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Identification of miRNAs Involved in Male Fertility and Pollen Development in *Brassica oleracea* var. *capitata* L. by High-Throughput Sequencing

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Abstract: MicroRNAs (miRNAs) are endogenously expressed, non-coding RNAs that play essential roles in numerous developmental and physiological processes in plants. However, there is a lack of data available on regulating fertility in cabbage (*Brassica oleracea* var. *capitata* L.). This study examined the expression of miRNAs during cabbage pollen development using high-throughput sequencing. Two small RNA libraries were constructed from flower buds from the cytoplasmic male-sterile (CMS) line and its maintainer fertile (MF) linee of cabbage, resulting in over 10 million sequence reads. A total of 211 new miRNAs and 7 conserved miRNAs were discovered. Eight randomly chosen miRNAs were found to have differential expression between CMS and MF, as determined by stem-loop quantitative real-time PCR (qRT-PCR). The expression patterns were similar to those detected with Illumina sequencing. Moreover, the possible targets of these putative miRNAs were inferred using a BlastX search and Gene Ontology (GO) analysis. The majority of targets have been indicated to be essential for the development of pollen or other plant parts. The discovery and characterization of miRNAs involved in pollen development in CMS and MF cabbage lines might drive research on the regulatory mechanisms of male sterility, leading to a better understanding of the crucial roles performed by miRNAs in this process.

Keywords: *Brassica oleracea*; miRNAs; male sterility; pollen development; high-throughput sequencing

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1. Introduction

Pollen formation in flowering plants comprises a complex series of activities governed by numerous genes [1,2]. Their growth and maturity require precise gene control at the transcriptional and post-transcriptional stages [3]. Pollen production has been linked to various distinct genes in recent years, and complete transcriptome investigations have revealed that the male gametophyte exhibits a complicated gene expression pattern [4–7]. The primary method of utilizing heterosis is male sterility [8]. Plant male sterility occurs when the male reproductive organs of a flowering plant are unable to produce fertile pollen or rupture, resulting in an abortion. Meanwhile, the female gametes can still develop normally, ensuring a smooth hybridization process [9,10]. There has been an increase in the number of studies conducted on pollen development in recent years, aiming to understand the molecular mechanism of male sterility in different plant species, including rice [11,12], soybean [13], cotton [14], and wheat [15]. MicroRNAs (miRNAs) are a type of small regulatory single-stranded non-coding RNA, typically between 20 and 24 nucleotides (nt) in length, that are ubiquitously present in eukaryotic organisms [16]. MiRNAs regulate their targets in higher plants by cleaving messenger RNA (mRNA) at virtually absolute complementarity sites to the miRNA sequence [17,18]. In particular, it has become clear that miRNAs play crucial roles in a broad range of biological processes, such as developmental regulation, signal transduction, hormone response, and stress tolerance [19,20]. In addition, there is mounting evidence that they play a regulatory role

in plant growth and development, specifically in the initiation of leaf sprouting [21], in flowering time regulation [22], in the accumulation of anthocyanins [23], in the response to the phytohormone abscisic acid [24], in root development [25], in cotton fiber elongation [26], in pollen or flower bud development [27,28], and so on. Researchers are now able to utilize sequence-based analysis as a powerful technique for uncovering previously unknown miRNAs in plants, especially those that are tissue-specific or have low abundance, due to the recent advancements in and affordability of high-throughput sequencing technologies [29–31]. To date, using experimental and computational methods, more than 38,000 miRNAs have been discovered in various plant species (release 22.1 by mirbase) [1].

Cabbage (*Brassica oleracea* var. *capitata* L.), one of the most important vegetable crops in the world, exhibits considerable heterosis, and male sterile lines have been widely used for hybrid seed production. Considering the economic significance of cabbage and its similarity to *Arabidopsis*, comprehensive genome sequencing projects have been undertaken, and mapping of the genome sequences has been completed [32]. In addition to the importance of cabbage, understanding the molecular processes that lead to cabbage pollen development is critical for controlling male fertility and exploiting heterosis. To date, certain genes have been identified as being involved in pollen development [33–37]. However, the miRNAs involved in cabbage pollen development remain unknown. Using computational analysis or high-throughput sequencing methods, only 10 miRNAs have been found in cabbage [38–40]. These miRNAs were expressed in the roots, stems, leaves, and cabbage flowers.

In the current study, we used high-throughput sequencing technology to sequence small RNA populations during cytoplasmic male sterility and maintain fertile lines during pollen development. Further analyses identified 7 known and 211 novel miRNAs based on sequence similarities or the secondary structure of their precursors. We used stem-loop quantitative real-time PCR to verify eight of these miRNAs in flower buds. In addition, we conducted a target function prediction analysis of miRNA using information from a BlastX search and the Gene Ontology (GO) database. The majority of these target genes are vital in plant pollen or anther development. These findings could offer useful knowledge on the possible regulatory function of miRNAs in forming cabbage CMS pollen. As a result, we can understand and explain the molecular mechanism underlying male sterility in cabbage.

2. Materials and Methods

2.1. Plant Materials, Sample Collection, and Total RNA Extraction

A male sterile system in cabbage (*Brassica oleracea* var. capitata L.), named '*Bo01-12A/B*' was used in this study. All floral organs of the '*Bo01-12A/B*' sister line show a completely consistent phenotype, except for the difference in pollen fertility. '*Bo01-12A*' is a cytoplasmic male sterile line (CMS) that lacks mature pollen, and '*Bo01-12B*' is the fertile maintainer line (MF) that generates normal mature pollen. The stable CMS line '*Bo01-12A*' was developed by eight generations of continuous backcrossing, with fertile '*Bo01-12B*' being self-crossed as its corresponding maintainer line. When reproducing the '*Bo01-12A/B*' line, half of the resulting offspring were found to be sterile, and the other half were fertile. The sterility traits in male offspring can be sustained over time. All plant materials used in this study were produced at the School of Horticulture's experimental farm at Anhui Agricultural University. During this investigation, three distinct types of samples were collected from 'Bo01-12A/B' plants during the flowering stage. These samples consisted of a combination of flower buds from 'Bo01-12A' and 'Bo01-12B' and the inflorescences exclusively from '*Bo01-12B*'. The flower buds were divided into five developmental stages based on longitudinal diameter and cytology results [33]. All specimens were pooled from 15 different plants, frozen quickly in liquid nitrogen, and stored at −80 °C until RNA extraction. Three biological replicates were performed. According to the manufacturer's protocol, total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA).

2.2. Small RNA Library Preparation and Sequencing

To prepare small RNA libraries, 1.5 µg of RNA per sample was employed as the input material, following the manufacturer's instructions for Illumina TruSeq Small RNA Prep Kits (LC Sciences, Hangzhou, China). To summarize, we ligated small RNAs to 3′ and 5′ RNA adapters. After ligating the adaptor to the RNA, it was transcribed into cDNA and then subjected to PCR. Then, fragments with 16–30 nucleotides (nt) were selected and analyzed using 15% PAGE. Once complete, the PCR products were purified, and the library was created. The small RNA libraries were sequenced using the Illumina Genome Analyzer according to the manufacturer's protocol (Beijing Genomics Institute, Beijing, China). All sequencing data were collected from 3 independent biological replicates and statistically analyzed.

2.3. Identification of Known and Novel miRNAs

After the raw sequence reads were extracted, they were filtered to remove adapter sequences, impurities, or sequences that were more than 15 to 30 nucleotides in length. The remaining sequences, ranging from 15 to 30 nucleotides, were fed into the ACGT101-miR software for miRNA prediction (edition 4.2; LC Sciences). Initially, the sequences were aligned to the RFam database (RFam contains ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), other ncRNAs, and repeats), and then Bowtie software was utilized to eliminate any clean reads that matched these sequences [41]. The sequencing data were compared to the Brassica database (BRAD) [2] using *Brassica oleracea* genome sequences. Any sequences that did not match were excluded. We used the miRBase database (miRBase version 22.1) [1] to compare the remaining sequences to those of all known plant miRNAs to determine which ones were conserved in *Brassica oleracea*. Candidates for conserved miRNAs were sequence pairs with no more than three mismatches. Simultaneously, the unmatched sequences were set aside as potential novel miRNA candidates.

The sequences were compared with the *Brassica oleracea* genome using a blasting technique to examine both new and established potential miRNAs. The flanking sequences were then utilized to forecast the hairpin folding structure of miRNA precursors, employing the Mireap method BGI, (http://sourceforge.net/projects/mireap/ (accessed on 3 August 2022)). Some requirements must be met by the non-coding sequence that makes up a putative miRNA precursor. The primary and crucial condition is a miRNA/miRNA* pair, which must be detected in high-throughput sequencing. The miRNA* sequence is the complementary reverse sequence to the target miRNA.

Additionally, the stem must contain both the miRNA and miRNA* sequences, and there must be no more than four mismatched nucleotides between them, with four continuous mismatches being unacceptable. Another requirement is that there should be no more than one asymmetric bulge in the miRNA/miRNA* duplex, and each asymmetric bulge must have no more than two bases. Fourth, a duplex with two nucleotide 3′ overhangs is formed when the miRNA and miRNA* are placed on opposite stem-arms (51). Fifth, a putative miRNA precursor requires a minimal folding energy (MFE) and minimal folding energy index (MFEI) less than -0.8 to differentiate itself from other short RNAs. Following the aforementioned thorough analysis, both conserved and new miRNAs were found in *Brassica oleracea*.

2.4. Stem-Loop Quantitative Real-Time PCR Assay

Stem-loop qRT-PCR is a high-sensitivity and -specificity method for detecting miRNAs in animals and plants [42,43]. To verify the existence and expression of the detected miRNAs, three unknown cabbage miRNAs and five established miRNAs were chosen for qRT-PCR. The stem-loop primers for the qRT-PCR tests were developed using [44]. Total RNA was extracted from floral buds of both lines using Trizol reagent (Takara, Japan) and was reverse transcribed using a PrimeScript RT reagent kit (Takara, Japan) according to the manufacturer's instructions. The qRT-PCR was performed with specific forward primers

and the universal primer using SYBR Premix Ex TaqII Kit (Takara, Japan) on CFX96TM Real-time System (Bio-Rad, Hercules, CA, USA). The 25 µL of PCR mixture included 2.0 µL of cDNA templates, 12.5 µL of 2 × SYBR Premix Ex TaqII, 1.0 µL of forward primer, 1.0 µL of reverse primer and 8.5 µL of ddH_2O. The amplification was performed as follows: 95 °C for 30 s, then 5 s at 95 °C and 30 s at 60 °C for 40 cycles. All reactions were performed with three biological replicates and GAPDH was used as an internal reference. All of the primers used in reverse transcription and PCR reactions are listed in Supplementary Table S1. The results were viewed using the CFX Manager Software (Bio-Rad, Hercules, CA, USA) and exported to Microsoft Excel. The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative transcript levels [45].

2.5. miRNA Target Prediction and Analysis

The potential targets of the identified miRNAs were predicted using the online software psRNATarget with default parameters [3]. The scoring system and the criteria established by [46] were then applied to each indicated target gene. Targets for microRNAs were deemed to be sequences with an aggregate score of fewer than 3.0 points. To acquire a deeper insight into the role and categorization of miRNA targets, a BlastX search was conducted against the NCBI protein database to assess the function of potential targets. The results of the GO classification were exhibited in three distinct hierarchies: cellular component, biological process, and molecular function [47].

3. Results

3.1. Morphological and Cytological Characterization of CMS and MF Lines

The sepals, petals, and pistils of both the cytoplasmic male sterile (CMS) and the maintainer fertility (MF) lines of cabbage exhibited normal development (Figure 1A,C). Nevertheless, the anthers of CMS lines were significantly different (Figure 1B,D) compared to the anthers of MF lines (Figure 1A,C). The developmental morphology of pollen was observed using the histochemical method. In the earliest phase of pollen development, the results revealed no significant differences between CMS and MF cabbage lines. The histochemical observation at the later stage of cabbage pollen development showed that tapetum cells gradually degenerated in MF lines, pollen cytoplasmic staining gradually deepened, and a large number of normal mature microspores were evenly distributed in the pollen sac (Figure 1C). However, the anther wall tissue of CMS lines degraded, the pollen sac shrank, the pollen content disappeared, the tapetum cells gradually disintegrated, and the pollen showed abortion (Figure 1D).

3.2. Overview Analysis of Sequences from Small RNAs in Cabbage Flower Buds

To find short RNAs that are differentially expressed during cabbage pollen development, two RNA libraries were created using flower buds from the MF and CMS lines. Deep sequencing of the libraries generated 6,866,663 and 6,895,083 raw sequence reads from MF and CMS cabbages, respectively (Supplementary Table S2). After omitting adapter sequences and filtering out low-quality reads, 5,158,080 (3,269,277 unique sequences) clean MF reads and 5,338,804 (3,269,277 unique sequences) clean CMS reads were retained for further analysis. We used NCBI GenBank and Rfam [4] to categorize and annotate the distinct small RNAs sequences. Among all unique small RNAs, 2.60% in MF and 2.14% in CMS flower buds matched non-coding RNAs, including rRNA, snRNA, snoRNA, and tRNA. The summaries of the various numbers and percentages of these various classes of small RNAs can be found in Table 1. Searching against miRBase 22.1 (http://www.mirbase.org (accessed on 14 June 2022)) identified 0.01% of miRNAs from MF and CMS.

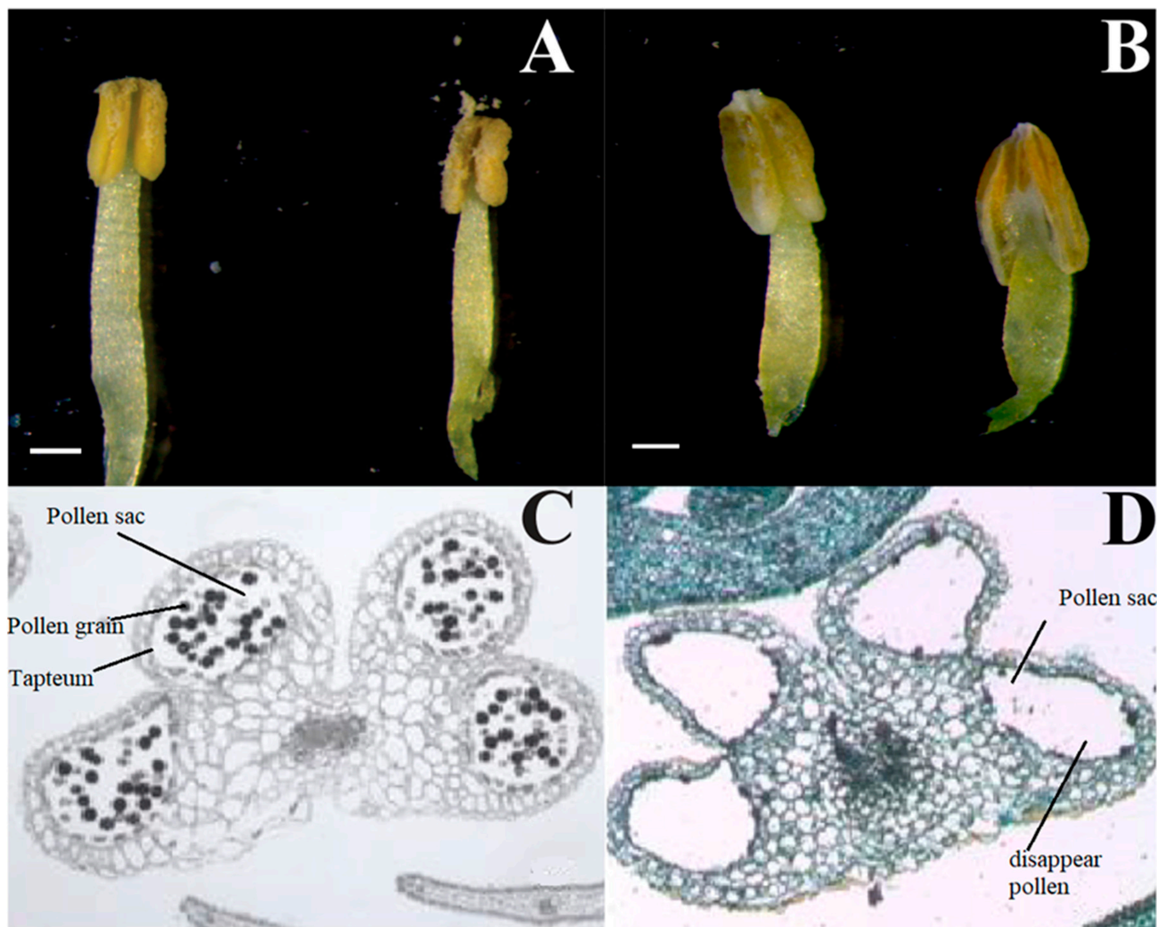

Figure 1. Phenotypic characterization and cytological characterization of the anther. Anther of MF line (**A**). Sterile anther of CMS line (**B**). Bars = 0.5 cm. Fertile anther of MF line (**C**). Sterile anther of CMS line (**D**).

Table 1. Distribution of small RNAs in cabbage flower buds from MF and CMS lines.

Category	MF				CMS			
	Unique	Percent	Redundant	Percent (%)	Unique	Percent	Redundant	Percent (%)
exon_antisense	89,744	2.84%	127,721	2.48%	84,956	2.60%	124,397	2.33%
exon_sense	101,550	3.21%	136,054	2.64%	97,802	2.99%	139,259	2.61%
intron_antisense	33,720	1.07%	40,735	0.79%	30,272	0.93%	38,399	0.72%
intron_sense	31,781	1.00%	46,920	0.91%	28,267	0.86%	45,752	0.86%
rRNA	75,067	2.37%	385,277	7.47%	63,993	1.96%	389,106	7.29%
snRNA	820	0.03%	3769	0.07%	817	0.02%	3792	0.07%
snoRNA	713	0.02%	3576	0.07%	689	0.02%	3168	0.06%
tRNA	5768	0.18%	169,111	3.28%	4612	0.14%	225,707	4.23%
miRNA	273	0.01%	8984	0.17%	303	0.01%	9426	0.18%
Others	2,825,652	89.27%	4,235,933	82.12%	2,957,566	90.47%	4,359,798	81.66%
Total	3,165,088	100.00%	5,158,080	100.00%	3,269,277	100.00%	5,338,804	100.00%

Regarding shared and unique small RNA reads, the MF and CMS libraries contained 35.52 and 37.71 percent of the total small RNAs, respectively. The two libraries shared only 26.77% of the total small RNAs (Figure 2A) and only a small fraction (6.37%) of unique small RNAs (Figure 2B). This revealed the similarity and diversity of small RNAs expressed in the sterile cabbage (CMS) line and its fertile maintainer (MF) during pollen development.

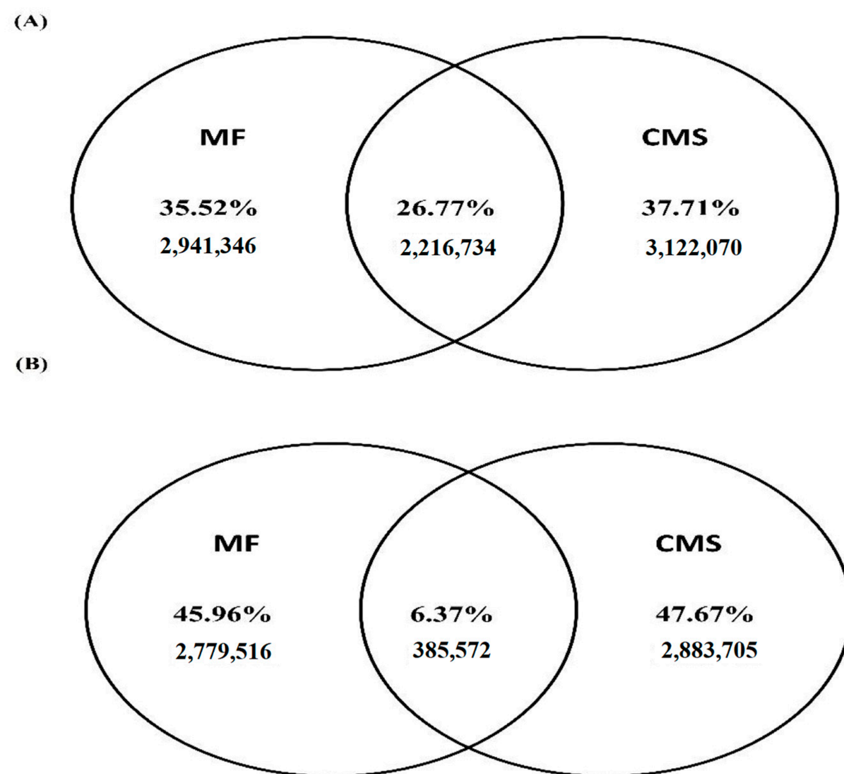

Figure 2. Distribution of common and unique small RNAs between MF and CMS libraries. (**A**) Total sRNA sequences. (**B**) Unique sRNA sequences.

Our library dataset also observed that the length dissemination of these small RNAs is not uniform (Figure 3). Most small RNAs for both libraries were 21–24 nt in length, the typical size range for Dicer-derived products. Consistent with the bulk of the previously published data on other plant species, the 24 nt-long RNAs were the most prevalent, accounting for 48.50% (MF) and 53.43% (CMS) of the total reads [48–50].

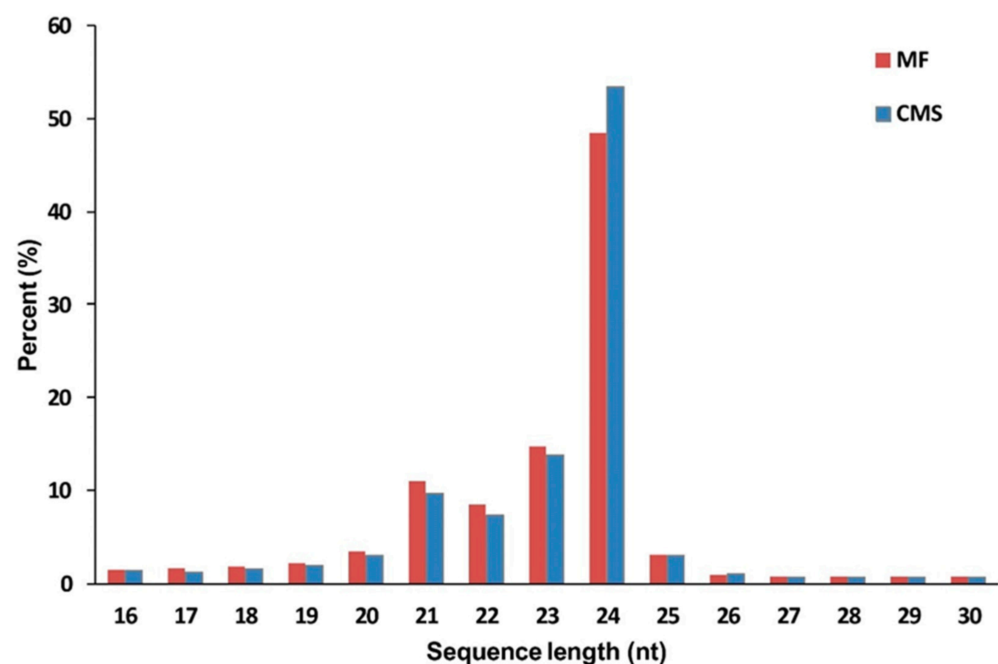

Figure 3. The size distribution of the small RNAs between MF and CMS libraries of cabbage.

3.3. Identification of Known miRNAs in Cabbage MF and CMS Line

To locate known miRNAs in cabbage floral buds, all mappable small RNA sequences were blasted against the latest miRBase repository (miRBase 22.1). Currently, the cabbage miRbase 22.1 database [1] contains 10 known miRNAs and 1 miRNA* from 6 miRNA families. The seven known bol-miRNAs (bol-miR157a, bol-miR171a, bol-miR172a, bol-miR172b, bol-miR398a-3p, bol-miR398a-5p, bol-miR824) in miRbase 22.1 were found by high-throughput sequencing in the study. Table 2 displays the data on all the established miRNAs in the MF and CMS libraries.

Table 2. Identification of known miRNAs in cabbage flower buds from they MF and CMS lines.

miRNA	Accession No.	miRNA-Seq	Count	
			MF	CMS
bol-miR157a	MIMAT0010169	TTGACAGAAGATAGAGAGCAC	1730	868
bol-miR171a	MIMAT0010170	TTGAGCCGTGCCAATATCACG	152	256
bol-miR172a	MIMAT0010171	AGAATCTTGATGATGCTGCAT	698	1100
bol-miR172b	MIMAT0010172	AGAATCTTGATGATGCTGCAT	59	87
bol-miR398a-3p	MIMAT0010174	TGTGTTCTCAGGTCACCCCTT	21	40
bol-miR398a-5p	MIMAT0010173	GGAGTGTCATGAGAACACGGA	23	35
bol-miR824	MIMAT0005597	TAGACCATTTGTGAGAAGGGA	3124	2908

miRBase database (miRNA Registry, Release 22.1, March 2018).

To some extent, the number of reads indicates miRNA enrichment [51]. Significant discrepancies exist in miRNA read frequencies between the two libraries (Table 2). For example, the expression of bol-miR157a and bol-miR824 were comparatively upregulated in the MF line compared to the CMS line, whereas bol-miR171a, bol-miR172a, bol-miR172b and bol-miR398a indicated down-regulation in the MF line comparative to the CMS line. Meanwhile, the expression level of bol-miR824 was significantly higher than other miRNAs in both libraries. Conversely, specific micro RNAs were expressed at a very low level, as seen by the small number of reads. The number of miRNAs differed between MF and CMS, suggesting that miRNA genes are transcribed differently during pollen development.

3.4. Identification of Novel miRNAs in Cabbage Flower Buds

The capability of miRNA flanking sequences to fold back into a hairpin form is critical for distinguishing potential miRNA from other short RNAs [52]. Consequently, all genomic loci that can fold into secondary structures generate sRNAs deemed miRNA candidates. The secondary structure of each miRNA was predicted and analyzed for a stable stem-loop hairpin. Therefore, 211 putative novel miRNAs were detected from different flower buds in cabbage. Supplementary Table S3 demonstrates the sequence traits and read count of each miRNA detected through high-throughput sequencing. The secondary hairpin structures of the representative miRNAs (bol-miR0001 and bol-miR0023) are shown in Figure 4.

The length distribution of these new candidates is concentrated between 20 and 24 nt, while the length of the precursors varies from 63 to 101 nt, in contrast to previous studies on other plants such as *A. thaliana*, *O. sativa* and *B. rapa* [39,53]. The minimal folding energy of these anticipated pre-miRNAs varied from −18 to −51.5 kcal/mol, which met the criteria of miRNA annotation.

To determine the expression level of miRNAs in a particular tissue, one could count all of the sRNA reads that can be mapped in the corresponding libraries [54]. This study revealed that the number of reads obtained for the predicted new miRNAs varied between 0 and 673, most of which were sequenced fewer than 10 times, as presented in Table S3. Although expressed at relatively low levels, most of the predicted new miRNAs were found to be expressed in the flower buds of both lines, which is consistent with the observation that non-conserved miRNAs tend to have lower expression levels than conserved miRNAs [46]. When comparing the expression levels of new miRNAs in the flower buds of CMS and MF

lines, only bol-miR0109-3p exhibited significantly high expression abundance, indicating its crucial function in cabbage pollen development. In addition, we discovered that several miRNAs were exclusively expressed in a single line; for instance, bol-miR0014-3p was only recognized in the sterile line (35 reads), but not in its maintainer line.

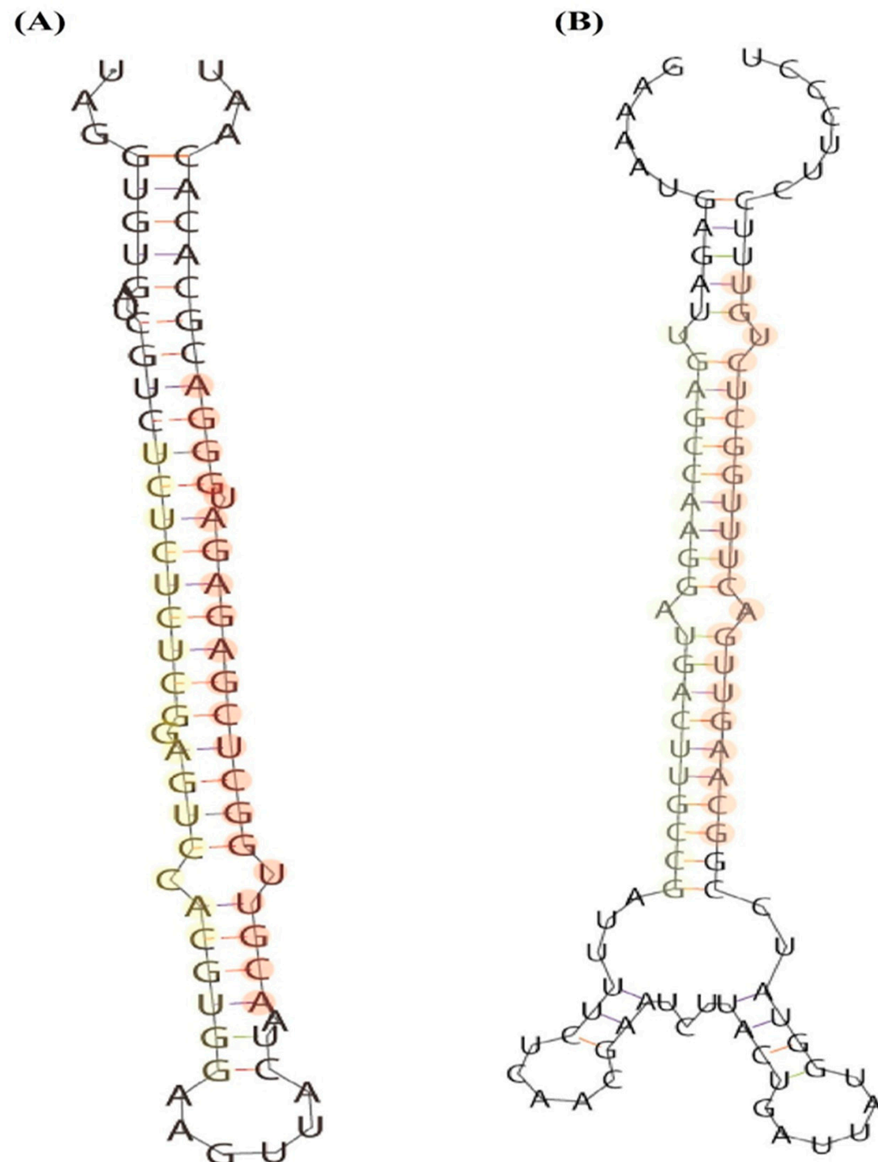

Figure 4. Predicted secondary structures of representative novel miRNAs in the flower buds of cabbage. (**A**) bol-miR0001. (**B**) bol-miR0023.

3.5. Expression Analysis of Known and Novel miRNAs in Cabbage CMS and MF Line

To confirm candidate miRNAs' presence and expression patterns during pollen formation, stem-loop qRT-PCR was performed on eight randomly selected miRNAs, including five conserved miRNAs (bol-miR157a, 171a, 172a, 824, 398a-3p) and three new miRNAs (bol-miR0023, 0101, 0142). These miRNAs were identified by qRT-PCR and demonstrated differential expression levels between the two lines (Figure 5). Among the eight differentially expressed miRNAs, bol-miR171a, bol-miR172a, bol-miR398a-3p, bol-miR0023 and bol-miR0101 were expressed at a relatively higher level in the CMS line compared with in the fertile line, whereas bol-miR157a, bol-miR824 and bol-miR0142 were expressed at a much lower level. Their expression patterns detected by qRT-PCR were comparable with

those obtained in the high-throughput sequencing results, indicating that the small RNA sequencing data were highly applicable in this research.

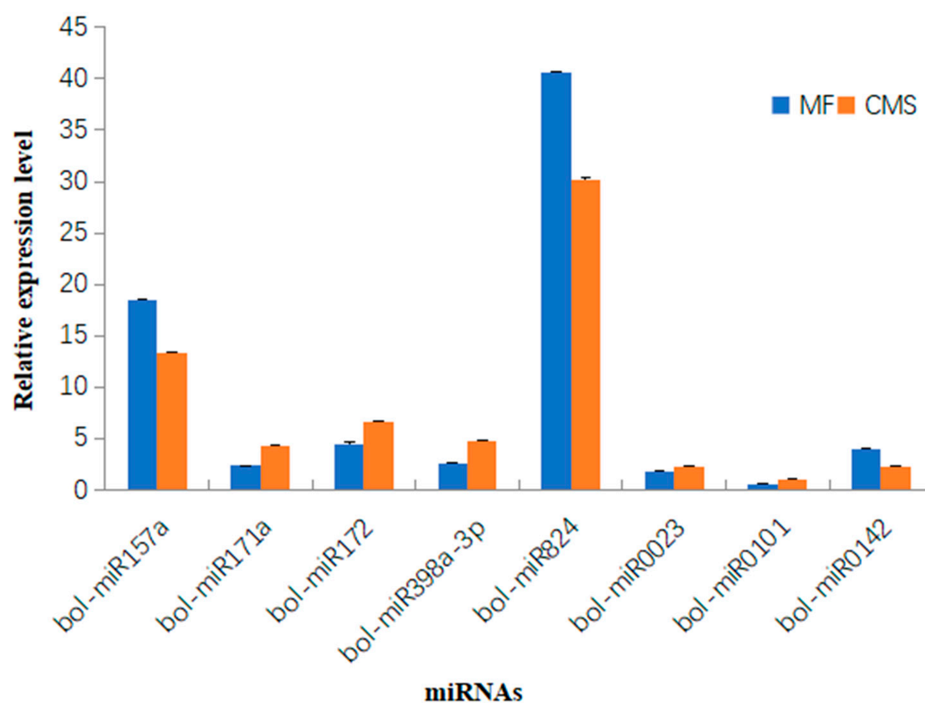

Figure 5. Expression analysis of 8 miRNAs in MF and CMS of cabbage by qRT-PCR.

3.6. Prediction and GO Function Analysis of miRNA Targets in Cabbage

To gain additional insights into the biological roles of newly discovered and previously identified miRNAs in cabbage, we utilized the psRNATarget program with default parameters (http://plantgrn.noble.org/psRNATarget/ (accessed on 4 October 2022)) to predict potential target genes. As a result, 54 and 763 potential target genes were found for 7 conserved and 211 novel miRNAs (Supplementary Table S4), with an average of 7.71 and 3.62 targets per miRNA molecule, respectively. They were subjected to a BlastX search against the NCBI protein database to annotate the target genes. A significant portion of the predicted target genes were found to be involved in various plant growth and development functions, including transcription factors, growth-regulating factors, F-box domain-containing proteins, AP2-like proteins, Zinc finger proteins, and members of the SBP-box gene family. These proteins play essential roles in plant development [55–57]. The fact that some miRNAs (bol-miR172a) have several target sites suggests that these miRNAs have distinct biological roles. Meanwhile, a single gene may potentially be targeted by several miRNAs. For example, a few novel miRNAs target F-box domain-containing proteins, including bol-miR0028-3p, bol-miR0072-5p and bol-miR0082-3p.

All identified targets in this research were subsequently submitted to GO analysis to examine these target genes' putative functions. Based on their biological and molecular functions, microRNA-targeted genes were organized into functional categories (Figure 6). The result of GO analysis revealed that 31 and 45 GO categories were enriched for novel and conserved miRNA targets, respectively. For conserved miRNAs, 139 genes participated in 9 cellular components, 78 genes were involved in 5 different molecular functions, and 311 genes were involved in 17 biological processes. According to classifications based on biological processes, the miRNA-target genes were associated with 17 biological processes. The most frequent term was "biological regulation", followed by "developmental process" and "reproductive process". For novel miRNAs, 1596 target genes were involved in 13 different cellular components, 721 target genes took part in 10 molecular functions, and 2681 target genes contributed to 22 biological processes (Supplementary Table S5). The

most frequent terms were “metabolic process”, “cell part”, and “catalytic activity”. These results suggest that cabbage miRNAs are involved in various physiological functions.

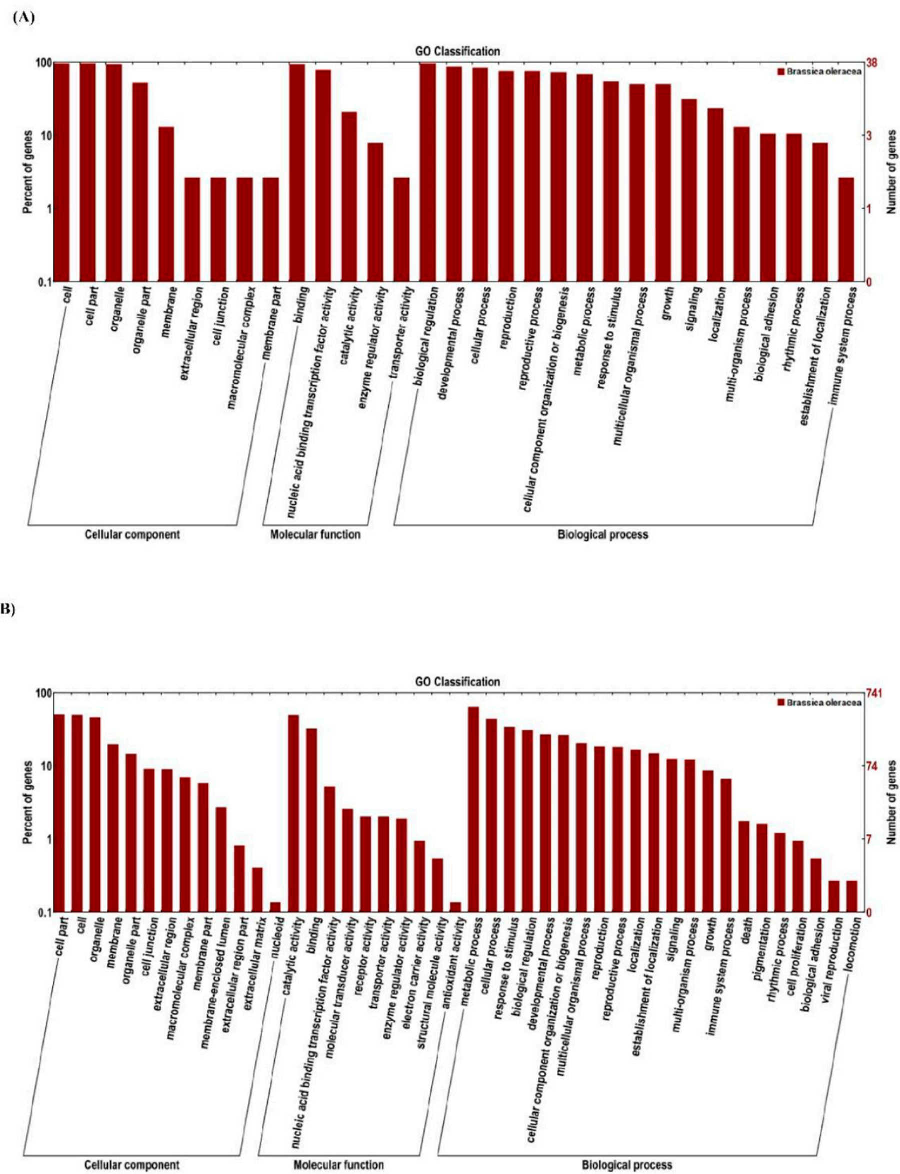

Figure 6. Gene categories and distribution of miRNAs targets in cabbage. (**A**) Gene categories and distribution of target genes for known miRNAs. (**B**) Gene categories and distribution of target genes for novel miRNAs.

4. Discussion

The role of small RNAs in regulating plant development, particularly in the process of anther and pollen formation, has been identified by researchers as significant [55,58]. However, the connection between miRNAs and pollen in *B. oleracea* has not been reported in any previous research. To identify miRNAs involved in the regulation of pollen development in *B. oleracea*, two small RNA libraries were constructed using the floral buds of a CMS line and its maintainer line. We found a substantially broader distribution of short RNAs (18–30 nt in length) using high-throughput sequencing, with 24 nt being the most abundant, followed by 21 and 22 nt. These results are consistent with previous research conducted on numerous other species, such as *Arabidopsis thaliana* [49], *Cucumis sativus* [59], *Medicago truncatula* [60], *Oryza sativa* [61] and *Zea mays* [62], suggesting that 24 nt is the most common length for small RNAs.

The detection of miRNAs in male-sterile plants such as maize, rice, *Brassica campestris* ssp. *chinensis*, *Brassica juncea*, and cotton has been documented previously [63,64]. This study is the first to utilize deep sequencing of flower buds from both CMS and maintainer lines in cabbage to discover miRNAs and their corresponding targets. The sequencing generated millions of unique sRNA raw reads, ranging from 16 to 30 nucleotides in length, resulting in the identification of 211 novel miRNAs, thereby increasing the number of known miRNAs in cabbage. Additionally, seven conserved miRNAs listed in miRBase 22.1 were also identified in this study.

Furthermore, some of the confirmed miRNAs and their target genes play a role in the regulation of pollen development [65,66]. These studies indicate that miRNA is significant in regulating plant pollen development, but there are few systematic studies on the miRNAs related to cabbage pollen development. The cabbage CMS line shares the same genetic makeup as its fertile maintainer line, making them valuable genetic resources for studying the mechanisms that lead to male sterility and pollen production. The use of high-throughput sequencing to examine the small RNA transcriptome has produced a vast amount of data, enabling the identification of conserved miRNAs and the exploration of novel miRNAs with high specificity and accuracy across various tissues, developmental stages, and environmental conditions. This study uses deep sequencing to perform comparative expression profiling of miRNAs during reproductive development in both the cabbage CMS and maintainer lines. This is the first study to attempt such an approach, which is essential in understanding how sRNA influences cabbage pollen development.

To study the molecular basis resulting in the cabbage male sterility, we constructed two small RNA libraries from the pollen development of CMS and its maintainer line pooled as one sample. The size and distribution of the millions of short RNAs read from the two libraries indicate that the 24 nt class is the most abundant, followed by the 22 nt and 18 nt classes. Similar findings were observed in *A. thaliana* [67], *Oryza sativa* [68], *Medicago truncatula* [69], *Arachis hypogaea* [70] and *Hevea brasiliensis* [71]. In the current study, we identified 211 novel miRNAs, increasing the number of distinct miRNAs in cabbage. This study detected seven conserved miRNAs registered in miRBase 22.1. Highly conserved miRNAs, as previously reported [72], are found in all organisms, are expressed at high levels, and are typically observed with members of the same family.

In contrast, non-conserved miRNAs may be unique to a single species, encoded in a single locus, and have low expression levels [73]. Our findings also provide for the number of reads anticipated for novel miRNAs being less than 100, which was in accord with the previous results. Notably, we could not detect the four newly identified miRNA (bol-miR9408, bol-miR9409, bol-miR9410, bol-miR9411) expressed in cabbage leaves from this high-throughput sequencing, perhaps implying that some miRNAs were specific to certain developmental stages, specific tissues, various growth conditions or different genotypes [38]. miRNAs are involved in numerous biochemical and physiological processes, responding to specific regulatory requirements at each developmental stage. It is plausible that iRNA classifications align with miRNAs' temporal and spatial expression patterns [74]. Our libraries comprise a mixture of flower bud samples from the CMS line and its fertile line. Consequently, the miRNA expression levels in our findings represent the development of cabbage pollen.

Stem-loop qRT-PCR is a technique that enables high-throughput detection and quantification of miRNA expression. This method offers a quick, sensitive, and specific means of profiling miRNA expression [43]. This study employed quantitative RT-PCR using a stem-loop primer to detect the presence of three novels and five previously identified miRNAs in CMS and viable lines. Furthermore, the miRNA expression patterns between CMS and its maintainer reflected the count number of sequences through deep sequencing, suggesting that the small RNA sequencing results were reliable in this study and that miRNA genes would be differentially transcribed during the development of cabbage pollen. Previous reports have suggested a miRNA pathway in plant pollen and anther development. For

example, Arabidopsis mature pollen contained miR157, miR171, miR172, and miR824 by utilizing 454 sequencings and the miRCURY array [65,75]. Microarray analysis identified miR171a and miR398 in loblolly pine male gametophyte and rice pollen, respectively [61,76]. Additionally, this investigation confirmed the existence and expression of these conserved miRNA families. Accordingly, many canonical miRNAs are evolutionarily conserved in various plant species; some have conserved functions in regulating the basic cellular and developmental pathways [77].

Understanding the control of miRNAs in plant growth requires the identification of target genes for miRNAs. In the current study, the prediction of probable target genes and GO analysis were carried out to evaluate and characterize the role of miRNA in the development of cabbage pollen. Some miRNAs whose targets have been established were found to play essential roles in the formation of floral organs and pollen. An instance is an interaction between bol-miR157 and the squamosa promoter binding protein-like (SBP) transcription factor, likely involved in gametophyte development [55]. Research has shown that bol-miR172 obstructs the functioning of AP2-like transcription factors by binding to them. These transcription factors are associated with regulating blooming time and developing floral organs in various plant species, such as Arabidopsis, maize, tobacco, and opium poppy [3,78–80]. Specific suppression of the mitochondrial electron transport chain has been shown to partially imitate the effects of bol-miR824 on MADs-box genes, which are involved in cytoplasmic homeostasis in CMS stem cells [81]. Both bol-miR0072 and bol-miR0082 target F-box proteins, which are crucial for controlling self-incompatibility as well as determining the identity of floral organs and floral meristems in plants [5,6], and in the control of the circadian clock, photomorphogenesis, and flowering time [80]; similarly, another study by Yan et al. (2015) reported that the important miRNA osa-miR528 might be involved in pollen sterility by targeting F-box proteins [81]. These findings support the notion that miRNAs are present in reproductive organs and might even contribute to the intricate regulatory network that governs pollen formation.

5. Conclusions

In conclusion, our research compared the expression profiles of the miRNAs derived from the CMS line of cabbage and its maintainer line of cabbage. Using high-throughput sequencing techniques, researchers identified several known and novel miRNAs that are expressed during the formation of cabbage flower buds. To verify the variance in the expression of several randomly picked miRNAs between the CMS and its maintainer line, the stem-loop quantitative real-time PCR analysis was employed. Analysis of predicted targets shows a clear role for miRNAs in both pollen development and male sterility. The present work establishes a firm basis for investigating miRNA-mediated regulatory networks in pollen formation. Additional research into the functional roles of these differentially expressed miRNAs and their targets is needed to shed light on the regulatory mechanisms that underlie cabbage's male sterility.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9040515/s1, Table S1: The miRNAs primers designed for stem-loop qRT-PCR. Table S2: The filtering for small RNA clean reads in the libraries of MF and CMS. Table S3: Novel miRNAs were identified from the MF and CMS lines in cabbage. Table S4: Predicted targets for identified candidate miRNAs. Table S5: GO analysis of target genes of known and novel miRNAs.

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