Small RNA and mRNA sequencing reveal roles of miRNAs involved in pomegranate female sterility

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Abstract: Female sterility is a key factor restricting plants reproduction. Our previous studies have revealed that pomegranate female sterility mainly arose from the abnormality of ovule development. MicroRNAs (miRNAs) play important roles in the ovule development. However, little is known about the roles of miRNAs in female sterility. Here, we used a combined high-throughput sequencing approach to unearth miRNAs and their targeted transcripts involved in female development. Finally, a total of 103 conserved and 58 novel miRNAs were identified. Comparative profiling indicated that the expression of 43 known miRNAs and 14 novel miRNAs were differentially expressed between functional male flowers (FMFs) and bisexual flowers (BFs), 30 known miRNAs and 9 novel miRNAs showed significant difference among different stages of BFs, 20 known miRNAs and 18 novel miRNAs exhibited remarkable expression differences among different stages of FMFs. Gene ontology (GO) analyses of 144 predicted targets of differentially expressed miRNAs indicated that 'reproduction process' and 'floral whorl development' processes were significantly enriched. miRNA-mRNA interaction analyses revealed six pairs of candidate miRNAs and its targets associated with female sterility. Interestingly, pg-miR166a-3p was accumulated whereas its predicted targets (Gglean012177.1 and Gglean013966.1) repressed in functional male flowers (FMFs), and the interaction between pg-miR166a-3p and its targets (Gglean012177.1 and Gglean013966.1) were confirmed by transient assay. A. thaliana transformed with 35S-pre-pg-miR166a-3p verified the role of pg-miR166a-3p in ovule development, which indicated pg-miR166a-3p’s potential roles in pomegranate female sterility. Our results provide new insights into molecular mechanisms underlying the female sterility at miRNA level.

Keywords: Pomegranate; MicroRNAs; reproduction process; andromonoecy; female sterility; ovule

1. Introduction

Female sterility is a widespread phenomenon in flowering plants restricting plants reproduction [1-3]. Abortion of either sexual organs is the initial step towards a sexually dimorphic specie [4]. Pomegranate (Punica granatum L.), one of the important deciduous fruit crops worldwide, is valued for its highly medicinal and economic values [5,6]. Pomegranate is characterized by the andromonoecy, two types of flowers are produced on the same plant, including the functional male flowers (FMFs) and bisexual flowers (BFs) [7,8]. FMFs, which are referred as “infertile” and “bell” flowers, have well-developed male parts, while abnormal female parts. They fail to set fruits and eventually fall from trees. Whereas, BFs, which are referred as “fertile” and “vase-shaped” flowers, have well-formed female and male parts and can set fruits [7,8]. Moreover, pomegranate flowers can appear to be solitary, paired, or clustered [8]. As our previously reported, the ratio of BFs was distinct
among different types of flowers. The ratio of BF's in clusters was noticeably fewer than that in solitary and paired ones [9]. Clustered terminal flowers contained more BF's than lateral flowers [9]. Although the proportion of FMFs is negatively correlated with crop productivity and yield [10], pomegranate female sterility may be helpful to optimize the allocation of limited nutrition resources to male and female function in the evolutionary eye [8]. In pomegranate, ovules are anatropous and bi-integument. FMFs' female sterility is closely related to abnormal ovule development, and their ovule development mainly ceased following the formation of the inner integument primordium [9].

miRNAs are endogenous 20-24 nt, non-coding RNAs that are derived from primary miRNA transcripts (pri-miRNAs) containing a stem-loop secondary structure [11,12]. They are known to suppress the expression of target genes at the post-transcriptional level via mRNA cleavage or translational inhibition [11,13]. In plants, miRNAs act crucial roles in plant organ development [14], stress tolerance [15], phytohormone signaling [16], growth phase change [17,18], and disease resistance [15]. The ovule development undergoes four main stages: initiation of primordia; regionalization of primordia into three regions: funiculus, chalaza, and nucellus; integuments development, and embryo sac development [19-21]. Lots of miRNAs have been shown to be important regulatory factors in ovule development [12,18]. For instance, miR167 is essential for correct patterning of gene expression during ovule development in A. thaliana, which regulates ovule growth by limiting ARF6 and ARF8 transcript expression domains in cells that will develop into integuments [22]. MiR166/165-insensitive phb-1d/+ mutant showed arrested outer integument in A. thaliana [23]. MiR165/6 regulate ovule development by restricting expression of the CLASS III HOMEO DOMAIN LEUCINE ZIPPER (HD-ZIP III) gene PHABULOSA (PHB) in specific regions [24]. PHABULOSA (PHB) and PHAVOLUTA (PHV) are expressed adaxially in the inner integument during ovule development [25]. Moreover, miR156 and miR157 regulate ovule development by targeting SPL/SBP box transcription factors [26,27].

The study of small RNAs in pomegranate has been reported previously [28-30]. However, the involvement small RNAs in pomegranate FMFs' ovule development ceasing has not yet been determined. We previously identified that the key stage of the termination of FMFs' ovule development was when its bud vertical diameter was 5.1-13.0 mm [9]. Genes influencing ovule development such as INO, ANT were digged out as candidate genes affecting pomegranate female sterility by RNA-seq [9]. To investigate miRNA-target modules involved in female sterility in pomegranate, sRNA-seq were conducted in pistils between three pairs of FMFs and BF's pistils: prior to, at the appearance of and subsequent to ovule inner integument primordium formation. Finally, candidate miRNA-target modules influence pomegranate female sterility were identified, which contributed to enhance our understanding of pomegranate ovule development.

2. Results

2.1. Deep sequencing of small RNAs of pomegranate fertile and sterile pistils

A total of 18 small RNA sequencing data were obtained which were deposited at the figshare database (https://figshare.com/articles/Small_RNA_sequencing_identify_miRNAs_involved_in_pomegranate_female_ sterility/9563579). Through sequencing of 18 accessions, we generated a total of 409.8 Gb clean reads, with the number of reads yielded from the 18 small RNA sequencing libraries ranged from 22.3 to 23.1 million. After aligning against the pomegranate reference genome, 84.8% to 89.6% of reads in 18 accessions were mapped to the pomegranate reference genome [31] (Table S1), with CG content varied from 47.1% to 50.1% (Table S1). The number of unique sRNAs for 18 accessions were ranged from 8.4 to 10.2 million, with 77% to 82.2% were mapped to the pomegranate reference genome [31] (Table S1). We found small RNAs of 20-24 nucleotides (nt) were dominant in all 18 sequencing libraries (Figure 1b), of which 24-nt length small RNAs showed a large percentage in the small RNA libraries (Figure 1b). Except for 24-nt length of small RNAs, 21-nt length small RNAs also showed a large percentage in the small RNA libraries (Figure 1b), suggesting the existence of post-transcription during pomegranate ovule development.
Figure 1. Schematic representation of sampling location and overview of the high-throughput sequencing data. (a) The locations with high ratio of FMFs and BFs and the external morphology of sampling stages. (b) Length distribution of small RNA reads in the sequencing samples. (c-e) Venn diagram showing unique and shared differentially expressed miRNAs. (c) differentially expressed miRNAs between FMFs and BFs, (d) differentially expressed miRNAs among FMFs development stages, (e) differentially expressed miRNAs among BFs development stages. FMFs, functional male flowers; BFs bisexual flowers; TNSI, TNSII, and TNSIII represent the BFs’ pistils when their vertical diameters were 3.0–5.0 mm, 5.1–13.0 mm, 13.1–25.0 mm, respectively; Similarly, the designations ATNSI, ATNSII, and ATNSIII were used to represent the FMFs’ pistils when their vertical diameters were 3.0–5.0 mm, 5.1–13.0 mm, 13.1–25.0 mm, respectively. Scale bars=1.0 cm.

2.2. Identifying miRNAs involved in pomegranate female sterility

A final set of 103 known miRNAs were identified from pomegranate BFs and FMFs’ pistils, including miR858, miR166/165, miR160, miR157, miR159, and miR408 families, etc. (Table S2). In addition, 58 novel miRNAs in pomegranate BFs and FMFs were also identified (Table S2). Among all identified miRNAs, 43 known miRNAs and 14 novel miRNAs were differentially expressed in comparison of FMFs and BFs (ATNSI_TNSI, ATNSII_TNSII, ATNSIII_TNSIII) (Table S3, Figure 1c), indicating the miRNAs related to pomegranate female sterility. A total of 30 of identified known miRNAs and 9 of novel miRNAs showed significantly different expression among different stages of BFs (TNSI_TNSII, TNSII_TNSII, TNSIII_TNSIII) (Table S4, Figure 1d). Moreover, a total of 20 known miRNAs and 18 novel miRNAs exhibited remarkable expression difference among different stages of FMFs (ATNSII_ATNSI, ATNSIII_ATNSI, ATNSIII_ATNSII) (Table S5, Figure 1e).

During different development stages of FMFs, 18 of the differentially expressed miRNAs showed significantly higher Seq-freqs in ATNSI than ATNSII and ATNSIII (Figure 2a), including two ATNSI specific miRNAs, pg-miR30 and pg-miR36 (Figure 2a). Seven of differentially expressed miRNAs showed significantly higher Seq-freqs in ATNSII than ATNSI and ATNSIII, including pg-miR444b.1 and pg-miR528-5p that were only identified in the ATNSII (Figure 2a). The other 13 differentially expressed miRNAs were up-regulated in ATNSIII than ATNSI and ATNSII, in which pg-miR6105b specifically expressed in ATNSIII and pg-miR166a-3p was abundantly expressed with reads count was greater than 100,000 during FMFs’ pistil development (Table S2, Figure 2a).
Figure 2. Sequencing frequency profiles of the known miRNAs and novel miRNAs (a) different expressed miRNAs among different stages of FMFs. (b) different expressed miRNAs among different stages of BFs. (c) different expressed miRNAs between FMFs and BFs. ATNSI-ATNSIII, FMFs’ pistils when their bud vertical diameters (BVDs) were 3.0–5.0 mm, 5.1–13.0 mm, and 13.1–25.0 mm, respectively. TNSI-TNSIII, BFs’ pistils when their BVDs were 3.0–5.0 mm, 5.1–13.0 mm, and 13.1–25.0 mm, respectively.

During different development stages of BFs, 15 of differentially expressed miRNAs exhibited up-regulated in TNSI than TNSII and TNSIII, of which pg-miR118, pg-miR7533a that were specifically expressed in TNSI (Table S2, Figure 2b). Moreover, 7 of differentially expressed miRNAs showed higher Seq-freqs in TNSII than TNSI and TNSIII (Figure 2b). The other 17 differentially expressed miRNAs manifested up-regulated in TNSIII (Figure 2b).

Among the 57 differentially expressed miRNAs between BFs and FMFs’ pistils, 15 showed differentially expressed in the comparison of TNSI and ATNSI (Figure 2c), in which pg-miR5671a, pg-miR7533a, pg-miR56, pg-miR528-5p and pg-miR7717a-5p showed specific expression in TNSI (Table S2, Figure 2c). In the comparison of TNSII and ATNSII, 10 miRNAs including pg-miR397a and pg-miR408-3p which were abundantly expressed showed up-regulated in ATNSII (Table S5, Figure 2c), on the contrary, 8 miRNAs including pg-miR160a-3p, pg-miR858b, pg-miR107 which were highly expressed exhibited significantly lower Seq-freqs in ATNSII than TNSII (Table S5, Figure 2c). Of the 27 differentially expressed miRNAs in comparison of ATNSIII and TNSIII, 11 miRNAs showed up-regulated in ATNSIII, in which pg-miR398b, pg-miR166a-3p and pg-miR902j-5p were highly expressed (Table S5, Figure 2c). The other 16 miRNAs showed down-regulated in ATNSIII (Table S5, Figure 2c).

2.3. Validation of expression patterns of miRNAs

To further confirm the miRNA sequencing results, the expression of 10 randomly selected miRNAs (pg-miR1514a-3p, pg-miR5671a, pg-miR4414 b, pg-miR408-3p, pg-miR398b, pg-miR398a-3p, pg-miR166a-3p, pg-miR397a, pg-miR167f-3p and pg-miR160a-3p) were validated using stem-loop qRT-PCR. The results showed that the consistency rate was 88%. The expression patterns of miRNAs were largely consistent with those determined by high-throughput sequencing, except for the expression level of pg-miR5671a between TNSII_TNSI, pg-miR398a-3p between ATNSII_ATNSI and pg-miR1514a-3p between TNSIII_TNSII showed opposite expression trend between qRT-PCR and deep sequencing (Figure 3), implying the reliability of miRNA sequencing data.
Figure 3. qRT-PCR validation of differential expression patterns of miRNAs between different stages. *P<0.05 and **P<0.01 (Student’s t-test).

2.4. Identification and analysis of miRNA targets

A total of 208 and 177 target genes were identified for the conserved miRNAs and novel miRNAs, respectively (Table S6), of which 144 were targeted by the differentially expressed miRNAs. Some novel miRNAs targeted the same gene families as conserved miRNAs (Table S6). For example, both pg-miR07 and pg-miR166a-3p targeted HD-ZIP genes, Gglean013966.1 and Gglean012177.1. Pg-miR01 and pg-miR858b targeted MYB family genes (Table S6).

To better understand the roles of miRNA in pomegranate female sterility, 144 targets of the differentially expressed miRNAs were used for GO analysis. A total of 19 biological processes, 11 molecular functions, and 2 cellular components were significantly enriched (correction value at P<0.01) (Figure S1, Table S7). Based on biological processes, the overrepresent terms were reproduction...
process (GO:0000003, GO:0022414, GO:0003006), post-embryonic development (GO:0009791), lignin
catabolic process (GO:0046274, GO:0046271, GO:0009808, GO:0009698) and floral whorl development
(GO:0048438, GO:0048437) (Figure S1a). Based on molecular function, cation binding (GO:0043169),
ion binding (GO:0046872, GO:0043167), copper ion binding (GO:0005507), transition metal ion
binding (GO:0046914) were prominently enriched (Figure S1b). As shown in Figure S1b, under the
category of cellular component, apoplast (GO:0048046, GO:0005576) was significantly enriched.

2.5. miRNA–mRNA interaction identification

To identify the potential miRNA–mRNA regulatory network that may related to pomegranate
female sterility, the expression patterns of 144 genes targeted by the differentially expressed miRNAs
were retrieved from the RNA-seq data (accession numbers SRX2735567-SRX2735584) (Table S8).
miRNAs are known to suppress the expression of target genes by mRNA cleavage or translational
inhibition [32]. To explore the roles of miRNA and mRNA interactions in female sterility, we studied
negatively correlation in miRNA-target modules (Table 1). We found pg-miR858b and pg-miRN01
co-repressed the expression level of Gglean012452.1 in ATNSI, and pg-miR858b and pg-miRN01
showed up-regulated in ATNSI between ATNSI and TNSI, whereas, Gglean012452.1 showed down-regulated in ATNSI (Table 1). Between ATNSII and TNSII, pg-miRN11 and pg-miR858b exhibited
higher seq-freqs in TNSII, their targets Gglean007369.1, Gglean014673.1, Gglean008242.1, Gglean018250.1, Gglean023939.1, and Gglean004315.1 showed opposite expression levels (Table 1). Pg-miR165a-3p and pg-miR444b.1 negatively regulated the expression of Gglean013966.1, Gglean012177.1 and Gglean003233.1 during the key stage of pomegranate female sterility (ATNSII_TNSII)
respectively (Table 1). Three negatively correlated miRNA-target modules were found between
ATNSIII and TNSIII (Table 1), including pg-miR166a-3p-Gglean012177.1, pg-miR166a-3p-
Gglean013966.1, and pg-miR952b-Gglean018134.1.Pg-miR858b, pg-miRN11, pg-miR165/166a-3p, pg-
mR444b.1, pg-miR952b and their targets were mined as candidate factors influencing pomegranate
female development. These results highlighted the complicated interactions between miRNAs and
mRNAs during pistil development in pomegranate.

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Table 1. Predicted mRNA targets of differentially expressed miRNAs in ATNS1_TNS1, ATNSII_TNSII
and ATNSIII_TNSIII.
As mentioned above, pg-miR166a-3p was highly active during the BFs and FMFs’ pistil development, and it was accumulated at a higher level in the pistil of FMFs than BFs (Figure 4a). A. thaliana plants transformed with pg-miR166a-3p driven by the CaMV35S promoter exhibited declining in plant biomass, clip size and seeds number (Figure 5a). The expression level of pg-miR166a-3p in different lines were detected (Figure 5b), L2 (with high expression level) and L3 (with relative low expression level) were selected to observe the development of buds and ovules and count the number of the seeds. The results showed that the number of meristems of inflorescence increased in transformants (Figure 5c), whereas the number of ovule primordia decreased significantly \((P < 0.01)\) (Figure 5c). The number of seeds was negatively correlated with the expression level of pg-miR166a-3p (Figure 5d-e). Taken together, the phenotypes observed in transgenic A. thaliana implied the role of pg-miR166a-3p in flower primordium and ovule development.

**Figure 4.** pg-miR166a-3p targeted *Gglean012177.1* was verified in vivo. (a) Expression level of pg-miR166a-3p, *Gglean012177.1* and *Gglean013966.1* during the FMFs development. (b) Vectors used in tobacco co-expression assays in each lane were shown in the table. (c) three kinds of overexpression vectors. (d) verification of the interaction between pg-miR166a-3p and *Gglean012177.1*. 
Figure 5. The phenotypes of 35S-pre-pg-miR166a-3p. (a) the external morphology of 35S-pre-pg-miR166a-3p and wild type A. thaliana, the white arrow showed the location of seeds. (b) Relative expression level of pg-miR166a-3p in different transgenic lines. (c) the internal morphology of 35S-pre-pg-miR166a-3p and wild type Arabidopsis. (d) seeds number of 35S-pre-pg-miR166a-3p line 2. (e) seeds number of 35S-pre-pg-miR166a-3p line 3.

In addition, pg-miR166a-3p and its predicted targeted genes Gglean012177.1 and Gglean013966.1 which were enriched in reproductive structure development process (GO:0048608) showed reciprocally expression patterns during BFs and FMFs’ pistil development (Figure 4a). The expression levels of Gglean012177.1 and Gglean013966.1, encoding the homeobox-leucine zipper (HD-ZIP) protein, gradually decreased from ATNSI to ATNSIII (Figure 4a), but HD-ZIP protein positively regulate ovule patterning and growth [33]. Collectively, the decreasing of HD-ZIP family genes (Gglean012177.1 and Gglean013966.1) and increasing of pg-miR166a-3p expression level from ATNSI to ATNSIII may related to the abnormal development of FMFs’ ovule. To verify the interactions between pg-miR166a-3p and two potential targets Gglean012177.1, Gglean013966.1 in vivo, a transient assay was conducted in tobacco leaves (Figure 4b and 4c, Figure 6a and 6b). The interactions were determined by the significant reduction of GFP fluorescent signal intensity that resulted from co-expression of pg-miR166a-3p with their target sites in Gglean012177.1 and Gglean013966.1. The results showed that both Gglean012177.1 and Gglean013966.1 could be mediated cleavage by pg-miR166a-3p (Figure 4c and 4d, Figure 6b and 6c). A modified target site (inactive site) and a perfect complementary site were used as negative and positive control respectively (Figure 4d, Figure 6c). These results indicated that pg-miR166a-3p can interact with its targets (Gglean012177.1 and Gglean013966.1) to affect the ovule development.
Figure 6. pg-miR166a-3p targeted Gglean013966.1 was verified in vivo. (a-b) three kinds of overexpression vectors. (c) verification of the interaction between pg-miR166a-3p and Gglean013966.1.

3. Discussion

Female sterility is one of the key factors restricting pomegranate yield [8]. Previous study has caved many transcription factors that may affect pomegranate pistil development, of which many were miRNAs targets [9]. In order to improve our understanding of pomegranate female sterility, we performed miRNAs expression profiles of developing FMFs and BF’s pistil in pomegranate, which was the first comprehensive analysis of miRNA expression pattern during pistil development in pomegranate. Based on the analyses of the differential expressed miRNAs in pistil between FMFs and BFs at different stages, we found that pg-miR858b and pg-miR01 harbored a strong association with pistil development at the early stage. pg-miR444b.1, pg-miR11, pg-miR166a/165a-3p and pg-miR952b may influence pomegranate integument development at later stages. Specifically, overexpression of pg-miR166a-3p in A. thaliana, exhibited declining in plant biomass, clip length and ovule number, implying the potential roles of pg-miR166a in pomegranate ovule development.

3.1. Newly identified miRNAs in pomegranate

Several conserved and novel miRNAs in pomegranate have been identified in previous studies through high-throughput small RNA sequencing [28-30]. Compared with the miRNAs expressed in fruit [30], a total of 28 known miRNAs identified in this study were conserved in pistil and fruit, while 67 known miRNAs were newly identified in pistil. Of the 28 conserved miRNAs, pg-miR160a-5p, pg-miR166a-3p, pg-miR167h, pg-miR168a, pg-miR159a, pg-miR171b and pg-miR319a-3p were abundantly enriched in both pistil and fruit, which indicated their pleiotropy in pistil and fruit development. The newly identified miRNAs including 1 up-regulated (pg-miR8712) and 7 down-regulated (pg-miR1514a-3p, pg-miR2592s-3p, pg-miR5671a, pg-miR7533a, pg-miR7717a-5p, and pg-miR7782-3p) miRNAs in comparison of ATNSI and TNSI, 4 up-regulated (pg-miR444b.1, pg-miR528-5p, pg-miR6300 and pg-miR5671a) and 3 down-regulated (pg-miR444b, pg-miR6161a and pg-miR8723a) miRNAs in comparison of ATNSII and TNSII, 7 up-regulated (pg-
miR1077-5p, pg-miR393h, pg-miR403c-5p, pg-miR4376, pg-miR5152-3p, pg-miR902j-5p, pg-miR952b) and 6 down-regulated (pg-miR1172.1, pg-miR170-5p, pg-miR4240, pg-miR5223, pg-miR842-5p and pg-miR8700) miRNAs in comparison of ATNSIII and TNSIII. Those miRNAs including pg-miR166a-3p, pg-miR8712, pg-miR444b.1, pg-miR528-5p, pg-miR6300 and pg-miR5671a, pg-miR1077-5p, pg-miR393h, pg-miR403c-5p, pg-miR4376, pg-miR5152-3p, pg-miR902j-5p and pg-miR952b, up-regulated in FMFs, may play important roles in pomegranate female sterility.

3.2. Potential roles of miRNAs in pomegranate female sterility

BFs of pomegranate have fertile stamens and carpel, whereas, FMFs have fertile stamens but arrested pistils (Figure 7). FMFs’ pistils mainly arrested after ovule inner tegument primordium formed [8,9]. Recently, increasing attention has been paid to the roles of miRNAs in ovule development, including A. thaliana [22], cotton [18], and rice [34]. In our study, a total of 161 miRNAs (including conserved and novel miRNAs) were identified in BFs and FMFs’ pistil.

Figure 7. Pomegranate miRNA-mediated interaction network during BFs and FMFs pistil development stages. I: ATNSI and TNSI, II: ATNSII and TNSII, III: ATNSIII and TNSIII.

miR858 has been reported to be highly expressed in cotton ovules at 0 and 3-days post anthesis, while its target gene GhMYB2 was poorly expressed [35], which indicated the roles of miR858 and GhMYB2 in cotton ovules development. Tomato myb21 Cas9 mutants showed female sterility, which indicated a function of MYB21 in tomato ovule development [36]. In this study, conserved pg-miR858b showed up-regulated at early stages in FMFs, and its predicted target Gglean012452.1, which was annotated as MYB2 homology gene, exhibited the opposite pattern of expression (Figure 7, Table 1), suggesting pg-miR858b and Gglean012452.1 may have impacts on pomegranate female sterility. Moreover, the novel miRN01 also showed up-regulated in ATNSI, and it targeted the same gene as miR858b (Figure 7, Table 1). Therefore, we speculated that miR858b and miRN01 may co-repress the expression of Gglean012452.1 to affect the development of pomegranate ovule development at early stages.

miR444b.1, miRN11, miR166a/165a and miR952b showed differentially expressed between BFs and FMFs at the later stages when morphological differences occurred (Figure 7). miR444b.1 showed differentially expressed between ATNSII and TNSII, and its target Gglean003233.1, encoded a leucine-rich repeat-containing protein, exhibited down-regulated in comparison of ATNSII and TNSII (Figure 7, Table 1). Leucine-rich repeat (LRR) proteins are involved in a number of biology processes, including seed and anther development [37,38]. Our study focused on the roles of LRR proteins in ovule development. In future, much work will be needed to show whether ‘pg-miR444b.1-Gglean003233.1’ regulate pomegranate female development. MiRN11, a 22-nt novel miRNA with a
relatively abundant read count in TNSII, was not expressed in ATNSII. Its target gene Gglean014673.1 was up-regulated in ATNSII compared with TNSII (Figure 7, Table 1). Gglean014673.1 encoded a KINβ1, homology of which was reported to be involved in regulation of sugar metabolism [39], and the latter participated in the determination of plant sexual reproduction [40]. Therefore, ‘miRN11-Gglean014673.1’ module may affect pomegranate female sterility by regulate sugar metabolism.

The miR166a/165a group has been reported to regulate carpel development [41] and its targets, class III homeodomain leucine zipper (HD-ZIP III) transcription factors, play important roles in ovule development. Loss of function of HD-ZIP III genes showed aberrant ovule development in Arabidopsis [33]. In this study, pg-miR166a-3p abundantly expressed at FMFs, its expression level gradually increased from ATNSI to ATNSIII. Its targets Gglean012177.1 and Gglean013966.1, belonging to HD-ZIP III family, showed the opposite expression patterns. The decrease of Gglean012177.1 and Gglean013966.1 expression level may be important factor affecting ovule development. Moreover, the phenotype of transgenic A. thaliana suggested that pg-miR166a-3p influence the development of ovules. Furthermore, pg-miR166a-3p has a capacity to bind to Gglean012177.1 and Gglean013966.1, as confirmed by transient expression. Overall, these results indicated roles of pg-miR166a-3p in modulating pomegranate female sterility.

3.3. miRNA targets regulating reproduction development involved in female sterility

It has been proposed that pomegranate FMFs developed with arrested ovules and stigma [8-9], which was involved in floral whorl development. In this study, GO enrichment of differentially expressed miRNA-targets between BFs and FMFs’ pistils revealed that the GO term of ‘reproduction development’ was significantly enriched (P < 0.01), suggesting genes involved in pomegranate female sterility. Additionally, Gglean019708.1 and Gglean029859.1 were predicted as the targets of pg-miR397a and pg-miR408a, respectively. Similarly, Gglean012177.1, Gglean013966.1 and Gglean031286.1 were predicted as pg-miR166a-3p’s targets. These targets were enriched in ‘reproduction development’ process (Table S8).

Among these targets, the homologous gene of Gglean029859.1, PLANTACYANIN, is mainly involved in the anther development and pollination [42], but its role in the pistil development need to be further confirmed in pomegranate. Gglean012177.1, Gglean013966.1 and Gglean031286.1 belong to the HD-ZIPIII gene family, and their expressions may affect the development of the adaxial tissue of lateral organs [43]. Gglean012177.1 is homologous to PHB gene, which was involved in the ovule patterning and growth [44], loss of function of PHB gene displays aberrant integument development [45]. PHV (homologous gene of Gglean013966.1) and PHB overlap in function and expression pattern. Both of them directly regulate integument development in A. thaliana [44]. REVOLUTA (REV) (homologous gene of Gglean031286.1) restrict outer-integument [45]. FMFs’ ovules development mainly ceased following the formation of the inner integument primordium [9]. Thus, regulation of those genes affecting integument development may lead to female sterility. Gglean012177.1 and Gglean013966.1 showed up-regulated in BFs’ pistils than FMFs’ pistils, homologous of them were reported to have impacts on integument development [45], indicating their roles in pomegranate female sterility.

4. Materials and Methods

4.1. Sample collection

In this study, pomegranate pistils were collected from 12-year-old ‘Tunisia’ (also named ‘Tunisiruanszi’) trees grown in nursery of the Zhengzhou Fruit Research Institute (CAAS) located in Zhengzhou, Henan, China, and managed with conventional methods. Solitary flowers located in perennial branches, which contained high ratio of BFs, were selected as the sampling location of BFs’ pistils. Lateral flowers located in annual branches, which were observed with high ratio of FMFs, were selected for FMFs’ pistils (Figure 1a). On the other hand, our previous studies have showed that the key stage for the termination of FMFs’ ovule development was during the bud vertical diameter (BVD) was 5.1–13.0 mm [9]. So BFs and FMFs’ ovary and stigma were cutted off from flowers as
pistils according to the BVD, which was measured using digital caliper. Pistils of FMFs at the pre-stage of FMFs’ ovules aborted (BVD 3.0–5.0 mm), the key stage of FMFs’ ovules aborted (BVD 5.1–13.0 mm), the stage post FMFs’ ovules aborted (BVD 13.1–25.0 mm) were collected for female sterility related miRNAs mining, pistils of BFs at the corresponding periods were selected as controls. Each stage with three biology repetitions.

In order to denote the accessions clearly and easily, the TNSI, TNSII, and TNSIII were used to represent the BFs’ (Bisexual flowers) pistils when their BVD was 3.0–5.0 mm, 5.1–13.0 mm, 13.1–25.0 mm, respectively. Similarly, the designations ATNSI, ATNSII, and ATNSIII were used to represent the FMFs’ (Functional male flowers) pistils when their BVD was 3.0–5.0 mm, 5.1–13.0 mm, 13.1–25.0 mm, respectively (Figure 1a).

4.2. RNA isolation, library construction, and sequencing

Total RNA was extracted from each sample using CTAB (Cetyltrimethyl Ammonium Bromide) method [46]. The concentration and integrity of total RNA were detected using NanoDrop 1000 (Thermo Fisher Scientific, Massachusetts, USA) and 1.5% agarose Electrophoresis.

The RNA with different size were isolated using polyacrylamide gel electrophoresis (PAGE). The 18-30 nt RNA was selected and recovery using a recovery kit following the manufacturer’s protocol. First- and second-strand complementary DNA (cDNA) was synthesized after adding the 5’ and 3’ adaptors. The double strands cDNA was amplified and then recovered using PAGE. The final libraries were sequenced using the Illumina HiSeq 2000 platform with 50 bp by IGENECODE (Beijing, China). Finally, more than 20 Mb sequence was generated for each sample.

4.3. Bioinformatic analysis

Low quality reads and adaptors from the raw reads were filtered using fastx toolkits [47]. Data filtering includes removing adaptor sequences, contamination and low-quality reads [low-quality reads: reads in length ≤ 15 nt, reads with <3 copies; and junk reads (≥80% A, C, G or T; ≥3 N; only A, C, or only G/T)] from raw reads. In view of the length of miRNA (20-24 bp), only reads with 20-24 bp in length and greater than 3 in depth was used to further analyses. The correlations among biological replicates were evaluated using coefficient package in R. The filtered reads for each sample were aligned against the pomegranate ‘Tunisia’ genome [47] using Bowtie2 program (Version 2.3.4.1) without mismatch [48]. Only mapped reads were used for further analyses. The conserved known miRNAs were identified with mirdeep2 using mirBase22.1 as the reference [49]. Novel miRNA was predicted based on the characteristics of hairpin structure of miRNA precursor using mirmap2 [49] through exploring the secondary structure by identifying the Dicer cleavage site and the minimum free energy of the small RNA tags unannotated in the former steps.

4.4. miRNA expression profiles and comparison between FMFs and BFs

Expression levels of known and novel miRNAs were estimated by reads count. Differential expression analysis of two development stages or two types of floral buds was performed using the DESeq2 R package (1.24.0) [50]. Log2 fold change ≥ 10.51 and adjusted P values ≤ 0.05 was used as the filter criterion of differential expression miRNAs according to previous reports [51-53]. We defined expression difference level using the following rules: extremely significant (**) if \( |P| \leq 0.5 \) or \( \log2\text{fold change} \geq -0.5 \) and adjusted \( P\text{-value} \leq 0.01 \); significant (*) if \( |P| \leq 0.5 \) or \( \log2\text{fold change} \geq -0.5 \) and \( 0.01 < \text{adjusted } P\text{-value} \leq 0.05 \); and otherwise insignificant.

4.5. miRNA-target prediction and association analysis with transcription analysis result

Target gene prediction of known and novel miRNA was performed by psRobot (v 1.2) and TargetFinder with default parameters [54,55]. The expression of the target genes at different development stage were estimated using RNA-seq analyses. The RNA-seq and miRNA-seq were performed using the same plant tissue samples [9].
4.6. miRNA function annotation

The conserved miRNAs were annotated by comparing the sRNA reads with the known plant miRNAs registered in miRbase (http://www.mirbase.org) [56]. GO analysis of target genes was conducted using the AgriGO database (http://bioinfo.cau.edu.cn/agriGO) [57].

4.7. Validation of miRNA expression in pomegranate female pistils by stem-loop qRT-PCR

To accurately evaluate the expression levels of miRNAs, stem-loop qRT-PCR were performed in our study [29, 58]. 5.8S was used as reference gene [29]. Total RNA was isolated from floral pistils at different development stage, using the approach mentioned above [46]. Quantitative real-time PCR (qRT-PCR) was performed with three biological replicates. All primers used in reverse transcription and qRT-PCR were provided in Table S9. The relative quantitative expression level was calculated by the 2ΔΔCt method [58].

4.8. Nicotiana benthamiana transient assay

The interaction between miRNAs and their predicted targets were verified by the transient assay in Nicotiana benthamiana [59-61]. Pg-miR166a-3p precursor were amplified and transferred into the PBI121-GUS vector with an empty vector (EV) as the control. The native target sites (5'-TTGGGATAGCCTGTCCCG-3' in Gglean013727.1 and 5'-CTCTGGGAGCTGGTCGCGG-3') in Gglean013566.1, modified target sites (5'-CTGAGCGAGGATAGCAGACGG-3') that could not be cleaved by pg-miR166a-3p, and perfect complementary sites (5'-TGCGGGAATGAACCTGTCCCGAG-3') were inserted into the MS4 vector (kindly provided by Dr Fengquan Tan, Huazhong Agricultural University, Wuhan, China) carrying green fluorescent protein (GFP) (Figure 4c and Figure 6b). Pg-miR166a-3p and its target sites were transformed into Agrobacterium strain GV3101 and co-infiltrated into the Nicotiana benthamiana leaves by a ratio of 4:1 (pre-pg-miR166a-3p-PBI121: target sites-MS4). The fluorescence was captured under a hand-held UV light (Beijing, China). Pre-pg-miR166a-3p-PBI121 was transformed to Columbia A. thaliana as reported method [62]. Wild type and transgenic A. thaliana were sampled from inner to outer rind when the first inflorescence flowers. The paraffin sections were stained with hematoxylin. The morphology of ovules of 35S::pre-pg-miR166a-3p were captured by Olympus DP71 (Olympus, Tokyo, Japan).

5. Conclusions

Our study provides a genome-wide comparison of miRNAs and their targets among different stages of FMFs’ pistils, among different stages of BFs’ pistils and between FMFs’ and BFs’ pistils at the same stage. Combined analyses of the small RNA and mRNA revealed that pg-miR858b and pg-miRNA1 affect pomegranate pistil development at the early stage (before inner integument primordium formation); pg-miR444b.1, pg-miRN11, pg-miR166/165a-3p and pg-miR952b influence pomegranate integument and embryo sac development (stages after inner integument primordium formation). In addition, pg-miR166a-3p and its predicted target genes Gglean0121771.1 and Gglean013966.1 showed reciprocally expression patterns. A. thaliana transformed with 35S-pre-pg-miR166a-3p verified the role of pg-miR166a-3p in ovule development, and the transient assay in Nicotiana benthamiana confirmed the interaction between pg-miR166a-3p and Gglean0121771.1 and Gglean013966.1. In conclusion, this study provides new insight into the miRNA-mediated network models that may regulate pomegranate female sterility.

Supplementary Materials: Supplementary materials can be found at attachment.

Author Contributions: SC and LC conceived the project and its components. LX, DJ and LC contributed to the acquisition of materials. LX and LC conducted the analysis of miRNA-Seq. XY and LC performed stem-loop qRT-PCR. XX, XY, HL and LC contributed to the transform of A. thaliana. DJ and LC performed the transient assay. LC wrote the paper. XL and KP helped the article proofread.
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**Conflicts of Interest:** The authors have no conflicts of interest to declare.

### Abbreviations

- **FMFs** Functional male flowers
- **BFs** Bisexual flowers
- **BVD** bud vertical diameter
- **TNSI** BF's pistils when their BVD was 3.0–5.0 mm
- **TNSII** BF's pistils when their BVD was 5.1–13.0 mm
- **TNSIII** BF's pistils when their BVD was 13.1–25.0 mm
- **ATNSI** FMFs' pistils when their BVD was 3.0–5.0 mm
- **ATNSII** FMFs' pistils when their BVD was 5.1–13.0 mm
- **ATNSIII** FMFs' pistils when their BVD was 5.1–13.0 mm

### References


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