Identification of miRNAs and their target genes in genic male sterility lines in *Brassica napus* by small RNA sequencing

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**Abstract**

**Background:** *Brassica napus* is the third leading source of edible oil in the world. Genic male sterility (GMS) lines provide crucial material for harnessing heterosis for rapeseed. GMS lines have been used successfully for rapeseed hybrid production in China. MicroRNAs (miRNAs) play crucial regulatory roles in various plant growth, development, and stress response processes. However, reports on miRNAs that regulate the pollen development of GMS lines in *B. napus* are few.

**Results:** In this study, 12 small RNA and transcriptome libraries were constructed and sequenced for the flower buds from the fertile and sterile lines of two recessive GMS (RGMS) lines, namely, “6251AB” and “6284AB”. At the same time, 12 small RNA and transcriptome libraries were also constructed and sequenced for the flower buds from the fertile and sterile lines of two dominant GMS (DGMS) lines, namely, “4001AB” and “4006AB”. Based on the results, 46 known miRNAs, 27 novel miRNAs on the other arm of known pre-miRNAs, and 44 new conserved miRNAs were identified. Thirty-five pairs of novel miRNA-3p/miRNA-5p were found. Among all the identified miRNAs, fifteen differentially expressed miRNAs with over 1.5-fold change between flower buds of sterile and fertile lines were identified, including six differentially expressed miRNAs between “4001A” and “4001B”, two differentially expressed miRNAs between “4006A” and “4006B”, four differentially expressed miRNAs between “6251A” and “6251B”, and ten differentially expressed miRNAs between “6284A” and “6284B”. The correlation analysis of small RNA and transcriptome sequencing was conducted. And 257 candidate target genes were predicted for the 15 differentially expressed miRNAs. The results of 5' modified RACE indicated that BnaA09g48720D, BnaA09g11120D, and BnaCnng51960D were cleaved by bna-miR398a-3p, bna-miR158-3p and bna-miR159a, respectively. Among the differentially expressed miRNAs, miR159 was chosen to analyze its function. Overexpression of bna-miR159 in *Arabidopsis* resulted in decreased seed setting rate, and shortened siliques, illustrating that miR159 may regulate the fertility and silique development in rapeseed.

**Conclusions:** Our findings provide an overview of miRNAs that are potentially involved in GMS and pollen development. New information on miRNAs and their related target genes are provided to exploit the GMS mechanism and reveal the miRNA networks in *B. napus*.

**Keywords:** *Brassica napus*, miRNAs, Genic male sterility, Pollen development, miR159, Silique development
can be divided into cytoplasmic male sterility (CMS) and genic male sterility (GMS) [1]. CMS is caused by
the interaction between the mitochondrial genome and nuclear genes [2, 3]; GMS is derived from natural muta-
tions in nuclear genes that control stamen development. GMS mutants always show advantages, such as complete
and stable male sterility and no potential negative cytoplasmic effect, compared with most CMS mutants. GMS
can be further divided into dominant GMS (DGMS) and recessive GMS (RGMS). In rapeseed, DGMS and RGMS
are widely used for hybrid rapeseed production [4]. Multiple-allele DGMS was generally accepted and was
proposed by Song et al. in 2005. This model presented multiple alleles in one locus inheritance to explain the
fertility heredity of a newly reported DGMS line 609AB. In this model, Mf, Ms, and ms are three alleles at the
same locus, with a relationship of Mf dominant over Ms and Ms over ms. The recessive allele is for normal ferti-
licity. The maintainers and restorers are easily screened. Thus, multiple-allele DGMS are widely used for hybrid
rapeseed seed production through the construction of a three-line hybrid system [5, 6].

Additionally, the recessive GMS (RGMS) systems have another distinct advantage. In RGMS systems, most
inbred lines can restore their fertility, so hybrids with strong heterosis are easily bred. Several RGMS lines
have been successfully commercialized in China, including S45AB [7], 117AB [8], and 9012AB [9]. For S45AB
and 117AB, approximately 50% of the fertile plants are required to be artificially removed before hybridizing the
rest (50% sterile ones) with restorer lines in hybrid production, because no complete maintainer line is available
[10, 11]. However, a three-line hybrid production system was developed for 9012AB [12], and this system has been
well documented [10, 13]. The RGMS line 9012AB has been used successfully for rapeseed hybrid production
in China. This male sterility was previously thought to be controlled by three independent genes (BnMs3, BnMs4,
and BnRf). In 2012, Dong et al. demonstrated a major modification of the sterility inheritance model in 9012A.
The modified inheritance model indicated that the male sterility was essentially controlled by two loci (BnMs3
and BnRf). The previously designated BnMs4 locus was just one allele of BnRf; it was then designated as BnRf∗,
which was designated in addition to BnRf (the allele from 9012A) and BnRf (the allele from temporary main-
tainer). The dominance relationship of the three alleles is in the following order: BnRf∗ > BnRf > BnRf. The BnRf
allele-specific molecular markers were identified; these markers would simplify the breeding process involving
this RGMS line [14].

Oilseed rape (Brassica napus, 2n=38, AACC), which has low erucic acid and glucosinolate contents, is the
third leading source of edible oil worldwide. In recent years, some conserved and novel miRNAs associated
with silique length [15], thickness of pod canopy [16], cadmium stress [17–19], flower organ development [20],
seed maturation [21, 22], cold stress [23], seed development, and oil synthesis [24] have been widely identified
in rapeseed. However, little information is available about the DGMS and RGMS occurrence at the post-transcrip-
tional level in rapeseed.

In this study, to systematically explore the roles of miRNAs and their targets involved in GMS occurrence dur-
ing pollen development in rapeseed, 12 small RNA and transcriptome libraries were constructed and sequenced
for the flower buds from the fertile and sterile lines of two RGMS lines (“6251AB” and “6284AB”). Meanwhile,
12 small RNA and transcriptome libraries were also constructed and sequenced for the flower buds from the fer-
tile and sterile lines of two DGMS lines (“4001AB” and “4006AB”). The aims of this study were to identify known
and potential novel miRNAs from the 24 libraries and to analyze the expression profiles of the miRNAs and their
targets in relation to DGMS and RGMS during rapeseed microspore development. The results would provide a
foundation for evaluating the important regulatory roles of miRNAs in pollen formation and GMS occurrence in
rapeseed and other crops.

Results

Analysis of small RNA library data sets and the small RNA profile

To identify miRNAs related to DGMS and RGMS during pollen development, the flower buds were collected
from the sterile (6251A, 6284A) and fertile (6251B and 6284B) lines of the RGMS lines. Meanwhile, the flower
buds were also respectively collected from the sterile (4001A, 4006A) and fertile (4001B and 4006B) lines of
the DGMS lines. Three biological replicates were conducted for each of the eight kinds of samples. Thus,
total of 24 sRNA libraries were constructed and deep-sequenced. The raw reads of the 24 sRNA libraries ranged
from 20.58 to 42.68 million (Table 1). The raw reads of the 24 sRNA libraries were uploaded to SRA data-
base of NCBI and 24 accession numbers were obtained, including SRX11350295, SRX11350296, SRX11350307,
SRX11350312, SRX11350313, SRX11350315, and SRX11350316 (https://dataview.ncbi.nlm.nih.gov/object/
PRJNA743414?reviewer=t674c02cj415380e8oldre45a). After removing the low-quality reads and contaminated
adapter sequences, the clean reads of the 24 sRNA libraries ranged from 19.77 to 41.61 million. The mapped
reads were further annotated against the Pfam database and subsequently divided into rRNAs, tRNAs, snRNAs,
snoRNAs, ta-siRNA, and others. The endogenous sRNAs
were identified as known and novel miRNAs. The average sRNA lengths of the three biological replicates for each sample were calculated, which showed the length distribution patterns of the sRNAs being similar to one another. In general, the majority of the small RNAs ranged from 21 nt to 24 nt in size. The 24 nt small RNAs were the most dominant, followed by 21 nt small RNAs (Fig. 1).

Identification of known and novel miRNAs in *B. napus*

To identify known miRNAs in *B. napus*, all mapped small RNA sequences were compared with the known mature bna-miRNA sequences deposited in the miRBase database 22.1. Forty-six small RNAs that have the same sequences with the known bna-miRNAs in miRBase were identified. The numbers of reads of the 46 known miRNAs in 24 libraries were listed in Additional Table S1. Among the 46 known miRNAs, bna-miR159a, bna-miR166a, and bna-miR166a showed very high expression levels.

To predict novel miRNAs in *B. napus*, BLAST analysis was conducted for all the mapped small RNAs to the *B. napus* genome sequence in *Brassica* database and known plant miRNAs in miRBase. The small RNAs that exactly map to the genome sequence but not the known plant miRNAs were classified as candidate novel miRNAs. Five criteria described in the Materials and Methods were used to search for novel miRNAs. As a result, 35 pairs of novel miRNA-3p/miRNA-5p were identified. The mature sequences, reads numbers, positions in chromosomes, precursor sequences and minimum free energy were listed in Table 2. The length distribution of the novel miRNAs was between 18 nt to 26 nt. The length of the novel miRNA precursors ranged from 51 nt to 300 nt with an average length of 154 nt. The minimum free energy ranged from $-240.26$ to $-9.9$ kcal mol$^{-1}$ with an average of $-70.14$ kcal mol$^{-1}$. The precursor sequences and secondary structures of the novel miRNA were shown in Additional file 1 (Table S2) and Additional file 2 (Fig. S1).

### Table 1: Overview of sRNA sequencing reads in *Brassica napus*

| Sample   | Raw reads | N% > 10% | Low quality | 5' adapter contaminant | 3' adapter null or insert null | With ployA/T/G/C | Clean reads |
|----------|-----------|----------|-------------|------------------------|-------------------------------|----------------|-------------|
| 4001B-1  | 24,481,718| 41       | 16,955      | 8590                   | 761,698                       | 14,436         | 23,679,998  |
| 4001B-2  | 27,695,084| 70       | 36,718      | 12,896                 | 698,693                       | 16,785         | 26,929,922  |
| 4001B-3  | 20,576,690| 38       | 16,651      | 10,078                 | 768,766                       | 9577           | 19,771,580  |
| 4006B-1  | 28,457,208| 131      | 44,382      | 14,481                 | 721,967                       | 14,232         | 27,662,015  |
| 4006B-2  | 27,355,185| 45       | 7473        | 9466                   | 895,573                       | 11,463         | 26,431,165  |
| 4006B-3  | 35,841,966| 57       | 25,238      | 16,152                 | 1,597,289                     | 18,301         | 34,184,929  |
| 6251B-1  | 22,307,540| 36       | 18,341      | 18,038                 | 533,864                       | 14,517         | 21,722,744  |
| 6251B-2  | 24,409,491| 46       | 15,603      | 27,336                 | 689,062                       | 7177           | 23,670,267  |
| 6251B-3  | 24,485,022| 34       | 10,518      | 24,084                 | 477,366                       | 16,924         | 23,956,096  |
| 6284B-1  | 25,552,984| 64       | 12,764      | 21,674                 | 567,970                       | 12,962         | 24,937,550  |
| 6284B-2  | 24,763,779| 34       | 16,616      | 23,880                 | 431,298                       | 15,913         | 24,276,493  |
| 6284B-3  | 25,378,477| 40       | 16,388      | 25,065                 | 1,546,184                     | 6044           | 23,784,756  |
| 4001A-1  | 21,968,633| 56       | 16,757      | 10,355                 | 723,740                       | 8680           | 21,209,045  |
| 4001A-2  | 26,445,702| 23       | 5505        | 11,592                 | 1,192,819                     | 7631           | 25,228,132  |
| 4001A-3  | 27,397,803| 96       | 35,289      | 12,605                 | 1,483,390                     | 12,457         | 25,853,966  |
| 4006A-1  | 23,437,578| 357      | 10,961      | 9403                   | 612,982                       | 14,712         | 22,789,163  |
| 4006A-2  | 42,675,389| 50       | 10,345      | 14,879                 | 1,018,244                     | 21,106         | 41,610,765  |
| 4006A-3  | 27,452,096| 93       | 40,026      | 12,496                 | 749,916                       | 14,657         | 26,634,908  |
| 6251A-1  | 21,484,268| 19       | 17,038      | 20,165                 | 462,483                       | 17,972         | 20,966,591  |
| 6251A-2  | 24,127,081| 56       | 12,533      | 20,730                 | 464,608                       | 24,999         | 23,604,155  |
| 6251A-3  | 21,608,183| 357      | 7763        | 13,126                 | 393,936                       | 10,376         | 21,182,625  |
| 6284A-2  | 23,525,443| 27       | 11,236      | 20,073                 | 746,946                       | 12,136         | 22,735,025  |
| 6284A-3  | 27,605,112| 37       | 14,315      | 23,792                 | 1,211,985                     | 12,779         | 26,342,204  |
| 6284A-3  | 29,689,151| 56       | 30,879      | 21,998                 | 753,037                       | 16,533         | 28,866,648  |
RNA sequences were compared to the known precursor sequences of bna-miRNAs in the miRBase database 22.1. Finally, 27 novel miRNAs on the other arm of known *B. napus* pre-miRNAs were identified. The miRNA sequences and the number of reads in 24 libraries were listed in Table 3.

**Identification of new conserved miRNA families and new miRNA members**

To identify new conserved miRNAs in *B. napus*, all mapped small RNAs were mapped to known plant miRNAs in miRBase and *B. napus* genome sequences. If the small RNAs can match known plant miRNAs with no more than three mismatches and can exactly map to *B. napus* genome sequences, then these small RNAs were initially classified as candidate new conserved miRNAs. Five criteria described in the Materials and Methods were used to strictly screen the candidate conserved miRNAs. As a result, 44 miRNAs (22 pairs of miRNAs) belonging to 15 miRNA families were identified (Table 4). Among them, bna-miR159b was a new miRNA member of bna-miR159 family. The rest of the 36 miRNAs (14 pairs of miRNAs) have not been previously reported as bna-miRNAs in miRBase; they show high sequence similarity to some of the known plant miRNAs. The bna-miR158a.1 and bna-miR158a.2 were identified for bna-miR158a member. The two pairs of bna-miR158a shared the same mature sequences. Their precursor sequences were highly similar with each other, and these sequences were from different loci of the *B. napus* genome. These two pairs of miRNAs were called sub-members. This type of sub-member was also observed for bna-miR159b and bna-miR408a. Four sub-members (bna-miR159b.1, bna-miR159b.2, bna-miR159b.3, and bna-miR159b.4) were
Table 2  Novel miRNAs identified in the fertile and sterile lines of *Brassica napus* by high-throughput sequencing

| miR_name         | Sequence                  | Len | Read | pre-position       | pre-len | MFE  |
|------------------|---------------------------|-----|------|--------------------|---------|------|
| bna-novel_1-3p   | CUUCCUCUCAACCAUUAUUGAUU  | 23  | 67   | chrA09:27889716.27889818 | 102     | −34.6|
| bna-novel_1-5p   | AUCAAUUGGUGGUUAGGUAGAGCC | 26  | 124  |                     |         |      |
| bna-novel_2-3p   | UGGCAUUGGUGUAAUUGAGUGU   | 22  | 190  | chrC04:42905032.42905108 | 76      | −26.9|
| bna-novel_2-5p   | CAUCAUCAUCACAGGCACCA     | 21  | 7    |                     |         |      |
| bna-novel_3-3p   | UCAUUGGUUGGCUCAUAUUGU    | 21  | 120  | chrC02:222515980.222516065 | 85      | −29.5|
| bna-novel_3-5p   | UCAUUGUGAUCGACGUGAGUU    | 22  | 12   |                     |         |      |
| bna-novel_4-3p   | AUUAUCGACACUGACAUCAUCALC | 21  | 106  | chrC08:2975781.2975915 | 134     | −80.8|
| bna-novel_4-5p   | UAACGUACUGUUGUAUACAUCC   | 20  | 52   |                     |         |      |
| bna-novel_5-3p   | UCAUUGUGGCUCAUAIUAGL    | 20  | 49   | chrC09:40959623.40959707 | 84      | −31.6|
| bna-novel_5-5p   | UCAUUGGCUCAUGUCCAUUAA   | 22  | 12   |                     |         |      |
| bna-novel_6-3p   | AAGGACUCUAAACAGAAUUGG    | 24  | 143  | chrC06:35249139.35249190 | 51      | −9.9 |
| bna-novel_6-5p   | AUAGGUCUACUGAAGGCAAUA   | 24  | 11   |                     |         |      |
| bna-novel_7-3p   | UGGCUUGGCUCCUCAUAAUCA   | 21  | 83   | chrA08:8293061.82931444 | 83      | −32.4|
| bna-novel_7-5p   | GUGAUAUGAGUGACGCAUGA    | 22  | 2    |                     |         |      |
| bna-novel_8-3p   | AUUCUAAUUGUAACUCAUULU   | 24  | 24   | chrA03:20065617.20065865 | 248     | −95.7|
| bna-novel_8-5p   | AAGGGUGUAGUAAUGGAGGCC   | 24  | 1    |                     |         |      |
| bna-novel_9-3p   | UUGGACUAGAGGGAACUUCCCU  | 21  | 1527 | chrA09:16465700.16465869 | 169     | −64.4|
| bna-novel_9-5p   | AGAUAUUCUCAUGUAUGCUAC   | 21  | 17   |                     |         |      |
| bna-novel_10-3p  | UGAAGUUCUUGCUAUUUCGAG  | 21  | 67   | chrA10:15442817.15442916 | 99      | −39.8|
| bna-novel_10-5p  | CGAAGAUAAGAAGCUGAAA    | 24  | 3    |                     |         |      |
| bna-novel_11-3p  | AACAGUGUUGAGUGCUCAUGUG  | 24  | 27   | chrA09:random:3088720.3089020 | 300     | −65.5|
| bna-novel_11-5p  | ACGAGUGGAGAAACUGAUCG    | 24  | 2    |                     |         |      |
| bna-novel_12-3p  | UUUUCAGGAACUUCUCUUULAU  | 23  | 44   | chrA05:random:416209.416319 | 110     | −29.5|
| bna-novel_12-5p  | AUUGGAAAGAUGUAGUAGCAGA | 22  | 6    |                     |         |      |
| bna-novel_13-3p  | UAAAGUAGACUCUGGUGACG    | 21  | 1163 | chrA03:20992442.20992727 | 285     | −240.3|
| bna-novel_13-5p  | GCUCACCGCGUCUCACUUAUA   | 21  | 1058 |                     |         |      |
| bna-novel_14-3p  | UCCGUUUCUGCUAGAAUAAUGAC | 24  | 22   | chrC04:45707769.45708016 | 247     | −148 |
| bna-novel_14-5p  | CAAAUAUCUACAGAAACGGAUA  | 24  | 23   |                     |         |      |
| bna-novel_15-3p  | AUAUGAGGGUCAAUAGGAGA   | 21  | 137  | chrAnn:random:33598332.33598540 | 208     | −139.1|
| bna-novel_15-5p  | UAUUGUAACCCUCAUAUAAGC  | 21  | 89   |                     |         |      |
| bna-novel_16-3p  | CUUAAGAUGUGCUAAUAACAGC  | 24  | 30   | chrC04:7124159.7124378 | 219     | −117.5|
| bna-novel_16-5p  | AUGUUUAUUGGUGCUUUAAGGUU | 25  | 7    |                     |         |      |
| bna-novel_17-3p  | ACAGAUAACUCUGGUAUAUCUG  | 21  | 15   | chrC01:random:3928762.3929012 | 250     | −164.8|
| bna-novel_17-5p  | GAAUAUAUCACUGUGCUUGUG  | 21  | 13   |                     |         |      |
| bna-novel_18-3p  | ACACUGACUGACUGACUAUUGC  | 24  | 17   | chrCnn:random:79954335.79954585 | 250     | −113.8|
| bna-novel_18-5p  | GUUGUACUAUUGUAACACGCGUGUAC | 26  | 3    |                     |         |      |
| bna-novel_19-3p  | UUGCACACUGUAUAUGAGUC   | 21  | 20   | chrA09:30966144.30966235 | 91      | −45.4|
| bna-novel_19-5p  | CUCUAUUAUCGUGCUUGCAUC   | 21  | 20   |                     |         |      |
| bna-novel_20-3p  | AAGAUACCGUGUCUUAACUUUGAU | 24  | 259  | chrC04:random:3818028.3818149 | 121     | −67.8|
| bna-novel_20-5p  | GUAAUAAGACCCGUAAUAUA   | 24  | 13   |                     |         |      |
| bna-novel_21-3p  | AAGCAGUUGUGGUUUUGGUUGAGA | 24  | 18   | chrA05:21620032.21620188 | 156     | −82.1|
| bna-novel_21-5p  | UUCAAAACCAUACAGAUGCUUUU | 24  | 24   |                     |         |      |
| bna-novel_22-3p  | GAUCAGUUGUUGUACUGUACC  | 21  | 445  | chrCnn:random:35712007.35712108 | 101     | −47.3|
| bna-novel_22-5p  | UGAACGUGACGACAGCAUCLU  | 20  | 3    |                     |         |      |
| bna-novel_23-3p  | UUGCUGUGGUUUUAAGGUAGU  | 21  | 55   | chrA06:23040126.23040236 | 110     | −52.8|
| bna-novel_23-5p  | ACCUCAAACACACAAAGAAGA  | 22  | 3    |                     |         |      |
| bna-novel_24-3p  | UGGUGUGUGCUUACUAUCAGC  | 20  | 40   | chrC02:8973302.8973545 | 243     | −93.1|
| bna-novel_24-5p  | AUGAUAACACCGGAAACAAA   | 20  | 26   |                     |         |      |
| bna-novel_25-3p  | AACUGUGUGAUAACUCUCAUGAG | 24  | 389  | chrC02:random:2214651.2214881 | 230     | −73.8|
identified for bna-miR159b, and two sub-members (bna-miR408a.1 and bna-miR408a.2) were identified for bna-miR408a. This phenomenon suggests that some MIRNA genes might be produced through a replication event from one origin to another one, which results in more copies of the miRNA group. Two members were identified for bna-miR319 and bna-miR398 families. Except the above mentioned five miRNA families, the rest of 10 miRNA families had only one miRNA member (Table 4).

The secondary structures of these new conserved miRNAs were shown in Additional file 2 (Fig. S1).

Expression profiling of differentially expressed miRNAs in sterile and fertile lines

The normalized expression levels of miRNAs were used for identifying differentially expressed miRNAs between the sterile line and the corresponding fertile line, such as “4001A” and “4001B”, “4006A” and “4006B”, “6251A” and “6251B”, and “6284A” and “6284B”, respectively (Fig. 2, Table 5). To further explore the miRNAs involved in the two DGMS lines, a Venn diagram analysis was conducted. The results indicated that two differentially expressed miRNAs (bna-miR319 and bna-miR398) were shared between the DGMS lines “4001AB” and “4006AB” (Fig. 3A). In addition, three differentially expressed miRNAs (bna-novel_34-5p, bna-miR408a-5p, and bna-miR398a-3p) were shared between the RGMS lines “6251AB” and “6284AB” (Fig. 3B). The novel miRNA “bna-novel_34-5p” was the only miRNA that was simultaneously differentially expressed in the DGMS and RGMS lines.

qRT-PCR was conducted to verify the expression profiles of these differentially expressed miRNAs in deep sequencing. Five differentially expressed miRNAs were chosen for qRT-PCR analysis. The results were consistent with those of deep sequencing. In qRT-PCR, miR158 was up-regulated in “4001B” flower buds compared with that in “4001A”. Novel_34 was greatly up-regulated in “4006B” flower buds (730-fold) compared with that in “4006A”. MiR159 and miR827 were both up-regulated in “6284A” flower buds compared with that in “6284B” (Fig. 4).

Table 2 (continued)

| miR_name | Sequence | Len | Read | pre-position | pre-len | MFE |
|----------|----------|-----|------|--------------|--------|-----|
| bna-novel_25-5p | CCAAAAGGGGUAUCAACAAAGUAAUUU | 26 | 1 | chrA03:669098..669269 | 171 | -83.8 |
| bna-novel_26-3p | UUGAUAACALGUAGCUCULUUU | 20 | 2089 | chrA07:19146929..19147018 | 89 | -34.7 |
| bna-novel_26-5p | AAGUCCCGUACGUUCAUGCUUG | 22 | 840 | chrA03_random:65177977..65178226 | 249 | -72.9 |
| bna-novel_27-3p | UUAACCGCUUUGUGAGCUCUUU | 22 | 367 | chrA06:4069612..4069740 | 128 | -56.1 |
| bna-novel_27-5p | AGACCUACACAACUCAGAAAAC | 21 | 47 | chrA07:19146929..19147018 | 81 | -42.5 |
| bna-novel_28-3p | UUCUGAGUCACGUGAACCACACCAUUGG | 24 | 426 | chrA06:4069612..4069740 | 128 | -56.1 |
| bna-novel_28-5p | UCAUGUGAGACGUUCAUGCUUG | 20 | 2 | chrA06:4069612..4069740 | 128 | -56.1 |
| bna-novel_29-3p | UCCUGAGACGUGAACCACACCAUUGG | 24 | 289 | chrA06:4069612..4069740 | 128 | -56.1 |
| bna-novel_29-5p | UUCUGAGACGUGAACCACACCAUUGG | 24 | 289 | chrA06:4069612..4069740 | 128 | -56.1 |
Target prediction and identification of differentially expressed miRNAs in sterile and fertile lines

A plant small RNA target analysis server (psRNATarget)-based analysis was performed to predict miRNA target genes with default parameters and a maximum expectation value of 3.5 (https://www.zhaolab.org/psRNA-Target/). A total of 560 transcripts were predicted to be targets of the 15 miRNAs (Additional Table S3). In addition, transcriptome sequencing was conducted using the same samples as sRNA sequencing (unpublished data). The differentially expressed and up-regulated mRNAs were predicted as the candidate targets for the differentially expressed and down-regulated miRNAs. At the same time, the differentially expressed and down-regulated mRNAs were predicted as the candidate targets for the differentially expressed and up-regulated miRNAs. As shown in Table 5. Thirty-eight candidate target genes were predicted for the six differentially expressed miRNAs between “4001A” and “4001B”. Eleven candidate genes were predicted for the two differentially expressed miRNAs between “4006A” and “4006B”. Twenty-seven candidate genes were predicted for the four differentially expressed miRNAs between “6251A” and “6251B”. One hundred and eighty-one candidate genes were predicted for the ten differentially expressed miRNAs between “6284A” and “6284B”.

To further demonstrate the potential target genes, 5’ modified RACE was performed using mixed samples from flower buds of the fertile lines (“6284B” and “4001B”). Three target genes were validated using 5’ modified RACE (Fig. 5).

Bn.A09.CSD1 (BnaA09g48720D) was cleaved by bna-miR398a-3p. Bn.A09.PPR (Bna-A09g11120D) was cleaved by bna-miR158-3p. Bn.Cnn.MYB (BnaCnng51960D) was cleaved by bna-miR159a.

Overexpression of bna-miR159a affected seeds and siliques development in Arabidopsis

Among all the differentially expressed miRNAs in the two DGMS and RGMS lines, bna-miR159a had the highest expression level. To reveal miR159 potential...
function, two constructs containing pre-miR159a-C6 and pre-miR159a-A7 were transformed to Arabidopsis, and corresponding MIR159OE-1 and MIR159OE-2 transgenic plants were obtained. Five and four lines were obtained for MIR159OE-1 and MIR159OE-2 in T₁, respectively. In wild-type plants of Arabidopsis, the

| Table 4 | Identification of new conserved miRNA families in Brassica napus |
|---------|---------------------------------------------------------------|
| bna-miRNA | Sequence | Len | Read | pre-position |
| bna-miR158a.1-5p | CUUGGCUAUGCUGUUGGAAAAG | 22 | 3884 | chrA08:2748114.2748220 |
| bna-miR158a.1-3p | UUUCCAAUGAAGAACAAGCA | 21 | 32,292 | |
| bna-miR158a.2-5p | CUUGGCUAUGCUGUUGGAAAAG | 22 | 3884 | chrC08:3581242.3581348 |
| bna-miR158a.2-3p | UUUCCAAUGAAGAACAAGCA | 21 | 32,292 | |
| bna-miR159b.1-5p | AGCUUGCUAUGCUGUUGGAAAAG | 22 | 258 | chrA02:9865184.9865001 |
| bna-miR159b.1-3p | UUUCCAAUGAAGAACAAGCA | 21 | 46,073 | |
| bna-miR159b.2-5p | AGCUUGCUAUGCUGUUGGAAAAG | 22 | 258 | chrA07_random:1944377.19444191 |
| bna-miR159b.2-3p | UUUCCAAUGAAGAACAAGCA | 21 | 46,073 | |
| bna-miR159b.3-5p | AGCUUGCUAUGCUGUUGGAAAAG | 22 | 258 | chrC02:19215807.19215624 |
| bna-miR159b.3-3p | UUUCCAAUGAAGAACAAGCA | 21 | 46,073 | |
| bna-miR159b.4-5p | AGCUUGCUAUGCUGUUGGAAAAG | 22 | 258 | chrC06:33954934.33954749 |
| bna-miR159b.4-3p | UUUCCAAUGAAGAACAAGCA | 21 | 46,073 | |
| bna-miR319a-5p | AGA GCU UCC UUG AGU CCA UUC | 21 | 27 | |
| bna-miR319a-3p | UUG GAC UGA AGG GAG CUC CUU | 21 | 4848 | |
| bna-miR319b-5p | GGA GAU UCU UUC AGU CCA GUC | 21 | 4 | chrC04:46407584.46407846 |
| bna-miR319b-3p | UUG GAC UGA AGG GAG CUC CUU | 21 | 4 | chrC04:46407584.46407846 |
| bna-miR391-5p | UUC GCA GGA GAG AUA GCG CCA | 21 | 110 | chrA10:10707678.10707812 |
| bna-miR391-3p | ACG GUA UCU CUC UCU GAC UGC | 21 | 237 | |
| bna-miR398a-5p | GGG UCG ACA UGA GAA CAC AUG | 21 | 141 | chrA03:2288822.2288945 |
| bna-miR398a-3p | UGU GUU CUC AGG UCA CCC CUG | 21 | 9870 | chrC02:10651723.10651921 |
| bna-miR398b-5p | GGA GUG UCA UGA GAA CAC GGA | 21 | 25 | chrC02:37793584.37793689 |
| bna-miR398b-3p | UGU GUU CUC AGG UCA CCC CUU | 21 | 145 | |
| bna-miR400-5p | UAAGAGAUAAUAUAAGCAC | 21 | 78 | chrA10:10707678.10707812 |
| bna-miR400-3p | GAUAUCCAAUAUCUAAUCA | 21 | 237 | |
| bna-miR408a.1-5p | GGG AGC CAG GGA AGA GGC AGU | 22 | 1232 | chrA05:478954.479121 |
| bna-miR408a.1-3p | UGC UUG UUC CCU GUC UCU CUC | 22 | 1002 | |
| bna-miR408a.2-5p | GGG AGC CAG GGA AGA GGC AGU | 22 | 1232 | chrCnn_random:8448205.8448064 |
| bna-miR408a.2-3p | UGC UUG UUC CCU GUC UCU CUC | 22 | 1002 | |
| bna-miR9554-5p | GAA GUA CCU GAA AUAA UC | 21 | 5 | chrA06:19718101.19718250 |
| bna-miR9554-3p | UCAUCCUCAUCCUACUCCU | 21 | 81 | |
| bna-miR9558-5p | AGA AGU GUC UGG CUU GCA ACA | 21 | 3 | chrC03_random:1702602.1702746 |
| bna-miR9558-3p | UUGCAAGCCAGACAUUCCUU | 22 | 8 | |
| bna-miR9559-5p | UUGGAAUUGGUGAUAUGUGG | 21 | 5 | chrA06:19552830.19552965 |
| bna-miR9559-3p | ACAAGAAGCCAAUCUCCAAUC | 21 | 3 | |
| bna-miR9560a-5p | ACAGUGGUGAAGAAAUAAUAGGU | 25 | 30 | chrA06:19552830.19552965 |
| bna-miR9560a-3p | UCAUCAUGUCUACCUCCUGCUG | 25 | 2 | |
| bna-miR9562-5p | ACUUAGCAUUUGAGAACAAAC | 21 | 4 | chrA02_random:1408210.1408358 |
| bna-miR9562-3p | UUAUUCACAACUCUAAUUC | 21 | 3 | |
| bna-miR9563a-5p | ACCCGUCUUCUAUACUUAC | 22 | 15 | chrA06:19552830.19552965 |
| bna-miR9563a-3p | UAAAGUUAAGAGACAGUUA | 22 | 17 | |
| bna-miR9568-5p | UGCGGUAUCUAAUGAAGGAGCU | 22 | 13 | chrA03:13274664.13274813 |
| bna-miR9568-3p | UCAUCAUGUCUACCUCCUGCUG | 22 | 2 | |
| bna-miR9569-5p | UGAGUAUAUCUUGGUGCUUG | 21 | 1198 | chrA06:19718101.19718250 |
| bna-miR9569-3p | ACACAGGAAACAUAUAACUCAA | 24 | 3509 | |

**Len** length of mature miRNA, **pre-position** the miRNA precursor sequences in chromosomes of Brassica napus, Read the total read count of all the small RNA libraries.
transcript of mature miR159a and its targets (AtMYB33 and AtMYB65) were detected in root, stem, rosette leaf, stem leaf, flower, and silique through qRT-PCR. The expression level of mature miR159a was the highest in silique (5875-fold), relatively lower in stem, stem leaf, and flower compared with that in root. The expression levels of AtMYB33 and AtMYB65 were very low and almost undetectable in silique, whereas they were relatively high in stem, stem leaf, and flower compared with that in the root (Fig. 6). In T2 transgenic plants, the transcripts of miR159a and its targets were detected in stem leaf from line 1 of MIR159OE-1 and mixed stem leaf from line 3 and line 4 of MIR159OE-2. The results indicated mature miR159a was overexpressed in MIR159OE-1 (4.04-fold) and MIR159OE-2 (13.6-fold) compared with that in WT. Meanwhile, the transcripts of AtMYB33 and AtMYB65 were suppressed in MIR159OE-1 and MIR159OE-2, especially in MIR159OE-2, compared with that in WT (Fig. 7).

The morphological characters of MIR159OE-1, MIR159OE-2, and WT were observed along with their development processes, especially in the flowering and fruiting periods. No significant difference was observed between transgenic and WT plants in the vegetative growth phase. However, during the reproductive growth period, in the T1 and T2 transgenic plants of MIR159OE-1 and MIR159OE-2, the seed setting rate decreased, and siliques became shorter compared with that in the WT (Fig. 8). The length of siliques from WT, MIR159OE-1, and MIR159OE-2 transgenic plants were measured. In T1, the siliques lengths of WT, MIR159OE-1, and MIR159OE-2 transgenic plants were measured. In T1, the siliques length of WT was approximately 13.4 mm, while in the MIR159OE-1 transgenic plants, the siliques lengths of line 1, line 4, and line 5 were 6.7 mm, 4.6 mm and 6.4 mm, respectively. And the siliques lengths of line 1, line 3, and line 4 of MIR159OE-2 were 5.4 mm, 5.6 mm, and 6.4 mm, respectively (Fig. 8E). In T2, the siliques length of WT was approximately 10.6 mm, while in the MIR159OE-1 plants, the siliques length of line 1 was 4.3 mm. The siliques lengths of line 3 and line 4 of MIR159OE-2 were 4.4 mm and 4.0 mm, respectively (Fig. 8F). These results indicated that overexpression of MIR159 resulted in significantly shorter siliques and reduced seed setting rate.

**Discussion**

MiRNAs, as the key post-transcriptional regulators, participate in various biological processes in plant. Recently, an increasing number of studies showed that
| Sample   | miR_name          | Read (A) | Read (B) | $\log_2 \text{FoldChange}$ | Candidate targets                                                                 |
|----------|-------------------|----------|----------|----------------------------|-----------------------------------------------------------------------------------|
| 4001     | bna-novel_4-3p    | 8.2      | 0.4      | 1.90                       | BnaC01g31810D,BnaA07g24350D,BnaC02g41520D,BnaA08g22150D,BnaA04g13020D,BnaA07g18960D,BnaA09g38810D,BnaC03g26470D,BnaC02g30320D,BnaA04g09990D,BnaA03g16600D,BnaA05g07140D,BnaA08g19190D |
| bna-miR390a/b/c | 26.8              | 11.1     | 1.32     |                            | BnaC03g02760D,BnaC01g03570D,BnaA05g23890D,BnaC03g53360D,BnaC01g22410D |
| bna-novel_31-5p | 10.7              | 55.2     | -2.16    |                            | BnaC01g21190D,BnaA01g17940D,BnaC03g3660D,BnaA03g31095D,BnaA09g01701D,BnaA10g2710D,BnaC01g212250D,BnaA03g38770D,BnaA02g29970D,BnaA02g18470D |
| bna-novel_34-5p | 0.6               | 624.0    | -8.16    |                            | BnaA09g14240D,BnaA05g30840D,BnaA01g24360D,BnaA07g34690D,BnaA03g14400D,BnaA05g09900D,BnaC01g09790D,BnaA08g26410D |
| bna-novel_1-3p | 0.1               | 8.1      | -2.64    | -                           |                                                                                  |
| bna-miR158-3p | 554.7             | 935.8    | -0.74    |                            | BnaA05g08940D,BnaA04g29320D |
| 4006     | bna-novel_34-5p    | 0.2      | 125.1    | -5.82                      | BnaA05g29360D,BnaA09g14240D,BnaA05g30840D,BnaA01g24360D,BnaA07g34690D,BnaC03g41430D,BnaA339560D |
| bna-novel_31-5p | 4.7               | 53.3     | -3.81    |                            |                                                                                  |
| bna-novel_28-3p | 5.2               | 38.3     | -2.48    |                            |                                                                                  |
| bna-miR408-5p | 23.8              | 60.6     | -1.15    |                            |                                                                                  |
| bna-miR398a-3p | 0                 | 5.1      | -2.10    |                            |                                                                                  |
| bna-miR398a-3p | 23.2              | 57.5     | -1.11    |                            |                                                                                  |
| 6284     | bna-miR394a/b      | 195.2    | 87.5     | 1.04                       | BnaA05g16600D,BnaA07g24160D,BnaA05g1890D,BnaA02g21710D,BnaA09g09250D,BnaA08g10740D,BnaA07g36430D,BnaC02g29160D,BnaA06g08380D,BnaC01g07190D,BnaA05g05020D,BnaA05g31320D,BnaC01g39490D,BnaC01g16400D,BnaA02g34270D,BnaC02g43190D,BnaA01g33370D,BnaC01g39860D,BnaA08g27810D |
| bna-novel_33-5p | 30.2              | 8.4      | 1.76     |                            |                                                                                  |
| bna-miR159a | 2320.1            | 1295.7   | 0.82     |                            |                                                                                  |
| bna-novel_9-3p | 114.4             | 68.3     | 0.75     |                            |                                                                                  |
| bna-miR398a-3p | 436.2             | 972.6    | -1.06    |                            |                                                                                  |
| bna-novel_3-3p | 3.1               | 23.4     | -2.43    |                            |                                                                                  |
| bna-novel_5-3p | 1.5               | 13.5     | -1.77    |                            |                                                                                  |
| bna-novel_1-3p | 0.9               | 7.5      | -1.72    |                            |                                                                                  |
| bna-novel_34-5p | 1.0               | 456.9    | -6.87    |                            |                                                                                  |

**Table 5** Differentially expressed miRNAs in "4001AB", "4006AB", "6251AB" and "6284AB" libraries and their candidate targets by sRNA sequencing and transcriptome analysis in *Brassica napus*.

Read (A) Read count in sterile lines (TPM), Read (B) Read count in fertile lines (TPM), $\log_2 \text{FoldChange}$ 

\( \text{FoldChange} = \frac{\text{Read (A)}}{\text{Read (B)}} \times 2^{\text{log}_2 \text{FoldChange}} \)
plant miRNAs were also involved in pollen and fertility development. In 2014, Jiang et al. identified 54 new conserved miRNAs and 25 pairs of novel miRNA/miRNA* in a GMS system of *B. campestris* ssp. *chinensis*. Eighteen differentially expressed miRNAs with over two fold change between flower buds of male sterile and fertile lines were identified; they might be involved in the pollen development process [25]. In 2017, Ma et al. verified that the overexpression of *MIR158* caused pollen abortion and reduced pollen vitality, which were caused by the degradation of pollen content from the binuclear microspore stage [26]. Dong et al. identified 85 known miRNAs and 120 novel miRNAs, which were expressed during rapeseed anther development in a novel recessive GMS system “CN12AB.” Moreover, 19 and 18 known miRNAs were found to be differentially expressed in 0.5–1.0 mm buds and in 2.5–3.0 mm buds between CN12A and CN12B, respectively. Among these, 14 miRNAs were up-regulated, and 23 miRNAs were down-regulated expressed in CN12A compared with those in CN12B [27]. In this study, to identify miRNAs and their targets involved in pollen development and GMS occurrence in rapeseed, 24 small RNA libraries and transcriptome libraries were constructed and sequenced for the flower buds from the fertile and sterile lines of two RGMS lines (“6251AB” and “6284AB”) and two DGMS lines (“4001AB” and “4006AB”). Based on the sequencing results, fifteen differentially expressed miRNAs with over 1.5-fold change between flower buds of male sterile and fertile lines were identified, including six differentially expressed miRNAs between “4001A” and “4001B”, two differentially expressed miRNAs between “4006A” and “4006B”, four differentially expressed miRNAs between “6251A” and “6251B”, and ten differentially expressed miRNAs between “6284A” and “6284B”. Among them, bna-novel_34-5p was common and differentially expressed between the fertile and sterile lines of “4001AB”, “4006AB”, “6251AB”, and “6284AB”. The results of previous studies and this study verified that miRNAs may play important regulatory roles in rapeseed pollen development and GMS occurrence.

MiR159 is conserved in many plants and is involved in multiple growth and development processes of plants. Allen et al. obtained a *mir159ab* double mutant in *Arabidopsis*, which showed pleiotropic morphological defects, including altered growth habit, curled leaves, small siliques, and small seeds [28]. Millar and Gubler verified that miR159 regulated anther development by regulating the expression of its targets, such as *MYB33* and *MYB65* [29]. Overexpression of miR159 caused the down-regulation of *MYB103* transcripts and earlier degeneration of the tapetum and aberrant pollen formation during anther development [30]. In radish, differential expression level of miR159 during anther development was observed among male sterile and maintainer lines. Increased levels of miR159 transcripts decreased the expression of *MYB101*, thereby inhibiting tapetum development and exine formation [31]. Chen et al. identified 17 differentially expressed miRNAs between long and short siliques of rapeseed, including miR159. Correlation analysis of miR159 and its targets suggested that miR159 repressed cell proliferation to control silique length [15]. Hu et al. found that the overexpression of Bra-*MIR159a* caused pollen abortion and abnormal pollen germination [32]. In this study, the differentially expressed miR159 in “6284A” and “6284B” was chosen to analyze its function during

![Venn diagram showing overlaps of significantly differential expressed miRNAs between different genic male sterility (GMS) lines of *Brassica napus*. A: Two differentially expressed miRNAs were shared between 4001AB and 4006AB lines. B: Three differentially expressed miRNAs were shared between 6251AB and 6284AB lines.](image-url)
anther development. The bna-miR159 was overexpressed in Arabidopsis and resulted in decreased seed setting rate, and shortened siliques, illustrating that miR159 may regulate the fertility and silique development of rapeseed. The results of previous reports and the results of the present study verified that miR159 and its target genes might be involved in the regulatory network of pollen development and male sterility and this module is conserved in plants.

**Conclusion**
A large number of miRNAs were identified during pollen development in the two DGMS and two RGMS lines by deep sequencing. These identified miRNAs included

![Fig. 4](image_url) The qRT-PCR analysis of differentially expressed miRNAs between the flower buds of A lines and B lines. The flower buds used for qRT-PCR analysis were collected from corresponding A lines or B lines.
27 novel miRNAs on the other arm of known pre-miRNAs, 44 new conserved miRNAs, and 35 pairs of novel miRNA-3p/miRNA-5p. Among all the identified miRNAs, 15 differentially expressed miRNAs with over 1.5-fold change between flower buds of male sterile and fertile lines were identified, including six differentially expressed miRNAs between “4001A” and “4001B”, two differentially expressed miRNAs between “4006A” and “4006B”, four differentially expressed miRNAs between “6251A” and “6251B”, and ten differentially expressed miRNAs between “6284A” and “6284B”. The qRT-PCR results of 5 differentially expressed miRNAs (miR158, novel_34, miR159, miR827, and miR398) were consistent with deep sequencing results. The association analysis of small RNA and transcriptome sequencing was conducted, and the analysis results indicated that 257 genes were predicted to be the candidate targets of 15 differentially expressed miRNAs. The results of 5’ modified RACE verified that Bn.A09.CSD1 (BnaA09g48720D), Bn.A09.PPR (BnaA09g11120D), and Bn.Cnn.MYB...
(BnaCnng51960D) were cleaved by bna-miR398a-3p, bna-miR158-3p, and bna-miR159a. Additionally, overexpression of bna-miR159 in Arabidopsis resulted in decreased seed setting rate, and shortened silique, illustrating that miR159 may regulate rapeseed fertility and silique development. All the results in our study would provide valuable clues for exploring miRNA-mediated regulatory networks in fertility development of GMS lines in B. napus.

Methods
Plant materials
“6251AB” and “6284AB” are two recessive genic male sterile (RGMS) lines of B. napus. The “6251A” and “6284A” are male sterile lines and the “6251B” and “6284B” are the fertile lines. Their male sterility is controlled by two loci (BnMs3 and BnRf) [14]. The original source of the two RGMS lines was “9012A”, which was identified by Chen et al. [9] and Sun et al. [33]. “4001AB” and “4006AB” are two dominant genic male sterile (DGMS) lines of B. napus. The “4001A” and “4006A” are male sterile lines and the “4001B” and “4006B” are the fertile lines. Their male sterility is controlled by three alleles (Mf, Ms, and ms) at the same locus [5]. The original source of the two DGMS lines was “Yi3A”, which was identified by Li et al. [34]. The above RGMS lines and DGMS lines used in this study have been grown for several generations. The progenies of the two RGMS lines are both segregated into sterile and fertile types during reproduction at a ratio of 1:1. While the two DGMS lines are segregated into sterile and fertile types at a ratio of 3:1. These plant materials were planted in the experimental farm of Zhuanghang comprehensive experimental station of Shanghai Academy of Agricultural Sciences. During flowering stage, mixed flower buds were respectively harvested from more than ten plants of the eight lines. The eight lines were “6251A”, “6251B”, “6284A”, “6284B”, “4001A”, “4001B”, “4006A”, and “4006B”. The eight kinds of samples were quickly frozen in liquid nitrogen and stored at −80 °C. Three independent biological replicates were collected for each kind of sample.

Small RNA library construction and sequencings
Total RNA for each kind of sample was extracted in three biological replicates using Trizol reagent (Invitrogen, USA). RNA samples with an OD260/OD280 ratio of 2.0 and a total content of more than 2 μg were qualified for small RNA library construction. Then, 24 sequencing libraries (three biological replicates respectively for “6251A”, “6251B”, “6284A”, “6284B”, “4001A”, “4001B”, “4006A”, “4006B”) were constructed using TruSeq-Small RNA Sample Preparation Kit (Illumina, USA) and then sequenced using Illumina Hiseq 2500/Miseq at Beijing Novogene Bioinformatics Technology Co. Ltd.
Clean reads were obtained by removing low-quality reads, N-containing fragments, and adapters. Then, the length of 18 to 30 nt clean reads were mapped to the *B. napus* genome sequence ([http://brassicadb.agridata.cn/brad/](http://brassicadb.agridata.cn/brad/)) using Bowtie2 with no mismatches allowed, more details were described in Niu et al. [35]. Unmapped sequences were removed.

The mapped small RNAs reads were aligned to known miRNAs in miRBase22.1. Modified software mirdeep2 [36] were used to predict the potential miRNAs and secondary structures. The ncrRNAs includes rRNAs, tRNAs, snRNAs, snoRNAs, and small genome repeat sequences were removed. The rest of sRNA sequences were aligned to *B. napus* NAT-siRNAs in PlantNATsDB to remove NAT-siRNAs. Then miREvo [37] and mirdeep2 [36] were used to predict novel miRNAs in *B. napus*. To reveal the differentially expressed miRNAs, the miRNAs expression was analyzed using the DESeq2 [38]. The online software of Venny 2.1.0 was used to draw Venn diagrams.

### Identification of conserved and novel miRNAs

The candidate sRNAs were mapped to all the known plant miRNA sequences from the miRBase database ([http://www.mirbase.org/](http://www.mirbase.org/)). The matched sRNAs with no more than three mismatches were considered as candidate conserved miRNAs, while the unmatched sRNAs were considered as candidate novel miRNAs. In addition,
Mfold software was used to predict the secondary structures of pre-miRNAs with the flanking sequences of the candidate small RNAs in the genome [39]. Five criteria must be met for identifying conserved and novel miRNAs [40, 41]. The sequences and lengths, read counts, and positions in chromosome were further analyzed for conserved and novel miRNAs.

**qRT-PCR**

Total RNA was treated with DNase I (Takara, Japan) to remove residual genomic DNA. For the qRT-PCR analysis of AtMYB33 and MYB65, AtACTIN2 gene was used as internal control. PrimeScript™ II reverse transcriptase (Takara) and oligo (dt) primers were used for first-strand cDNA synthesis. For the qRT-PCR analysis of mature miRNAs, U6 was used as internal control gene, and the first cDNA was synthesized using a miRNA First Strand cDNA Synthesis kit (Stem-loop Method) (Sangon Biotech). The qRT-PCR reactions were performed in a MyiQ2 qRT-PCR detection system (Bio-Rad, www.bio-rad.com/) using iQ SYBR Green supermix (Bio-Rad) [42]. Each experiment was conducted in three biological replicates, and the same sample was performed in three technical replicates. Relative expression levels of miRNAs and their target genes were quantified by using the 2-ΔΔCt method [43]. All the primers used for qRT-PCR analysis are listed in Additional file 1: Table S4.

**5’ modified RACE analysis**

A mixture of flower buds from the fertile lines (“6284B” and “4001B”) was used for total RNA isolation. The 5’ modified RACE was performed using a FirstChoice™ RLM-RACE Kit (Invitrogen, USA). Total RNA was directly ligated to the 5’ RACE oligo. The first-strand cDNA synthesis and the two rounds of PCR reactions were conducted following the manufacturer’s instructions. The PCR products containing the target gene bands were ligated into pGEM-T Easy Vector (Promega, USA) for sequencing [25]. The primers are listed in Additional file 1: Table S4.

**Vector construction and plant transformation**

Two precursor sequences of bna-miR159a, which were located in A7 and C6 chromosomes of *B. napus* were designated as pre-miR159a-C6 and pre-miR159a-A7, respectively (Additional file 1: Table S5). The 411 nt and 470 nt genomic fragments containing pre-miR159a-C6 and pre-miR159a-A7 were amplified from *B. napus* using gene specific primers with endonuclease cleavage sites Sma I and Sal I. Then the fragments were cloned into pCAMBIA1301 binary vector with CaMV 35S promoter. The two vectors were designated as p35S::MIR159a-C6 and p35S::MIR159a-A7, which were introduced into *Agrobacterium tumefaciens* strains GV3101 and further transformed into *Arabidopsis* by floral dip. The inflorescences were dipped in the *Agrobacterium* solution containing sucrose and Silwet-77 for 2 min. The infected plants were cultivated for 48 h in the dark environment and then transferred to greenhouse [44]. The seeds were harvested and screened by germination on MS medium containing 25 mg/l hygromycin. The T1 and T2 hygromycin-resistant seedlings were transplanted and grown in greenhouse. Seeds from each transgenic plant were harvested separately. The corresponding *Arabidopsis* transgenic plants were designated as MIR159OE-1 and MIR159OE-2. The transgenic and wild-type plants were cultivated in the greenhouse under the same environment. The phenotypes of T1 and T2 transgenic plants were observed and recorded. Their silhouette length data were collected from more than three plants for each transgenic line. More than 30 siliques were measured for each plant.

**Abbreviations**

GMS: Genic male sterility; RGMS: Recessive genic male sterility; DGMS: Dominant genic male sterility; CMS: Cytoplasmic male sterility.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s12870-021-03306-w.

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Not applicable.

**Authors’ contributions**

J-XJ and L-YY conceived the project and research plan. J-XJ finished the manuscript. J-XJ, P-FX and Y-JL analyzed the sequencing data, performed the experiments and drafted the manuscript. X-RZ, J-YZ, and J-FZ planted and observed the DGMS plants. Y-LL, M-YJ, and W-RW planted and observed the RGMS plants. All authors have read and approved the final manuscript.

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**Availability of data and materials**

The raw reads of the 24 sRNA libraries were uploaded to SRA database of NCBI and 24 accession numbers were obtained, including SRRX11350295, SRRX11350296, SRRX11350307, SRRX11350312, SRRX11350313, SRRX11350315, and SRRX11350316 (https://dataview.ncbi.nlm.nih.gov/object/PRJNA743414?revie
The raw transcriptome data will also be further utilized to excavate differentially expressed miRNAs in the fertile and sterile lines of RGMS and DGMS line.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References
1. Kaul MLH. Male sterility in higher plants. Berlin: Springer; 1988.
2. Hanson MR, Bentolila S. Interactions of mitochondrial and nuclear genes that affect male gameteophyte development. Plant Cell. 2004;16:154–69.
3. Chase CD. Cytoplasmic male sterility: a window to the world of plant mitochondrial-nuclear interactions. Trends Genet. 2007;23:81–90.
4. Fu TD. Breeding and utilization of rapeseed hybrid. 2nd ed. Wuhan: Hubei Science and Technology Press, 2000.
5. Song LQ, Fu TD, Tu JX, Ma CZ, Yang GS. Molecular variation of multiple allele inheritance for dominant genic male sterility gene in Brassica napus L. Theor Appl Genet. 2006;113:55–62.
6. Liu J, Lu W, He QB. Genetic analysis and molecular mapping of gene associated with dominant genic male sterility in rapeseed (Brassica napus L.). Gene Genomics. 2008;30(9):523–32.
7. Pan T, Zeng FY, Wu SH, Zhao Y. A study on breeding and application GM5 line of low eruci acid in rapeseed (B. napus). Oil Crop China. 1988;3:5–8.
8. Hou GZ, Wang H, Zhang RM. Genetic study on genic male sterility (GMS) material no.117A in Brassica napus L. (in Chinese). Acta Agric Shanghai. 2004;20(1):30–2.
9. Chen FX, Hu BC, Li QS. Discovery and study of genic male sterility (GMS) material 9012A in Brassica napus L. (in Chinese). Acta Agric Univ Pekinen.
10. Ke LP, Sun YQ, Hong DF, Liu PW, Yang GS. Identification of AFLP markers linked to one recessive genic male sterility gene in oilseed rape, Brassica napus. Plant Breed. 2005;124:367–70.
11. Yi B, Chen Y, Lei S, Tu J, Fu T. Fine mapping of the recessive genic male—sterile gene (Bnms1) in Brassica napus L. Theor Appl Genet. 2006;113:643–50.
12. Chen FX, Hu BC, Li QS, Chen WS, Zhang ML. Genetic studies on GM5 in Brassica napus L. Inheritance of recessive GMS line 9012A (in Chinese). Acta Agron Sin. 1996;22:431–8.
13. Xiao L, Yi B, Chen YF, Huang Z, Chen W, Ma CZ, et al. Molecular markers linked to Bnrf: a recessive epistatic inhibitor gene of recessive genic male sterility in Brassica napus. Euphytica. 2008;164:377–84.
14. Dong FM, Hong DF, Xie YZ, Wen YP, Dong L, Liu PW, et al. Molecular validation of a multiple-allelic recessive genic male sterility locus (Bnrm) in Brassica napus L. Mol Breed. 2012;30:193–205.
15. Chen L, Chen L, Zhang X, Liu T, Niu S, Wen J, et al. Identification of miRNAs that regulate silique development in Brassica napus. Plant Sci. 2018;269:106–17.
16. ChenZY, Huo Q, Yang H, Jian HJ, Qu CM, Lu K, et al. Joint RNA-Seq and miRNA profiling analyses to reveal molecular mechanisms in regulating thickness of pod canopy in Brassica napus. Genes (Basel). 2019;10(8):591–607.
17. Zhou ZS, Song JB, Yang ZM. Genome-wide identification of Brassica napus microRNAs and their targets in response to cadmium. J Exp Bot. 2012;63(12):4597–613.
18. Jian HJ, Yang B, Zhang AX, Ma JQ, Ding YR, Chen ZY, et al. Genome-wide identification of microRNAs in response to cadmium stress in oilseed rape (Brassica napus L.) using high-throughput sequencing. Int J Mol Sci. 2018;19(5):1431–47.
19. Fu Y, Mason AS, Zhang YF, Lin BG, Xiao ML, Fu DH, et al. MicroRNA- mRS expression profiles and their potential role in cadmium stress response in Brassica napus. BMC Plant Biol. 2019;19(1):570–89.
20. Wang TY, Ping XK, Cao YR, Jian HJ, Gao YM, Wang J, et al. Genome-wide exploration and characterization of mRF72/euAP2 genes in Brassica napus L. for likely role in flower organ development. BMC Plant Biol. 2019;19(1):336–50.
21. Körbes AP, Machado RD, Guzman F, Almerao MP, de Oliveira LFV, Loss-Morais G, et al. Identifying conserved and novel microRNAs in developing seeds of Brassica napus using deep sequencing. PLoS One. 2012;7(11):e006633. https://doi.org/10.1371/journal.pone.006633.
22. Huang D, Koh C, Feurtao JA, Tsang EW, Cutler AJ. MicroRNAs and their putative targets in Brassica napus seed maturation. BMC Genomics. 2013;14:140.
23. Megha S, Basu U, Joshi RK, Kav NNW. Physiological studies and genome-wide microRNA profiling of cold-stressed Brassica napus. Plant Physiol Biochem. 2018;132:1–17.
24. Wei W, Li G, Jiang X, Wang Y, Ma Z, Niu Z, et al. Small RNA and degrada- dome profiling involved in seed development and oil synthesis of Brassica napus. PLoS One. 2018;13(10):e0204998. https://doi.org/10.1371/journal.pone.0204998.
25. Jiang JX, Jiang JJ, Yang YF, Cao JS. Identification of microRNAs potentially involved in male sterility of Brassica campestris ssp. chinesis using microRNA array and quantitative RT-PCR assays. Cell Mol Biol Lett. 2013;18(3):416–32.
26. Ma ZW, Jiang JX, Hu ZW, Lv TQ, Yang Y, Jiang JJ, et al. Over-expression of mRF158 causes pollen abortion in Brassica campestris ssp.chinesis. Plant Mol Biol. 2017;93:313–26.
27. Dong Y, Wang Y, Jin FW, Xing LJ, Fang Y, Zhang ZY, et al. Differentially expressed miRNAs in anthers may contribute to the fertility of a novel Brassica napus genic male sterile line CN12A. J Integr Agric. 2020;19(7):1731–42.
28. Allen RS, Li J, Stahle MI, Dubroué A, Gubler F, Millar AA. Genetic analysis reveals functional redundancy and the major target genes of the Arabidopsis mRF159 family. Proc Natl Acad Sci U S A. 2007;104(41):16371–6.
29. Millar AA, Gubler F. The Arabidopsis GAMYB-like genes, MYB33 and MYB65, are microRNA-regulated genes that redundantly facilitate anther development. Plant Cell. 2005;17:705–21.
30. Zhan ZB, Duan H, Wang Y, Wu ZR, Wang Y, XU WH, Wang RH, et al. Identification of microRNAs and their target genes explores microRNA-mediated regulatory network of cytoplastic male sterility occurrence during another development in radish (Raphanus sativus L.). Front Plant Sci. 2016;7:1054–69.
31. Hu Z, Sheng X, Xiang X, Cao J. Evolution of MIR159/m19 genes in Brassica campestris and their function in pollen development. Plant Mol Biol. 2019;101(6):537–50.
32. Sun CC, Zhao H, Wang WR, Li YL, Qian XF, Fang GH. Inheritance and utilization of a recessive genic male sterile two-type line 2011A8B in Brassica napus L. (in Chinese). Acta Agric Shanghai. 2004;20(1):30–2.
33. Li SL, Qian YX, Wu ZH. Inheritance of genic male sterility in Brassica napus and its application to commercial production (in Chinese). Acta Agric Shanghai. 1985;12:1–12.
34. Niu CD, Li HY, Jiang LJ, Yan MJ, Li CY, Geng DL, et al. Genome-wide identification of drought-responsive microRNAs in two sets of Malus from interspecific hybrid progenies. Hortic Res. 2019;6(1):75–92.
36. Friedländer MR, Mackowiak SD, Li N, Chen W, Rajewsky N. miRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. Nucleic Acids Res. 2012;40:37–52.
37. Wen M, Shen Y, Shi S, Tang T. miRfEvo: an integrative microRNA evolutionary analysis platform for next-generation sequencing experiments. BMC Bioinformatics. 2012;13:140–9.
38. Love M, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15(12):550–700.
39. Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 2003;31:3406–34.
40. Meyers BC, Axtell MJ, Bartel B, Bartel DP, Baulcombe D, Bowman JL, et al. Criteria for annotation of plant MicroRNAs. Plant Cell. 2008;20:3186–90.
41. Jiang JX, Lv ML, Liang Y, Ma ZM, Cao JS. Identification of novel and conserved miRNAs involved in pollen development in *Brassica campestris* ssp. *chinensis* by high-throughput sequencing and degradome analysis. BMC Genomics. 2014;15:146–58.
42. Jiang JX, Bai JJ, Li SX, Li XR, Yang LY, He YK. HTT2 promotes plant thermotolerance in *Brassica rapa*. BMC Plant Biol. 2018;18:127–35.
43. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods. 2001;25(4):402–8.
44. Clough SJ, Bent AF. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. 1998;16:735–43.

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