiosperms[9].

Juglans mandshurica Maxim is a plant of the genus *Juglans* in the Juglandaceae. It is a valuable timber tree species in northern China. It is also one of the three-hardwood forest in Northeast China[10]. In the plant of the genus *Juglans*, there are two main types of plant reproduction, and the ratio of protogyny to protandry groups is roughly 1:1[11]. Due to the different opening times of male and female flowers, when the protogyny female flowers are opening, the protandry male flowers are in the pollination stage, and when the protogyny male flowers are pollinating, the protandry female flowers are in the opening stage[12–14]. In low-density populations, the ratio of protogyny to protandry of *Juglans mandshurica* may be biased, which can lead to a lack of mating partners in areas dominated by a single form[15]. This phenomenon will lead to insufficient pollination time between male and female flowers, which seriously

affects the pollination and fruit set rates, thus leading to a decrease in the fruit set and seed quality of *Juglans mandshurica*.

MicroRNAs (miRNA) are a class of small non-coding RNAs of 20-24nt in length, which are derived from single-stranded RNA hairpin pre-cursor (Dicer) in animals and from Dicer-like1 (DCL1) in plants[16–18]. miRNA in plants are mainly used to control many biological functions by targeting complementary sequences and regulating the expression of target genes[19, 20]. miRNA are genetically very conserved in plants and play a crucial role in many vital developmental processes in plants, such as plant flower bud differentiation, fruit development, hormone synthesis, etc. Numerous studies have shown that miRNA can affect plant cell development and differentiation by directly silencing the expression of their target genes, and that differentially expressed miRNA can also indirectly affect plant flower development by affecting related hormone synthesis and other signaling pathways, leading to pistil or stamen abortion and consequently to sex differentiation[21]. Therefore, the identification of key miRNA species and their functions in the plant life cycle can provide new directions for the study of plant development and sex differentiation. For example, in *Cymbidium ensifolium*, differential expression of two families, miR160 and miR396, can target ARFs and GRFs, to regulate flower development[22]. The miR156/157 and miR160 families have distinct roles in *Juglans regia* L floral bud differentiation. Research on miR396 and miR408 has demonstrated the influence of these two families on the sexual differentiation of *Xanthoceras sorbifolium* Bunge[23]. However, there are fewer studies on miRNA-mediated sex differentiation and floral bud differentiation in *Juglans mandshurica*. In this study, male and female flower buds of the hermaphroditic *Juglans mandshurica* at the stage of physiological differentiation and morphological differentiation were used as test material.

Therefore, in this study, the male and female flower buds of the hermaphrodite *Juglans mandshurica* were used as test material at physiological and morphological stages of differentiation. MiRNA sequencing and transcriptome deep sequencing of male and female flower buds using Illumina sequencing technology. Analysis of miRNA expressed by male and female flower buds at different developmental stages and prediction of target genes for different differential miRNA. Combined with transcriptomic data analysis, we identified key miRNA associated with sex differentiation in *Juglans mandshurica* and constructed a primary regulatory network to provide reference information for the study of sex differentiation mechanisms in *Juglans mandshurica*.

2. Results

2.1. Mapping of sRNA in the buds of *Juglans mandshurica*

To identify novel miRNA and to investigate the role of miRNA in sex differentiation and gynoecium-androecium transformation of *Juglans mandshurica*, we constructed 12 small RNA libraries (three biological replicates per sample) in four types of protogyny female buds (T1), protogyny male buds (T2), protandry female buds (M1) and protandry male buds (M2). Deep sequencing of the small RNA library yielded average raw small RNA reads for the four *Juglans mandshurica* groups of 70099677, 42177432,

32725495 and 43655127 respectively. After removing the adapters and removing low quality sequences, reads in the 18-36nt range in length were selected (Clean Reads). A total of 20.0 G of clean data were obtained, with the average values for the four types being 38132413, 33541027, 25967944 and 35423485 (Table S1), which average of 73.70% of original data.

The distribution of sequence lengths before and after de-duplication showed that the sRNAs obtained were of different lengths, with the T1 group having the most abundant 33-nt RNA before de-duplication, while the other three groups had the most abundant 24-nt RNA. After de-duplication, all four groups of sRNAs were mainly distributed between 21-24nt in length, with 24nt RNA being the most abundant (Additional file 1). The number of unique sequences shared between different subgroups was counted at both the total and unique sequence levels. The total number of sequences in the four libraries was 316263574 (Fig. 1-A), and the total number of sequences after de-duplication was 1043444 (Fig. 1-B). The Clean Reads were compared to the reference genome and the results of the comparison are shown in Table S2, where on average more than a third of the sequences in the four sample sets were mapped to the reference genome. This indicates that the quality of the miRNA sequencing data is reliable and can be used for subsequent analysis. These sequences are distributed on 16 chromosomes of *Juglans mandshurica*, with chr01 being the most abundant and chr12 the least abundant. (Fig. 1-D)

Using Blast, Unique Reads were matched against the Rfam13 database to screen for the four known classes of ncRNA (rRNA, tRNA, snRNA and snoRNA). Reads that did not match the four types of ncRNA mentioned above were compared using Blast with mature miRNA sequences from plants in the miRBase22 database, from which conserved miRNA were screened. As a final summary of all the comparison results (Table S3), most of the RNAs were concentrated in the UNKNOWN category, with the second most abundant category being rRNA (Fig. 2). This suggests that there may be many more non-coding RNAs in the unknown category.

A total of 310 known miRNA and 278 novel miRNA were detected in the 12 libraries based on the comparison results of the miRBase 22.1 database and the miRDeep2 prediction results (Table S4). miRNA are generally conserved in plants and conserved miRNA have been identified in a total of 109 miRNAs among known miRNA and have been shown to be conserved in 68 plants. The largest miRNA family was MIR166 with 30 miRNA members, followed by MIR159, MIR167 and MIR156 with 24, 21 and 16 miRNA members, respectively (Fig. 1-E). PCA principal component analysis was performed on 12 replicates of samples from four groups of *Juglans mandshurica* (Fig. 1-C) and the results showed that replicates clustered together within the *Juglans mandshurica* group, indicating a low degree of variation between them, while the large variation between the *Juglans mandshurica* groups indicated significant differences between samples and could be used for differential expression analysis.

This sequencing was performed to analyze the base preference of pecan miRNA. The analysis showed a preference for uracil (U) for the first base of miRNA 19-23nt in length, adenine (A) for the first base of miRNA 24nt in length, and guanine (G) for the first base of miRNA 25nt in length (Fig. 3-A). Analysis of the base preferences for each site of the miRNA showed that U was relatively common in the first two

positions and cytosine (C) in the first three positions. Bases 23 and 24 showed a strong preference for U and C, and base 25 showed a strong preference for G (Fig. 3-B). This is in agreement with the results of previous studies.

2.2. miRNA differential expression analysis

According to the screening criteria for differentially expressed genes in small RNAs of *Juglans mandshurica* buds, we obtained a comparative library of *Juglans mandshurica* DEGs: M1vsM2:107 differential miRNA, T1vsT2:85 differential miRNA; T1vsM1, 46 differential miRNA ; T2vsM2:65 differential miRNA, and counted the common and unique sequences between the groups (Fig. 4-A). A total of 172 differential conserved miRNA were found in four groups of 12 libraries, and the abundance of differential miRNA was counted (Fig. 4-B). Clustering analysis was performed on the four groups of differential genes (Fig. 5).

2.3. Target gene prediction and GO, KEGG enrichment analysis

To better understand the function of DEGs, we predicted their target genes. A total of 638 genes were predicted as target genes and 2999 binding sites for miRNA to target genes were predicted (Table S5). The target genes were aligned with GO, KEGG, eggNOG, NR, Swissprot databases and blast to the Arabidopsis genome for observation of their function (Table S6). Target gene prediction revealed a total of 107 differentially expressed miRNA targeting 1022 potential mRNA loci in the M1vsM2 subgroup, with an average of 9.55 per mRNA/miRNA. Among the miRNA miR159b-3p and miR159a target the most mRNAs (32). Most miRNA target multiple loci, suggesting that a single miRNA can regulate multiple target genes. However, a single target gene can also be targeted by multiple miRNA. A total of 621 mRNAs can be regulated by more than one miRNA, with 12 mRNAs being targeted by 11 miRNA. According to the taxonomic annotation of the GO functions of DEG in M1vsM2, a total of 2954 functions can be observed, of which 2117 are Biological Processes (BP), 352 are Cellular Components (CC) and 485 are Molecular Functions (MF) (Table S7). The results showed that M1 and M2 were significantly enriched for 178 terms, of which 147 terms belonged to biological functions, 4 terms to cellular components and 27 terms to molecular functions. The most enriched term was binding (GO:0005488) while the enriched terms included stamen development (GO:0048443), androgen development (GO:0048466), and floral organ development (GO:0048438). This suggests that these genes may play a dominant role in M1vsM2 sexual differentiation (Fig. 6-A).

T1vsT2 had 85 differentially expressed miRNA targeting 906 mRNA loci with an average of 10.66 mRNA/miRNA. Of these miRNA annotated to target genes, miR159a-3p targeted the most miRNA (37), followed by miR159b-3p.1 (34). A total of 604 mRNAs could be regulated by more than 1 miRNA. Based on the results of the categorical annotation of GO functions in T1vsT2 (Table S7), it can be observed that a total of 2765 functions were annotated in this group, including 1980 biological functions, 307 cellular components and 477 molecular functions. Screening at a corrected P value (FDR) < 0.05, T1vsT2

significantly enriched a total of 238 terms. Of these, BP had 20, CC had 3 and MF had 26. The most enriched term was metabolic process (GO:0008152), followed by binding (GO:0005488) and cellular metabolic process (GO:0044237) (Fig. 6-B).

There were 46 differentially expressed miRNA targeting 416 mRNA loci in T1vsM1, with an average of 9.04 mRNAs/miRNA. Among these miRNA, miR159b-3p.1 and miR159c targeted the most mRNAs (34), followed by miR159a (33). A total of 247 mRNAs could be targeted by more than 1 miRNA. The categorical annotation results of GO functions showed that a total of 1653 functions were annotated in this group, including 1180 for BP, 183 for CC and 280 for MF (Table S7). Analysis of the significance of significant enrichment of GO functions showed that 163 functions were significantly enriched, including 130 biological functions, 5 cellular components and 19 molecular functions. The most enriched were cellular metabolic processes (GO:0044237), binding (GO:0005488) and organic cyclic compound binding (GO:0097159) (Fig. 6-C).

There were 65 differentially expressed miRNA targeting 656 mRNA loci under the T2vsM2 grouping, with an average of 10.09 per mRNA/miRNA. miR172b-5p targeted the most mRNAs (30), followed by miR156l (25). A total of 401 mRNA could be regulated by more than 1 miRNA, of which 12 mRNAs could be regulated by 11 miRNA. According to the categorical annotation results of GO functions, it can be observed that a total of 2476 functions were annotated in this group, including 1779 biological functions, 272 cellular components and 425 molecular functions (Table S7). 154 functions were screened for significant enrichment by GO function analysis, including 136 biological functions, 3 cellular components and 15 molecular functions. The most enriched term was metabolic process (GO:0008152), followed by binding (GO:0005488) and cellular metabolic process (GO:0044237) (Fig. 6-D).

The results of our differential expression analysis and GO and KEGG enrichment analysis for both protogyny and protandry types showed that there were 28 significantly differentially expressed miRNA, including 21 up-regulated genes and 7 down-regulated genes. GO enrichment analysis showed that a total of 299 functions were significantly enriched, with the most enriched function being cellular processes (GO:0009987), followed by metabolic processes (GO:0008152) and binding (GO:0005488).

KEGG enrichment analysis of the four groups (Table S8) showed that the most enriched target gene pathway in all four groups was Plant hormone signal transduction (ko04075), followed by Protein processing in endoplasmic reticulum (ko04141) and MAPK signaling pathway - plant (ko04016). Meanwhile, KEGG enrichment analysis of both protogyny and protandry types as a whole showed that the most enriched target gene pathways were Plant hormone signal transduction (ko04075), Amino sugar and nucleotide sugar metabolism (ko00520), and MAPK signaling pathway - plant (ko04016). This indicates that the target genes of the above pathways play a crucial role in the formation of both sex differentiation and heterosexualism mechanisms in *Juglans mandshurica*.

These target genes contain many transcription factors that are directly or indirectly involved in sex differentiation. For example, the SPL family targeted by miR156, the AP2 family targeted by miR172, the MYB family targeted by miR159 and miR171 can be used to regulate GA synthesis and signaling by

targeting the SCL gene family, and the ARF gene family regulated by the miR160/167 family can regulate growth hormone signaling, among others.

2.4. qRT-PCR for validation of gene expression

To verify the expression pattern of the high-throughput sequenced miRNA, we randomly selected nine miRNA for real-time fluorescence PCR analysis (Table S9). The expression trends of the selected miRNA were consistent with the sequencing results (Fig. 7), indicating that the sRNA sequencing results were reliable.

2.5. Key miRNA screening

Based on the results of differential gene expression, GO and KEGG enrichment analyses, combined with other plant sex differentiation studies, we inferred key *miRNA* that have an important influence on sex differentiation and the mechanism of heterozygous heterozygosity formation in *Juglans mandshurica* (Table 1), and constructed a key miRNA-mRNA targeting regulatory network using cytoscape (Fig. 8).

Table 1
Four groups of key differentially expressed miRNA.

Group	miRNA	DEM	Log2 Fold Change
M1vsM2	miR159c-3p	Down	-4.182016741
	miR399a	Down	-3.921752724
	miR319a	Down	-3.133464842
	miR171f-5p	Down	-2.485869043
	miR172d	Down	-1.630965443
	miR164-3p	UP	8.294286129
	miR167b	UP	6.595958558
	miR156b	UP	6.455034958
	miR156d-3p	UP	5.339716891
	miR171a	UP	4.132868324
	miR167c	UP	3.641872628
	miR396t	UP	2.903334354
T1vsT2	miR319a	Down	-4.000611515
	miR159c-3p	Down	-3.305003284
	miR319b	Down	-3.086782755
	miR172d	Down	-2.073500685
	miR167a	UP	2.921788545
	miR167b	UP	2.901437113
	miR171a	UP	2.771554322
	miR396t	UP	2.409984627
	miR319	UP	2.401037572
	miR166b	UP	2.274164037
	miR5139a	UP	2.039594749
T1vsM1	miR166c	Down	-5.189040491
	miR164a	UP	2.779404952
T2vsM2	miR166c	Down	-5.189040491
	miR164-3p	UP	10.00090635

Group	miRNA	DEM	Log2 Fold Change
	miR171c-5p	UP	6.912390786
	miR171b	UP	3.745594554

2.6. Combined analysis of miRNA sequencing and transcriptome sequencing

The miRNA-predicted target genes were compared with the significantly differentially expressed mRNAs in the transcriptome data to find the corresponding mRNAs (Fig. 9), which were subjected to GO functional enrichment analysis. The results showed that M1vsM2 enriched the most genes for the function of metabolic process (GO:0008152), T1vsT2 enriched the most genes for the function of regulation of biological process (GO:0050789), T1vsM1 enriched the most genes for the function of regulation of biological process (GO:0007275), T2vsM2 for the cellular macromolecule metabolic process (GO:0044260). All four groups were significantly enriched for functions related to sex differentiation, organic matter biosynthesis process (GO:1901576), reproduction (GO:0003006), flower development (GO:0009908), etc. This suggests that these miRNA and mRNAs play a key role in the direction of sexual differentiation.

3. Discussion

Sexual differentiation in plants has gradually become a popular area of academic research in recent years, but research into the mechanisms of sexual differentiation in heteromorphic plants is currently lacking in depth. miRNA are an important class of regulatory molecules in plants and play an important role in the process of plant sex differentiation[24]. Expression of miRNA and their regulated target genes significantly affects the development and transformation of plant sexual organs[25, 26].

In this study, sRNA libraries were constructed for the protogyny female buds, protogyny male buds, protandry female buds and protandry male buds of *Juglans mandshurica*. Clean data pair ratios indicated good sequencing quality. The results of the analysis showed that there were significant differences between protandry and protandry female and male flower buds. miRNA length distribution statistics showed that miRNA of 24nt length were the most abundant in *Juglans mandshurica*. The analysis of base preference was consistent with previous studies. 12 libraries were constructed from three replicates of four sets of samples, and 310 known miRNA and 278 novel miRNA were detected. 172 DEGs were found, and 638 target genes and 2999 target gene cleavage sites were predicted.

Sexual differentiation and heterozygosity are not only directly regulated by genes, but hormonal regulation also plays a key role in the reproductive process. In the GO functional enrichment analysis of differential miRNA target genes in male and female buds of *Juglans mandshurica*, many genes were assigned to plant floral organ development, stamen and pistil development, and many genes were assigned to biological processes related to reproductive development. In the KEGG pathway enrichment

analysis, Plant hormone signal transduction enriched the largest number of target genes. For example, in this study, miR159 and miR319 together regulated the GAMYB gene, and GAMYB could respond to GA signaling[27]. In *Cucumis sativus* L, miR159 has been shown to regulate the ratio of male to female flowers by regulating the GAMYB gene family, and silencing GAMYB1 leads to a reduced ratio of male to female flowers in *Cucumis sativus* L[28]. miR319 is highly similar to miR159 in *Arabidopsis*. The present sequencing showed that TCP4 and MYB33 could be targeted by miR159 and miR319, while miR167 was repressed in expression by TCP4 and MYB33[29], thereby promoting the expression of ARF6 and ARF8, two key genes for flower formation. miR167 and miR319 affect FT gene expression by targeting FTIP1[30–33]. miR319 and miR159 are highly expressed in the pistil and relatively low in the stamen, while miR167 is expressed in the opposite direction, suggesting that these three miRNA families play a key role in the sexual differentiation of *Juglans mandshurica*. Previous studies have shown that miR319 is expressed at low levels and in low abundance in plants[34–37]. However, the present study measured relatively high miR319 expression, which is different from previous findings. miR156 targets the SPL gene family to regulate flower development and sexual differentiation[38, 39]. In *Arabidopsis*, SPL3/4/5 can directly activate MADS box genes such as LFY, FUL and AP1 to promote flowering, and SPL9 can positively regulate the flowering promoters FUL, SOC1, and AGL42, which can also activate the transcription of miR172, thereby increasing the expression of miR172 and inducing flowering in plants [40]. miR172 can target AP2[41, 42] and remove the inhibitory effect of AP2 on FT, ultimately forming the miR156-SPL-miR172-AP2 regulatory pathway[43]. The miR156 family was all relatively under-expressed in pistils and over-expressed in stamens, as measured by the current sequencing, while miR172 was the opposite, consistent with previous reports in other species. ARFs, AFBs and TIRs as growth hormone response factors are growth hormone signaling F-box proteins and transit inhibitor response proteins regulated by miR160 and miR390, miR167 and miR393 families respectively. The expression of these four miRNA is significantly lower in female flowers than in male flowers. It is hypothesized that these four miRNA families play an important role in pistillate development by indirectly regulating the secretion of growth hormone, thereby promoting the sex differentiation of *Juglans mandshurica* flowers and facilitating the conversion of male to female flowers in *Juglans mandshurica*. This is consistent with results from studies in plants such as *Arabidopsis*[44][45], *Solanum lycopersicum* L[46, 47], *Cymbidium ensifolium*[48] and *Fragaria × ananassa* Duch[49]. miR399 in rice can influence SPLs, anther wall lignin synthesis and flavonoid pathways together with miR156 to affect male fertility[50]. miR396 targets the growth hormone releasing factor GRF and thereby regulates the level of plant growth hormone secretion, which can reduce pistil fertility[51–53].

The miR167, miR164 and miR171 families are significantly differentially expressed in *Juglans mandshurica*, with very high fold change values when comparing a protogyny and protandry flower buds. The miR164-targeted NAC gene family has been reported in *Juglans regia* L, suggesting that it may affect the formation and development of pistillogenetic, staminogenic and leaf bud primordia in *Juglans regia* L[54]. miR171 affects genes related to gibberellin and growth hormone transduction in cucumber[55], and is involved in another development in tomato, where overexpression renders male flowers sterile[56, 57].

This evidence leads us to speculate that miR167, miR164 and miR171 may be key miRNA families for heteromorphic differentiation in *Juglans mandshurica*.

Sex determination in plants is a complex biological process in which environment, age, genes and hormone secretion all influence sex differentiation[58]. miRNA play a key role in plant sex differentiation by suppressing the expression of target genes. In this study, it is hypothesized that miRNA affect sex differentiation by regulating the expression levels of target genes and thus hormone secretion in *Juglans mandshurica*, and that they form the heterosexualism between monoecious to dioecious plants.

4. Materials and Methods

4.1.Plant Material Collection and Preparation

The test materials were collected on 2 April 2021 from male and female flower buds of *Juglans mandshurica* in Liaodong, China. The protogyny was named C and the female buds were named T1 with three replicates of C1, C2 and C3 for female buds; the male buds were named T2 with three replicates of C4, C5 and C6 for male buds; the protandry was named X and the female buds were named M1 with three replicates of X1, X2 and X3 for female buds; and the male buds were named M2 with three replicates of X4, X5 and X6 for male buds. The total number of samples was 12. They were snap frozen in liquid nitrogen, stored at -80°C in a refrigerator.

4.2.Plant Material Collection and Preparation

Total plant RNA was extracted using the TRNzol Universal Kit, RNA-specific agarose electrophoresis was used to check for RNA degradation and contamination, RNA concentration was checked using a spectrophotometer and RNA integrity was checked using an Agilent 2100 Bioanalyzer system.

4.3. Small RNA library construction and sequencing

Small RNA library construction and sequencing were performed at Personalbio Technology (Shanghai, China). The special structure of the 3' and 5' ends of the small RNA is used to add adaptors to it and reverse transcribe it into cDNA. Libraries were quality checked using the Agilent High Sensitivity DNA Kit and quantified using the Quant-iT Pico GREen dsDNA Assay Kit. Finally, sequencing was performed in Single-End mode using the Illumina platform. Adapters and low-quality sequences were removed from the raw data and clean reads(Total reads) of 18-36nt were selected for subsequent analysis. The number of clean reads is counted and identical sequences in the sample are de-duplicated and their frequency counted, called Unique reads, for subsequent analysis. In Total Reads and Unique Reads, the number of sequences that are common and unique to different groupings is counted. Bowtie was called using mapper.pl in the MirDeep2 program to match Unique Reads to the reference genome database, and sequences matched to the reference genome were used to participate in subsequent analyses.

4.4. miRNA identification and analysis

Unique reads were matched against the Rfam13 database (<http://rfam.sanger.ac.uk/search/>) using Blast to screen rRNA, tRNA, snRNA and snoRNA from small RNA libraries, and the remaining unmatched data were matched against miRbase22 using Blast. The remaining unmatched data were compared against the miRbase22 database (<http://www.mirbase.org/>), and the number of mismatches in the above screening should be less than 2. Finally, to summarise the annotation of all small RNAs compared to other RNAs and to provide a unique annotation for all small RNAs, the available annotation results are listed in this order: known miRNA > novel miRNA > rRNA > tRNA > snRNA > snoRNA.

In this study, the frequency of different bases in the first position of miRNA of different lengths and the frequency of different bases in each position of all miRNA were counted separately. Using Blast to compare the miRBase database for close relatives of *Juglans mandshurica*, we observed the conservation of the compared miRNA between species. Conserved miRNA were searched for families using miFam.dat. Based on the number of sequences compared to conserved miRNA, the Reads Count values of miRNA were counted and their CPM values were calculated. According to the criteria for the identification of plant miRNA based on sequencing sources published by optimised over 2018 Axtell et al[59, 60]. The number of miRNA obtained from this sequencing was counted. The expression of conserved miRNA in each sample was collated and differentially expressed miRNA (DEGs) were analyzed using DESeq (version 1.18.0), with the screening criteria of differential expression significance (p-value) and fold change in expression (fold change) > 2 or < 0.5. Finally, all differentially expressed genes of each group were obtained and bi-directional clustering analysis of genes and samples was performed using the Pheatmap software package in R(4.2.1).

4.5.miRNA target gene prediction and analysis

The UTR sequence at the 3' end of *Juglans mandshurica* was used as the target sequence and the differentially expressed miRNA sequence was predicted for the target gene using psRobot.tat. Gene Ontology (GO) enrichment analysis was performed on the target genes by first mapping all target genes to each term in the Gene Ontology database, counting the number of target genes in each term, and calculating the term with significant enrichment of target genes using hypergeometric distribution, with FDR < 0.05 considered as significant enrichment. Using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database for target gene enrichment analysis, we counted the number of target genes contained at different levels of each KEGG pathway and identified the major metabolic and signaling pathways in which the target genes were involved. To identify the major biological functions performed by target genes associated with DEGs, the biochemical metabolic pathways and signaling pathways involved (q value < 0.05). Key miRNA were also identified based on sRNA expression, enrichment analysis results and progress in studying related miRNA in other closely related species.

4.6. Quantitative real-time fluorescence PCR assay

Nine differentially expressed miRNA were randomly selected and their expression profiles were examined by QRT-PCR to verify the accuracy of this sRNA sequencing. Total RNA was extracted using the TIANGEN kit, reverse transcribed to cDNA using the PrimeScript kit and Oligo (DT), and PCR reactions were

performed on a real-time PCR instrument by adding PCR reaction solution formulated according to the reaction system. Reaction procedure: 95°C for 5min, 95°C for 15sec, 60°C for 30sec, 40 cycles. Relative quantification of gene expression was calculated using the $2^{-\Delta\Delta CT}$ method using U6 as an internal reference gene and compared to miRNA sequencing expression.

4.7. Combined analysis of miRNA sequencing and transcriptome sequencing

Finding differential miRNA-differential mRNA target relationships based on analysis of the *Juglans mandshurica* bud transcriptome data, combined with predicted differential miRNA target genes and transcriptome differences. Diagrammed with cytoscape and functional enrichment analysis of mRNAs that are differentially targeted by miRNA.

5. Conclusion

The two main types of reproduction are heteromorphic *Juglans mandshurica*, protogyny and protandry we have studied the sRNA sequencing of the female and male inflorescences of the female and male inflorescences of the protogyny and male inflorescences of the protandry, respectively. Sex differentiation and dioecious heterozygous differentiation of *Juglans mandshurica*, from a miRNA perspective. Sex differentiation and dioecious heterosexuality in *Juglans mandshurica* from a miRNA perspective. The sequencing results identified 310 known miRNA and 278 novel miRNA, screened 172 differentially conserved miRNA, and predicted a total of 638 target genes using PSRobots. GO enrichment analysis showed that these target genes are mainly involved in biological functions related to reproduction and flower development in *Juglans mandshurica*, and KEGG enrichment analysis showed that the most enriched target gene pathway is Plant hormone signal transduction. These target genes are involved in the signaling of hormones such as GA, IAA, etc. For example, GAMYB1 is targeted by miR159, ARF by miR390, GRF by miR393, etc. Nine differentially expressed miRNA were randomly validated by QRT-PCR, demonstrating the accuracy of this sequencing method, and combined with transcriptome sequencing to construct a primary regulatory network for the sex differentiation mechanism of P *Juglans mandshurica*.

In conclusion, our study on the sequencing of miRNA from male and female flower buds of *Juglans mandshurica* and the identification of miRNA related to sex differentiation of *Juglans mandshurica* will help to elucidate the mechanism of sex differentiation of heteromorphic *Juglans mandshurica*, and this study will also provide theoretical support for other studies on *Juglans mandshurica*.

Declarations

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Authors' contributions Jingze Li conceived and designed the experiments, performed the experiments, analyzed the data, prepared the graphs and tables, and drafted and revised the manuscript. Mengmeng

Zhang, carried out the experiments, analyzed the data and approved the final draft. Jingwen Wu conceived and designed the experiments and approved the final draft. Baiting Qin prepared the graphs and tables, and drafted and revised the manuscript. Chunping Liu conceived and designed the experiments, approved the final draft, Lijie Zhang conceived and designed the experiments, analyzed the data, prepared the figures and tables, wrote or reviewed the draft paper, and approved the final draft.

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Availability of data and materials

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors. The sequencing reads are deposited in the National Center for Biotechnology Information under the BioProject number PRJNA975084

Ethics approval and consent to participate

***Juglans mandshurica* Maxim.** material was collected from the Eastern Liaoning Province, China. This plant species is widespread in China and it is not classified as an endangered species. No licence is required for the collection of plant material. Plant material was preserved in accordance with the institutional guidelines of the College of Forestry, Shenyang Agricultural University. All methods were carried out in accordance with relevant guidelines and regulations

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

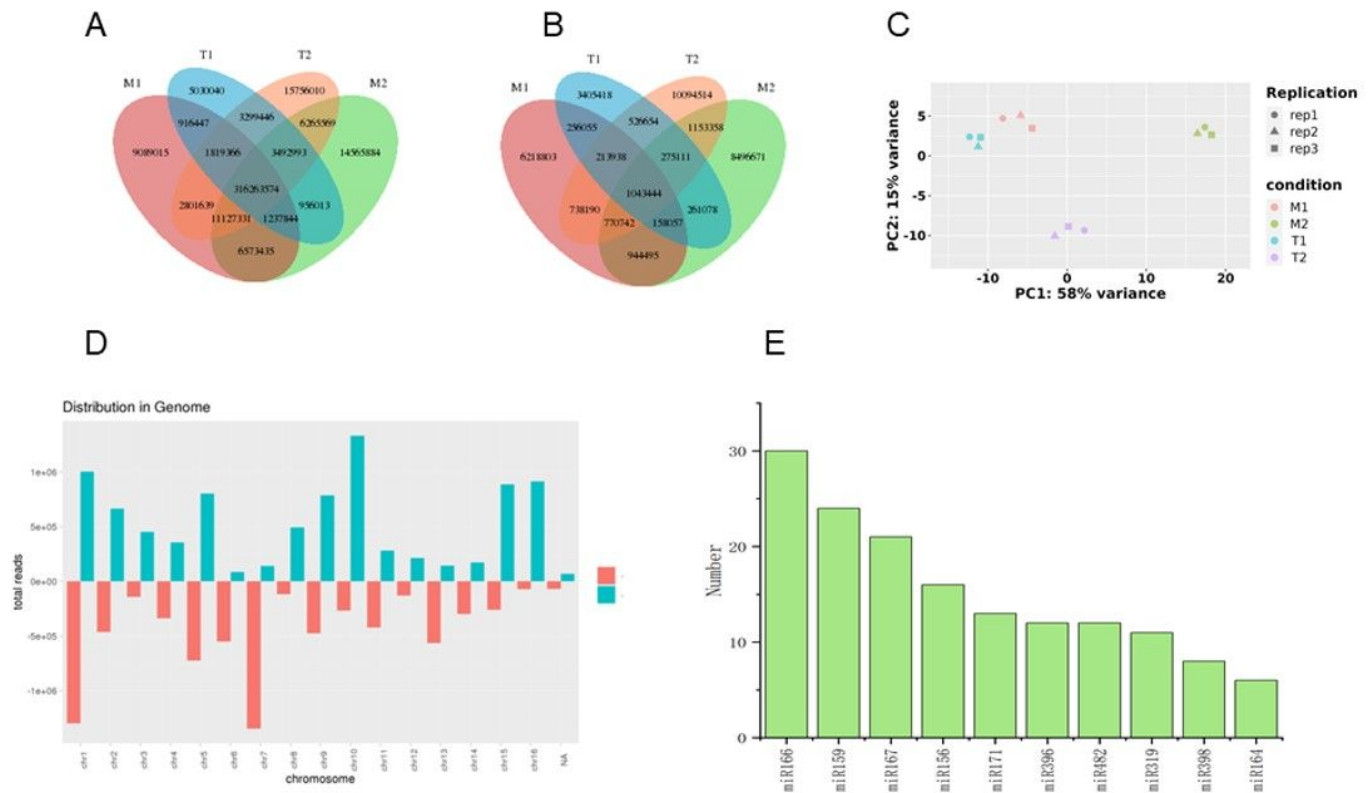


Figure 1

(A): Venn diagram of the total number of sequences, with the intersection being the total number of sequences common to both sets of samples. (B): Venn diagram of de-duplicated sequences between groups. (C): Principal component analysis of 12 libraries with 3 replicates from 4 groups of *Juglans mandshurica* samples (D): genomic distribution diagram, with the horizontal axis being the chromosome number, +/- indicating the direction of sequence alignment to the chromosome, and the vertical axis being the abundance of sequences aligned forward/reverse to each chromosome. (E): number of members of the top 10 conserved miRNA families identified.

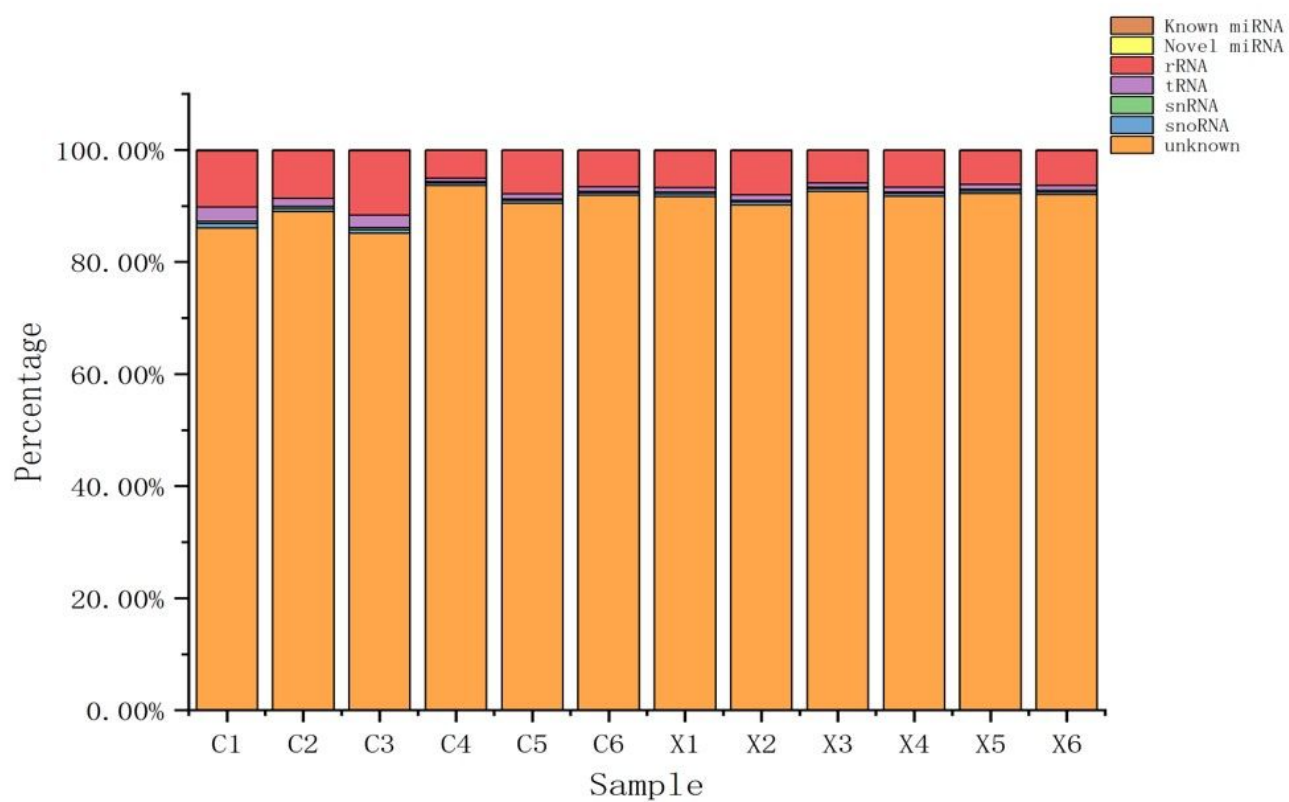


Figure 2

small RNA classification statistics chart

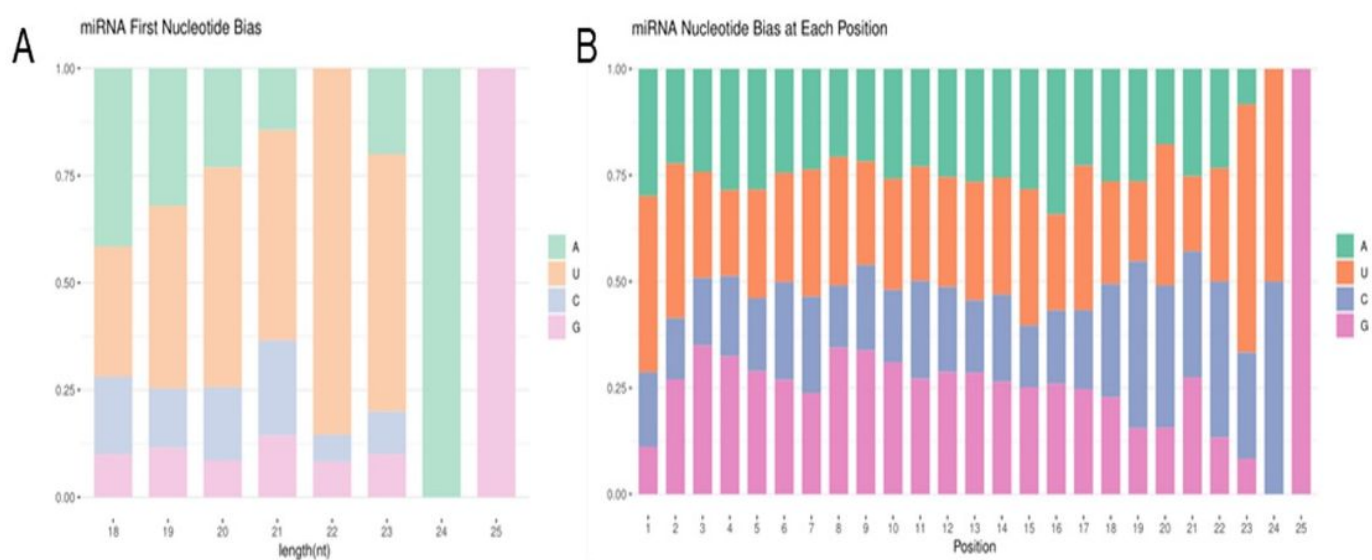


Figure 3

(A): Preference statistics for the first base of miRNA of different lengths; (B): Base preference statistics for each site of miRNA.

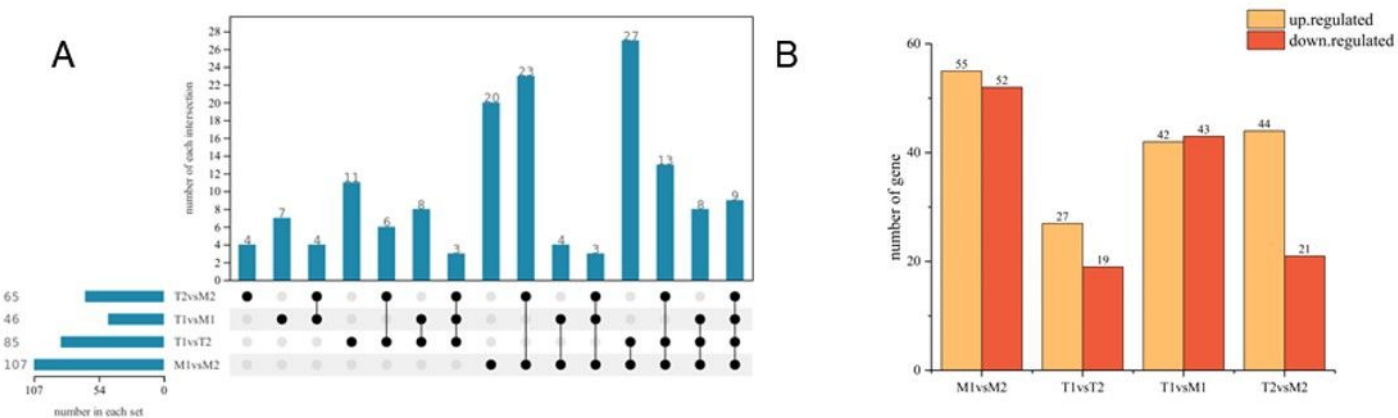


Figure 4

Number of differentially expressed miRNA in male and female buds (A): Upset plot of differential miRNA in four groups. (B): Mode and number of differential miRNA regulation in each group, red is up-regulated, yellow is down-regulated.

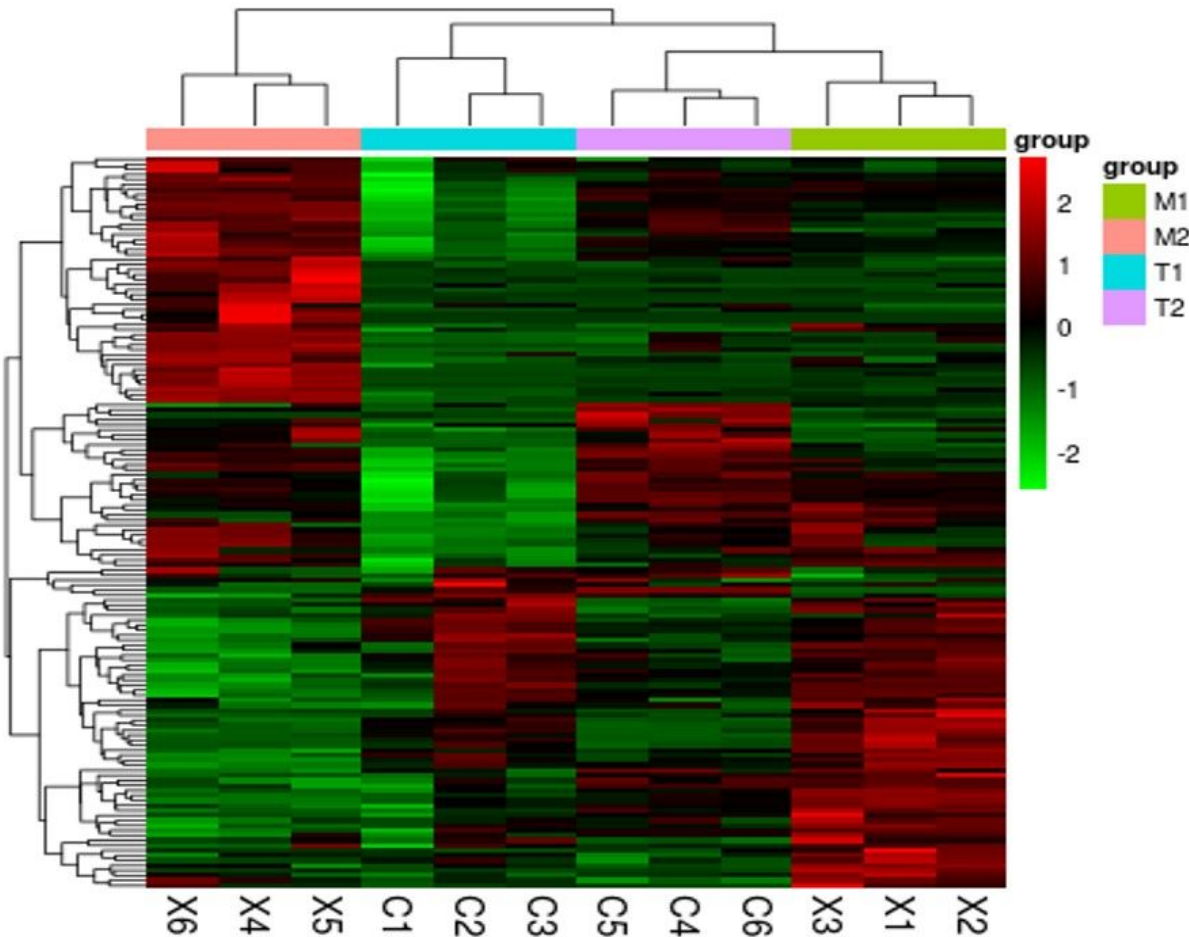


Figure 5

Heat map of differentially expressed miRNA clustering, with miRNA represented horizontally, one sample per column, high expression miRNA in red and low expression miRNA in green.

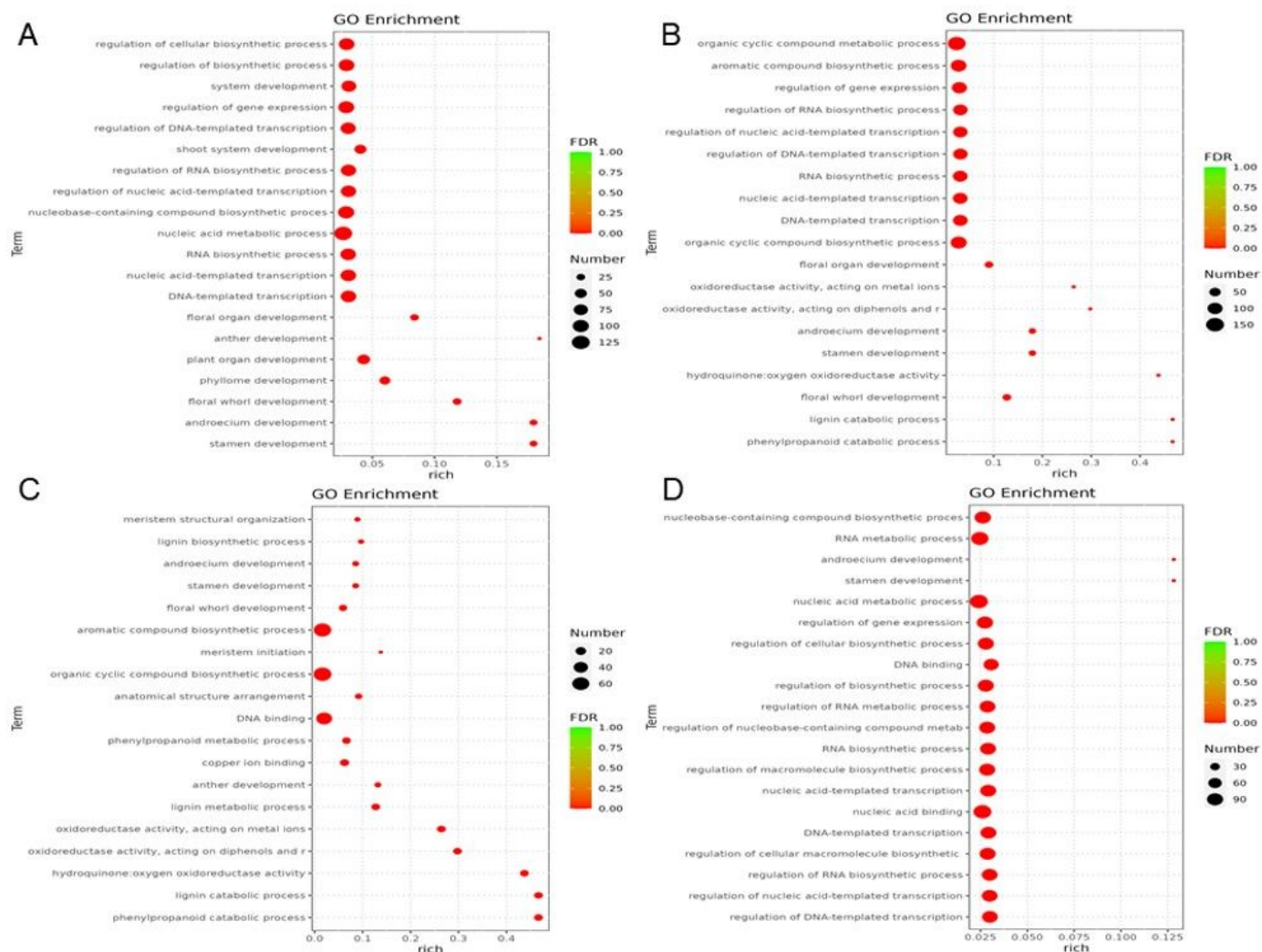


Figure 6

GO enrichment analysis (A): M1vsM2; (B): T1vsT2; (C): M1vsT1; (D): M2vsT2.

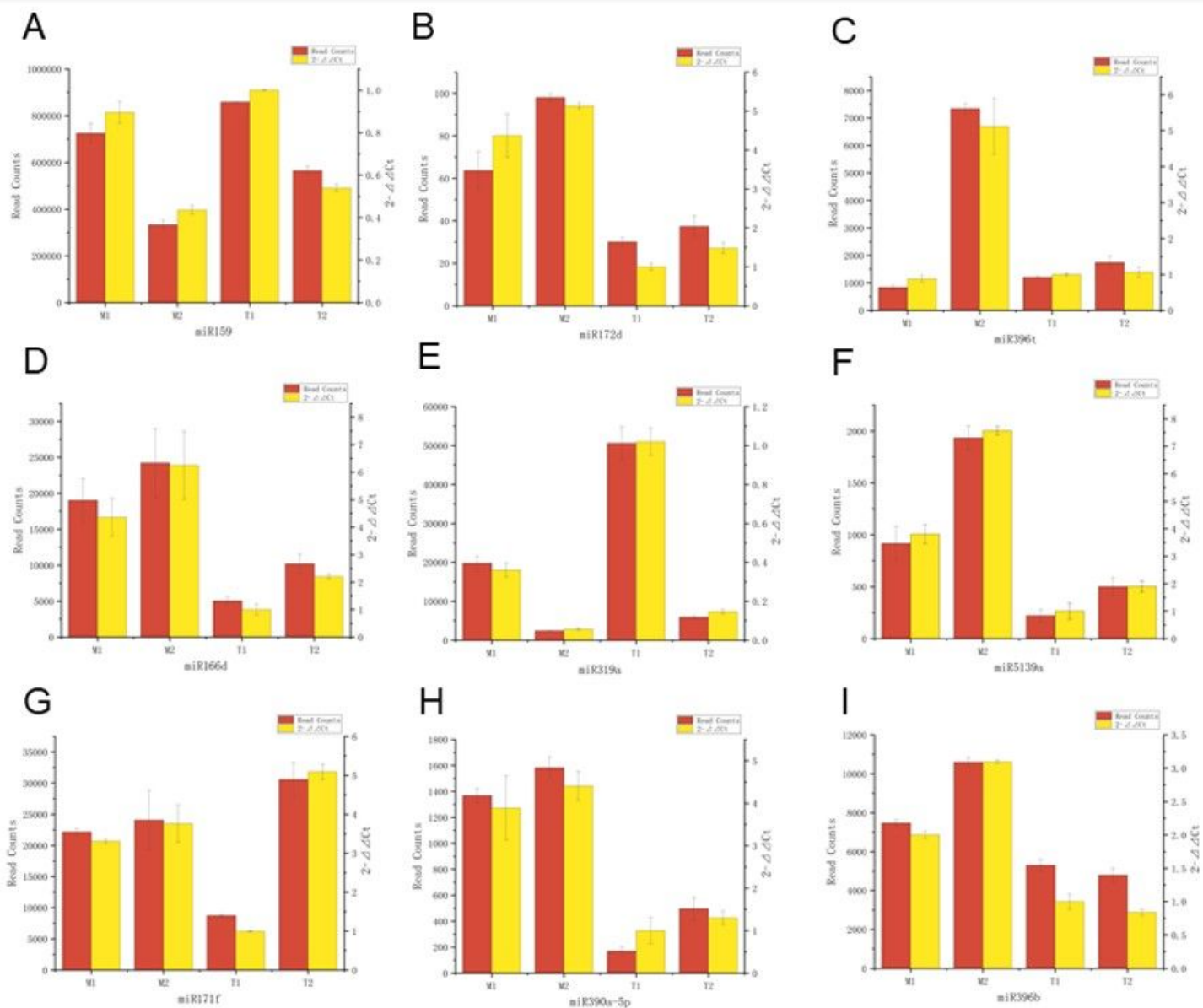


Figure 7

(A-I) Validation of nine randomly selected differentially expressed genes derived from miRNA-seq using qRT-PCR.

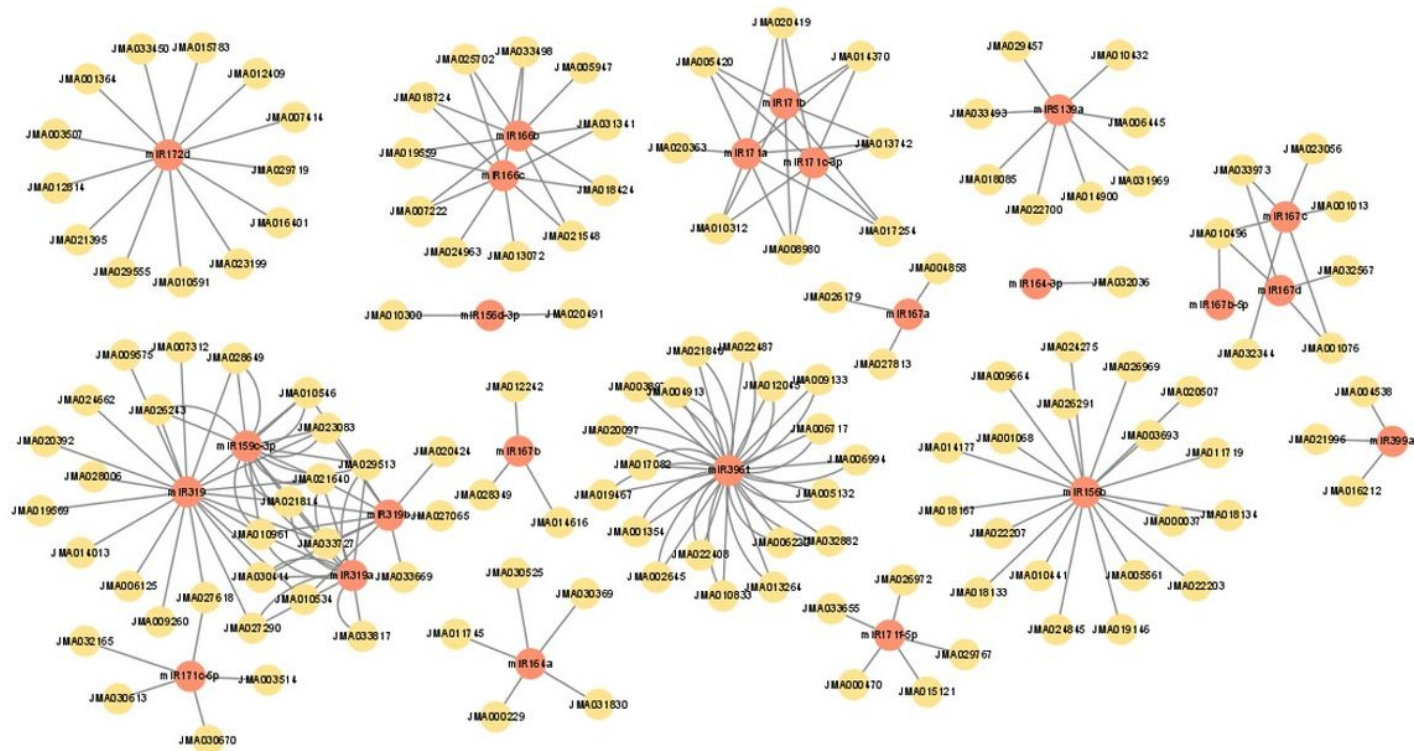


Figure 8

A network regulatory map of key miRNA and their target genes. The network contains 161 nodes and 221 edges. Red ovals represent miRNA and yellow ovals represent target genes.

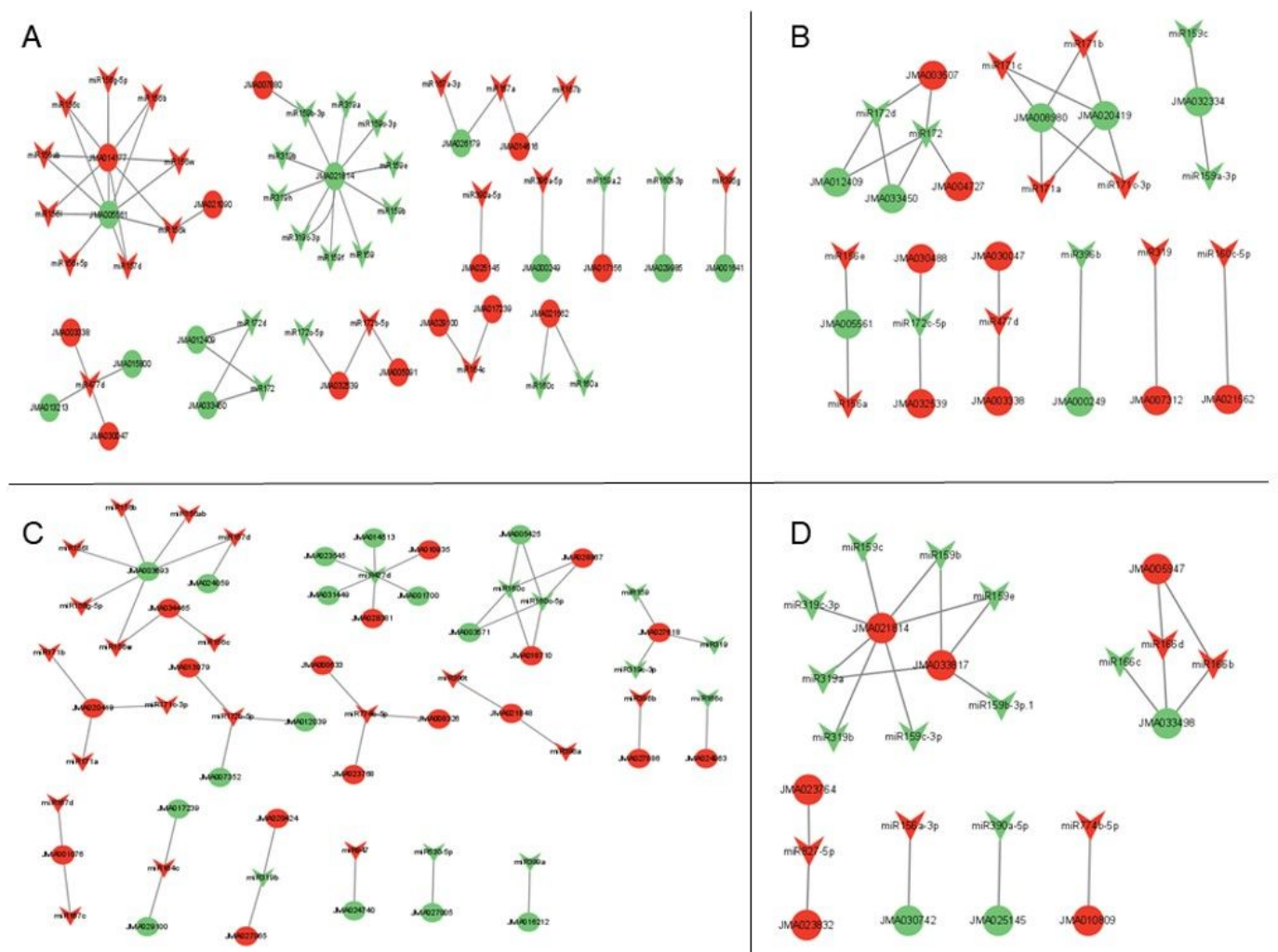


Figure 9

Plots of combined miRNA and transcriptome analysis, (A): M1vsM2; (B): T1vsT2; (C): T1vsM1; (D): T2vsM2. V represents significantly differentially expressed miRNA; circle represents differentially expressed mRNAs. red represents up-regulated, green represents down-regulated.

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