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Short communication

IDENTIFICATION OF microRNAs POTENTIALLY INVOLVED IN MALE STERILITY OF *Brassica campestris* ssp. *chinensis* USING microRNA ARRAY AND QUANTITATIVE RT-PCR ASSAYS

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Abstract: microRNAs (miRNAs) are a class of newly identified, noncoding, small RNA molecules that negatively regulate gene expression. Many miRNAs are reportedly involved in plant growth, development and stress response processes. However, their roles in the sexual reproduction mechanisms in flowering plants remain unknown. Pollen development is an important process in the life cycle of a flowering plant, and it is closely related to the yield and quality of crop seeds. This study aimed to identify miRNAs involved in pollen development. A microarray assay was conducted using the known complementary sequences of plant miRNAs as probes on inflorescences of a sterile male line (*Bcajh97-01A*) and a fertile male line (*Bcajh97-01B*) of the *Brassica campestris* ssp. *chinensis* cv. 'Aijiaohuang' genic male sterility sister line system (*Bcajh97-01A/B*). The results showed that 44 miRNAs were differently expressed in the two lines. Of these, 15 had over 1.5-fold changes in their transcript levels, with 9 upregulated and 6 downregulated miRNAs in inflorescences of '*Bcajh97-01A*' sterile line plants. We then focused on 3 of these 15 miRNAs (miR158, miR168 and miR172). Through computational methods, 13 family members were predicted for these 3 miRNAs and 22 genes were predicted to be their candidate target genes. By using 5' modified RACE, 2 target genes of miR168 and 5 target genes of miR172 were identified. Then, qRT-PCR was applied to verify the existence and expression patterns of the 3 miRNAs in the flower buds at five developmental stages. The results were generally consistent with those of the microarray. Thus, this study may give

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Abbreviations used: BLAST – Basic Local Alignment Search Tool; qRT-PCR – quantitative reverse-transcription polymerase chain reaction; RACE – rapid amplification of cDNA ends

a valuable clue for further exploring the miRNA group that may function during pollen development.

Key words: *Brassica campestris*, Chinese cabbage, *Brassica rapa*, Microarray, microRNA, Pollen development, Quantitative RT-PCR, 5' modified RACE, Male sterile line, Male fertile line

INTRODUCTION

The correct temporal and spatial expression of the full set of genes in cells allows normal plant growth and development. However, it remains unclear how cells control gene expression in different tissues at different times. Scientists have been attempting to elucidate the regulatory mechanisms of gene expression, and one of the most crucial discoveries in recent decades is that of microRNAs (miRNAs) [1]. These are ~22-nucleotide (nt), noncoding, small regulatory RNA molecules that are widely present in plants and animals. They have also been found in some viruses [2-4]. miRNAs regulate the expression patterns of their target genes at the post-transcriptional level by degrading target mRNAs or by reducing the translation of target genes [5-7]. Since the first miRNA was identified in a nematode (*Caenorhabditis elegans*) in 1993 [8], miRNAs have been found in various species and identified as being involved in a wide range of biological processes, including leaf development and polarity [9], lateral root formation [10], floral organ identity [6], signal transmission [11], plant nutrient homeostasis [12-13], and plant responses to biotic and abiotic stresses [13-15].

Pollen development is essential for plant reproduction and crop production. Extensive attempts have been made to determine the mechanism of pollen development, which may involve multiple regulators and complex regulatory networks. Whether miRNAs play any role in pollen development remains unknown. Previous studies have demonstrated the presence of many miRNAs in *Arabidopsis thaliana* pollen. Using 454 sequencing technology, 33 known miRNAs have been identified in *A. thaliana* mature pollen, and quantitative RT-PCR analysis has revealed that 17 miRNAs are more abundant in the pollen than in the leaves [16]. miRNA expression profiles have also been analyzed in *A. thaliana* pollen and inflorescences using miRNA array assays, and the profiles show 26 miRNAs that are differently expressed in the two materials. Most of them are downregulated in mature pollen [17]. Furthermore, research on sperm cells and pollen in *A. thaliana* using Illumina sequencing has verified the presence of 83 and 75 miRNA families, respectively [18]. Borges *et al.* also illustrated and summarized the miRNA families detected in sperm cells and pollen in their review paper [19].

In recent years, most studies on miRNA functions in the plant sexual reproductive process have focused on the model plant *A. thaliana*. However, *A. thaliana* cannot embody economic value. *Brassica campestris*, an important Cruciferous species, is an economically important vegetable crop that exhibits

a wide range of morphological diversity in areas such as plant type, leaf morphology and head formation, thereby generating a variety of subspecies and reflecting this species' immense breeding potential. Studies on *B. campestris* miRNAs would be more valuable to the agricultural industry than studies on *A. thaliana*.

A. thaliana and *B. campestris* are Cruciferous species that have high sequence homology. They share many conserved miRNAs. Computational and/or experimental identification revealed six miRNAs (miR158, miR391, miR824, miR825, miR827 and miR840) and two small RNAs (small-85 and small-87) that are *Arabidopsis–Brassica* lineage-specific small RNAs [20]. However, some studies have suggested the presence of genetic divergences between the two species in terms of miRNA evolution. He *et al.* cloned 237 small RNAs and found that 65% perfectly or almost perfectly matched the *A. thaliana* genome. The remaining 35% (83/237) of unmatched small RNAs were likely to be specific to *Brassica.* For example, bra-miR1885, which can be induced specifically by Turnip mosaic virus (TuMV) infection and has been found to target TIR-NBS-LRR class disease-resistant protein-coding gene sequences [21], was identified to be a *Brassica*-specific novel miRNA [22]. Bna-miR1140, a newly discovered candidate miRNA family, has also been found to be possibly unique to *Brassica* [23]. The discovery of such *Brassica*-specific miRNAs suggests that these kinds of miRNA may play important and specific roles in regulatory processes in *Brassica*. Therefore, these conserved and species-specific *Brassica* miRNAs are worthy of further study.

This study aimed to determine whether miRNAs play important regulatory roles in pollen development in an important vegetable crop, the Chinese cabbage (*B. campestris*). We used a plant miRNA microarray to screen miRNAs that may be involved in pollen development using the inflorescences of the *B. campestris* ssp. *chinensis* cv. 'Aijiaohuang' genic male sterility sister line (*Bcajh97-01A/B*). Results indicated that 15 miRNAs had significant expression differences in the inflorescences of the sterile line and fertile line plants. Through computational methods, 13 family members were predicted for the 3 miRNAs and 22 genes were predicted to be their candidate target genes. A total of 7 target genes were demonstrated using 5' modified RACE. We then verified the expression abundance of 3 selected miRNAs (miR158, miR168 and miR172) at 5 pollen developmental stages using qRT-PCR. This study may serve as an important basis for further exploring the group of miRNAs that may be involved in pollen development.

MATERIALS AND METHODS

Plant materials

'*Bcajh97-01A/B*' is a genic male sterile A/B line (sister line) of Chinese cabbage (*B. campestris* ssp. *chinensis* syn. *B. rapa* ssp. *chinensis* cv. 'Aijiaohuang'). Male sterility is controlled by a pair of nuclear recessive genes (*MSMS*) [24-25].

The fertile line (*Bcajh97-01B*) is the maintainer line of the sterile line (*Bcajh97-01A*). The progenies of the A/B line are segregated into sterile and fertile types during reproduction with a 1:1 ratio. This procedure has been used for more than 10 years and the character of male sterility has been shown to be steadily maintained. The plants were grown on the experimental farm of Zhejiang University.

Plant samples were collected, including inflorescences and flower buds at different stages. The inflorescences contained freshly opened flowers and unopened flower buds. The flower buds were classified into five developmental stages (stages I to V), depending on the longitudinal diameter. These five stages represented the five developmental stages of pollen development, as determined based on cytological observations [24]. Stage I flower buds (< 1 mm) corresponded to the onset of microspore mother cell formation. Stage II flower buds (1.2 to 1.6 mm) represented the meiosis period. Stage III flower buds (1.8 to 2.2 mm) corresponded to the tetrad period. Stage IV flower buds (2.4 to 2.8 mm) represented the uninucleate pollen period. Stage V flower buds (3.0 to 3.4 mm) were the largest flower buds that were about to open. At this stage, opened anthers with mature pollen can be observed. The materials were frozen in liquid nitrogen immediately after harvesting and stored at -80ºC for subsequent analysis.

miRNA microarray assay

Total RNA isolation and microarray experiments were performed by LC Sciences (USA, http://www.lcsciences.com). The plant miRNA microarray assay used 853 unique probes that represented 853 unique mature miRNAs from 21 plant species. All the mature miRNA sequences were obtained from Sanger miRBase Release 12.0 (http://www.mirbase.org/). The miRNA probe sequences were designed to be complementary to the mature miRNA sequences. Mature miRNA names were used as probe names. Four repeats were conducted for each probe. The ratio of the two sets of detected signals (log2 transformed, balanced) in the two inflorescence samples were calculated, as were the *p*-values of the *t*-test for each probe. Differentially detected signals were those whose *p*-values were less than 0.01. The following detailed experiment procedure is the μParaflo miRNA microarray assay [26-28].

The assay started with 2 μg of total RNA samples which were 3'-extended with a poly(A) tail using poly(A) polymerase. An oligonucleotide tag was then ligated to the poly(A) tail for later fluorescent dye staining. Two different tags were used for the two RNA samples in dual-sample experiments. Hybridization was performed overnight on a μParaflo microfluidic chip using a micro-circulation pump (Atactic Technologies, Houston, USA). On the microfluidic chip, each detection probe consisted of a chemically modified nucleotide coding segment, complementary to the target microRNA (from miRBase, http://www.mirbase.org/) or control RNA, and a spacer segment of polyethylene glycol to extend the coding segment away from the substrate. The detection

probes were made by *in situ* synthesis using PGR (photogenerated reagent) chemistry. The hybridization melting temperatures were balanced by chemical modifications of the detection probes. Hybridization used 100 l 6xSSPE buffer consisting of 0.90 M NaCl, 60 mM Na_2HPO_4 and 6 mM EDTA (pH 6.8) containing 25% formamide at 34ºC. After RNA hybridization, tag-conjugating Cy3 and Cy5 dyes were circulated through the microfluidic chip for dye staining. Fluorescence images were collected using a GenePix 4000B laser scanner (Molecular Device, USA) and digitized using Array-Pro image analysis software (Media Cybernetics). Data were analyzed by first subtracting the background and then normalizing the signals using a locally weighted regression filter (LOWESS). For two-color experiments, the ratio of the two sets of detected signals (log2 transformed, balanced) and *p*-values of the *t*-test were calculated. Differentially detected signals were those whose *p*-values were less than 0.01.

Computational identification of miRNAs

miRNAs are a kind of highly conserved small RNA. Thus, potential miRNAs can be easily identified in different plant species using a comparative method. The non-redundant mature miRNAs of *A. thaliana* were used as query sequences to search the conserved homologous miRNAs in *B. campestris* against the NCBI databases (GSS, HTGS, RNA sequence, genomic sequences and EST) and Brassica Database (http://brassicadb.org/brad/) using BLASTN. Secondary structures of candidate pre-miRNAs were predicted with the web-based software MFOLD 3.1 (http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi) [29-30]. The following criteria were used in the prediction [31-32]:

1. No more than two mismatches were allowed for the candidate mature miRNA sequences compared with the corresponding known *A. thaliana* mature miRNA sequences.
2. The candidate pre-miRNA sequences had to be able to fold into stem-loop structures.
3. 22 nt mature miRNAs fell on the stem part.
4. miRNAs had to have less than six mismatches with the opposite miRNA* in the other arm of the structure.
5. Any miRNA precursor with secondary structures had to have higher negative minimal free energies and a higher minimal free energy index than other types of RNAs.
6. The content of A+U had to be between 30 and 70%.
7. No loop or break was allowed in the miRNA sequences.

Prediction of miRNA targets

The potential target genes were predicted in two ways. The first method is used on the basis of the speculation that miRNA target genes may be conserved in *A. thaliana* and *B. campestris*. The candidate target genes in *B. campestris* were obtained by searching for homologous genes of *A. thaliana* target genes in the Brassica Database. Moreover, psRNA Target (http://plantgrn.noble.org/psRNATarget/), a small RNA target analysis server for plant studies, was used

to predict the potential target genes [33]. Because the *B. rapa* de novo scaffolds assembly v1.1 (2008-08-30) cds is included in the preloaded transcript/genomic library of the psRNA Target analysis server, this transcript library was chosen for the target searching assay. The default parameters were set in all the search steps.

5' modified RACE analysis

Five tissues (root, stem, leave, inflorescence and silique) of the fertile line (*Bcajh97-01B*) were used for RNA isolation. Total RNA was extracted using the mirVana kit (Ambion, USA). Then a mixture of total RNAs was obtained by mixing an equal amount of total RNA from the five tissues. The mixture was subjected to a purification of poly(A^+) RNA using an Oligotex mRNA Kit (Qiagen, Germany). The 5' modified RACE was performed using a GeneRacer Kit (Invitrogen, USA). Poly(A^+)-enriched RNA (250 ng) was directly ligated to the GeneRacer RNA oligo without calf intestinal phosphatase and tobacco acid pyrophosphatase treatment. The first-strand cDNA was synthesized using Cloned AMV RT and the oligo dT primer. For the first round of PCR, 1 μl of cDNA, GeneRacer 5' primer and the gene-specific outer primer were used. For the second round of PCR, 1 μl of the first round PCR product as template, together with the GeneRacer 5' nested primer and the gene-specific inner primer were used. The gel-purified PCR products were then ligated into pGEM-T Easy Vector (Promega, USA) for sequencing. The primers are listed in Suppl. Table 1 in Supplementary material at http://dx.doi.org/10.2478/s11658-013-0097-9.

qRT-PCR assay

Total RNA was extracted using a mirVana Kit (Ambion, USA). According to the procedures provided by the manufacturers of the miRNA cDNA Synthesis Kit (TaKaRa, Japan), 1 μg of total RNA for each sample was polyadenylated with ATP by poly(A) polymerase and then the poly(A)-tailed total RNAs were reverse-transcribed using PrimeScript RTase using a universal adaptor primer (containing oligo-dT). qRT-PCR was performed on a Bio-Rad CFX96 machine. About 1 μl of 50 ng·μl^{-1} cDNA was mixed with 10 μl of 2× SYBR Green mix (TaKaRa, Japan) and 8 pmol of each miRNA-specific primer and universal reverse primer in a final volume of 20 μl. Reactions were performed by an initial incubation at 95ºC for 30 s, followed by 40 cycles at 95ºC for 5 s and 60ºC for 20 s. The relative expression levels of miRNAs were quantified using the $2^{-\Delta\Delta Ct}$ method [34]. The 5.8S rRNA of *B. campestris* was used as the internal control gene. Three biological repeats were conducted. Two-tailed, unpaired *t*-tests were performed comparing miRNA relative expression values in flower buds from '*Bcajh97-01A*' and '*Bcajh97-01B*'. The primers for qRT-PCR are listed in Suppl. Table 2.

RESULTS

Results of miRNA array assay

The signal intensity was analyzed to identify the miRNA expression levels. The ratios of the two sets of detected signals (log2 transformed, balanced) in the two inflorescence samples were calculated, as well as the *p*-values of the *t*-test for each probe. The miRNA array assay identified a total of 44 miRNAs that were differently expressed when '*Bcajh97-01A*' and '*Bcajh97-01B*' inflorescences were compared. Of these, 22 were upregulated in inflorescences of '*Bcajh97-01A*' sterile line plants and the other 22 were downregulated compared with the levels for inflorescences of '*Bcajh97-01B*' fertile line plants (Suppl. Fig. 1). When the fold change of the relative expression level was set as 1.5 and the p value < 0.01 was set for screening, 15 miRNAs showed significantly different expression levels. Nine of these were upregulated and the remaining 6 were downregulated in inflorescences of '*Bcajh97-01A*' sterile line plants compared with the levels in '*Bcajh97-01B*' fertile line plants (Fig. 1). The expression levels of these 15 miRNAs were statistically significantly different with a *p*-value < 0.01 (Suppl. Table 3).

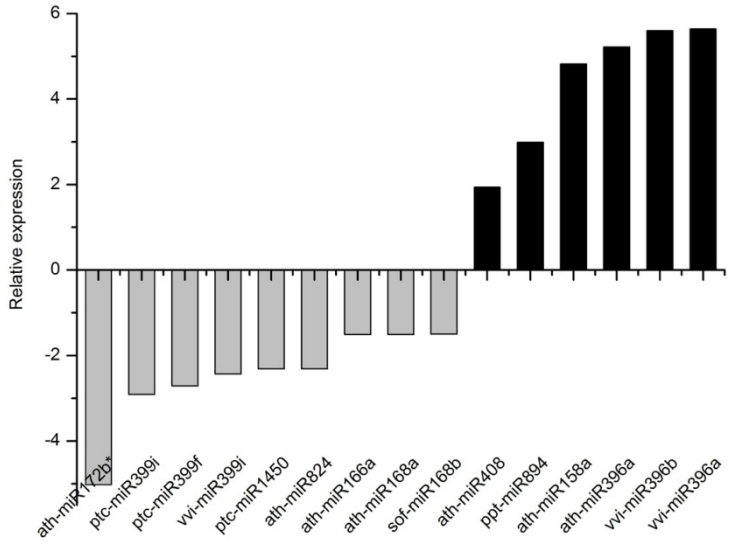

Fig. 1. Fifteen miRNAs in the array showed significant expression differences between '*Bcajh97-01A*' and '*Bcajh97-01B*' inflorescences. The Y-axis represents the fold change of relative abundance between the '*Bcajh97-01A*' and '*Bcajh97-01B*' inflorescences. The grey columns represent the miRNAs enriched in '*Bcajh97-01A*' inflorescences, while the black columns represent the miRNAs enriched in '*Bcajh97-01B*' inflorescences. ath: *Arabidopsis thaliana*; ptc: *Populus trichocarpa*; vvi: *Vitis vinifera*; sof: *Saccharum officinarum*; ppt: *Physcomitrella patens*.

Table 1. Information about miRNAs identified in *Brassica campestris*.

miRNA	GenBank Acc. No.	miRNA (5'-3')	NM (nt)	LM	LP	S	GC (%)	MFEI	miR Base
bra-miR158a	CT015463.1	TTCCAAATGTA GACAAAGCA	1(a)2(b)	20	97	3'	38.1	0.9	no
bra-miR158b	A05: 21937277-21937536	TCCCAAATGTA GACAAAGCA	0(a)1(b)	20	103	3'	41.7	1.0	no
bra-miR168a	DU984956	TCGCTTGGTGC AGGTCGGGAA	0(ab)	21	139	5'	57.6	0.9	no
bra-miR168b(3)	AC232514.1	TCGCTTGGTGC AGGTCGGGAA	0(ab)	21	133	5'	57.6	0.9	no
bra-miR172a	AC189388.2	AGAATCTTGAT GATGCTGCAT	0(ab)	21	125	3'	36.8	1.1	yes
bra-miR172b/b*	A10: 16831523 -16831640	AGAATCTTGAT GATGCTGCAT	0(ab)	21	133	3'	36.8	1.1	yes
		GCAGCACCATT AAGATTCACA	0(b*)	21	133	5'	36.8	1.1	yes
bra-miR172c	AC189648.2 (DX011558.1) (DX078594)	GGAATCTTGAT GATGCTGCAT	0(e)	21	103	3'	43.7	1.1	no
bra-miR172d	AC189648.2	TGAATCTTAAT GATGCTGCAT	2(abe)	21	103	5'	43.7	1.1	no
bra-miR172e	DU979540.1 (ED520082)	GGAATCTTGAT GATGCTGCAT	0(e)	21	86	3'	43.7	1.1	no
bra-miR172f	ED525000.1	GGAATCTTGAT GATGCTGCAT	0(e)	21	116	3'	43.7	1.1	no
bra-miR172g	ED516998.1	GGAATCTTGAT GATGCTGCAT	0(e)	21	109	3'	43.7	1.1	no
bra-miR172h	ED526567.1	GGAATCTTGAT GATGCTGCAT	0(e)	21	91	3'	43.7	1.1	no
bra-miR172i	DX034851.1	GGAATCTTGAT GATGCTGCAT	0(e)	21	99	3'	43.7	1.1	no

NM – number of mismatches compared with the corresponding miRNA family members (given in brackets) of *Arabidopsis thaliana*; LM – length of mature miRNA; LP – length of precursor miRNA; S – arm side of the hairpin secondary structure in which mature miRNA is located; MFE – minimum folding free energy; AMFE – adjusted minimum folding free energy; MFEI – minimum folding free energy index; miRBase – whether the miRNA family or member has been added to miRBase.

Identification of parts of *B. campestris* miRNA sequences using a comparative method

Non-redundant mature miRNAs of *A. thaliana* were used as query sequences to search the conserved homologous miRNA sequences with all available *B. rapa* sequences from the NCBI and Brassica databases. Three *A. thaliana* miRNAs (ath-miR158a, ath-miR168a and ath-miR172b*) were chosen because these three may also function in fertilization (unpublished data). Thirteen potential family members were found for the three miRNAs in *B. campestris*: bra-miR158a, bra-miR158b, bra-miR168a, bra-miR168b, bra-miR172a, bra-miR172b, bra-miR172c, bra-miR172d, bra-miR172e, bra-miR172f,

bra-miR172g, bra-miR172h and bra-miR172i (Table 1). Two members of the miR158 family (bra-miR158a and bra-miR158b) were found and the bra-miR158a sequence was observed to be the same as the corresponding sequences of two *A. thaliana* members (ath-miR158a and ath-miRNA158b), whereas bra-miR158b had a base difference. bra-miR168a was noted in miRBase in *B. rapa* and bra-miR168b was discovered in three pieces of continuous tandem repeats in genomic sequence (AC232514.1). Ten members of the miR172 family (including bra-miR172b*) and their located genome sequences were predicted. Stem-loop structure analysis and other parameter values were performed for all the predicted miRNA members and the results all concurred with the pre-established criteria described in the Materials and Methods section.

Target genes of conserved miRNA families may be conserved in different plants

We searched for the potential target genes using BLASTX against the NCBI and Brassica databases. A plant small RNA target analysis server, psRNA Target, was also used. The results showed 6 potential target genes for the bra-miR158 family, 5 potential target genes for the bra-miR168 family and 11 potential target genes for the bra-miR172 family (Suppl. Table 4). The potential functions of these target genes were analyzed based on Gene Ontology, InterPro domain, KEGG and Swissprot annotation. The candidate target genes for a given miRNA family tended to belong to the same gene family. For example, six candidate target genes were found for the miR158 family. Three of them (Bra027656, Bra026882 and Bra028267) are PPR repeat-containing protein family genes and two (Bra024786 and Bra024785) are transferase family genes. The candidate target gene At1g64100 of miR158 in *A. thaliana* is also a PPR repeat-containing protein family gene. For the bra-miR168 family, five candidate target genes were found in *B. campestris*. Two of them (Bra032254 and Bra014136) belong to the AGO protein family. In *A. thaliana*, the candidate target genes of miR168 were also AGO protein family genes. For the bra-miR172 family, 8 out of 11 candidate target genes are AP2 transcript factor family genes: Bra017809, Bra011741, Bra020262, Bra002510, Bra000487, Bra011939, Bra012139 and Bra007123. The corresponding *A. thaliana* homologous genes of these target genes are also listed. Based on the above analysis, we concluded that potential target genes of conserved miRNA families may be conserved in different plants.

To further demonstrate the potential target genes, 5' modified RACE was performed using mixed samples consisting of equal amounts of RNA isolated from root, stem, leaf, inflorescence and silique tissue of the fertile line '*Bcajh97-01B*'. A total of 7 potential target genes were validated using 5' modified RACE (Fig. 2). The candidate target genes Bra032254 (homologous to AT1G48410, AGO1) and Bra014136 (homologous to AT5G43810, AGO10) were identified as cleaved by miR168. Five target genes of miR172 were also identified, including Bra017809 (homologous to AT4G36920, AP2), Bra011741 (homologous to AT4G36920, AP2), Bra020262 (homologous to AT5G60120, TOE2),

Bra002510 (homologous to AT5G60120, TOE2) and Bra011939 (homologous to AT2G28550, TOE1). There were two cleavage sites for Bra011741 and three for Bra002510. For the other five target genes, only one cleavage site was found, between the tenth and eleventh nucleotides of the miRNA/target complementary regions (Fig. 2).

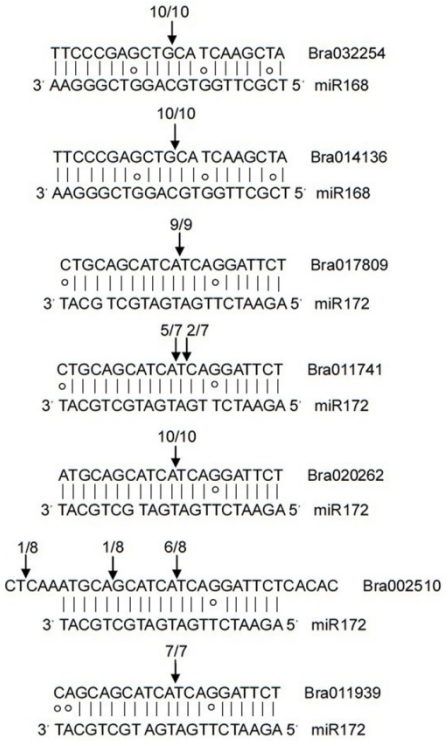

Fig. 2. 5' modified RACE detection of predicted miRNA target genes in *B. campestris*. The cleavage sites of 7 target mRNAs are shown. The arrows indicate the cleavage sites of the target mRNA. Watson-Crick pairing (vertical dashes) and G:U wobble pairing (circles) are indicated. The cloned sequences that were found to match the correct gene sequences and have 5' ends centered on the miRNA complementary site and nearby 10 nucleotides were counted.

The expression level of mature miRNAs during the five pollen development stages

To further confirm the miRNA array results and analyze the miRNA expression profiles, poly(A)-tailed qRT-PCR was performed on four selected miRNAs (bra-miR158a, bra-miR168a, bra-miR172b* and bra-miR172b) in flower buds at five stages of development. Three biological repeats were conducted. Two-tailed, unpaired *t*-tests were performed analyzing the relative expression levels of the four miRNAs in flower buds from '*Bcajh97-01A*' and '*Bcajh97-01B*'.

In the miRNA array assay, ath-miR158a, which has the same nucleotide sequences as bra-miR158a, was shown to be downregulated in inflorescences of

'*Bcajh97-01A*' compared with the expression levels in '*Bcajh97-01B*'. In qRT-PCR assay, as shown in Fig. 3, the expression level of bra-miR158a was very significantly different between the flower buds of '*Bcajh97-01A*' and '*Bcajh97-01B*': $p = 0.0083$ at stage II, $p = 0.0068$ at stage III, and $p < 0.0001$ at stage IV. Also, a significant difference was found at stage V ($p = 0.0126$). Although the fold-changes in the relative expression of bra-miR158a were not high, the expression differences were statistically significant, except at stage I. The expression levels mainly increased with flower bud growth. In general, the expression trend of bra-miR158a was consistent with that of ath-miR158a in the miRNA array.

ath-miR168a was upregulated in the '*Bcajh97-01A*' inflorescences in the miRNA assay. bra-miR168a, which had the same nucleotide sequences with ath-miR168a, was predicted in *B. campestris*. In the qRT-PCR assay, the expression differences of bra-miR168a mainly appeared at stage V, when the flower buds were about to open with mature anthers containing fully mature pollen. A highly significant difference was found at stage V ($p = 0.0006$). However, there were no significant differences from stage I to stage IV.

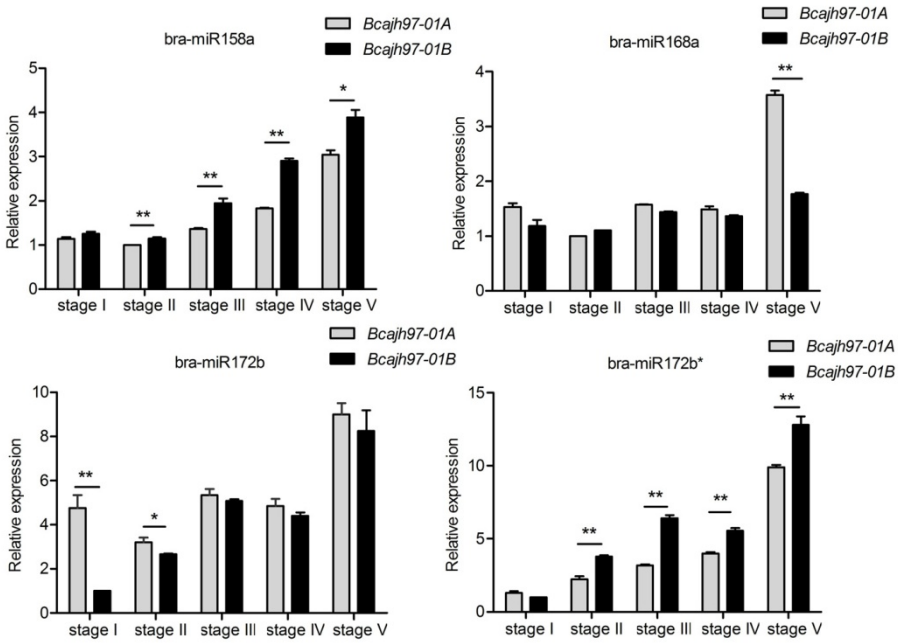

Fig. 3. Relative qRT-PCR expression analysis of four miRNAs in flower buds of '*Bcajh97-01A*' and '*Bcajh97-01B*' plants. The Y-axis shows the fold change of expression level. The X-axis indicates developmental stage of the flower buds of the sterile line (grey bars, *Bcajh97-01A*) and fertile line (black bars, *Bcajh97-01B*) plants. The relative expression abundance of each miRNA was measured in three biological replicates and is represented as the means ± SEM. Error bars are shown. The *p*-value was obtained from the unpaired *t*-test with a two-tailed test. $p < 0.05$ is considered statistically significantly different (asterisk, *) and $p < 0.01$ is considered highly significantly different (double asterisk, **).

ath-miR172b* was found to be upregulated in the '*Bcajh97-01A*' inflorescences in the miRNA array assay. However, in the qRT-PCR assay, its corresponding miRNA in *B. campestris*, bra-miR172b* was found to be downregulated in '*Bcajh97-01A*' flower buds except in stage I, when microspore mother cells were starting to form. The expression levels were highly significantly different from stage II to stage V. As with the expression pattern of bra-miR158a, the expression levels of bra-miR172b* increased through the five developmental stages of flower buds.

In the qRT-PCR analysis, it was obvious that bra-miR172b was upregulated in '*Bcajh97-01A*' flower buds at stage I compared with the levels in '*Bcajh97-01B*'. This might be attributed to the formation of microspore mother cells. Its relative expression abundance was highly significantly different at stage I ($p = 0.0004$) and significantly different at stage II ($p = 0.0255$). Although bra-miR172b and bra-miR172b* belong to the miR172 family, they showed opposite trends of expression pattern (Fig. 3).

DISCUSSION

Diverse miRNAs exist in pollen

This study aimed to identify potential miRNAs involved in pollen development in *Brassica campestris* ssp. *chinensi*s. Interestingly, numerous earlier studies have demonstrated that many miRNAs exist in *Arabidopsis thaliana* pollen. In our miRNA array assay, of the 15 screened miRNAs that we speculated to be involved in pollen development, 7 miRNA families (miR158, miR166, miR168, miR172, miR396, miR399 and miR824) also reportedly exist in *A. thaliana* mature pollen [16]. Four out of the 8 miRNA families (miR158, miR172, miR396 and miR166) were again detected in *A. thaliana* pollen and inflorescences using microarray assays [17]. Our qRT-PCR results further verified the existence and expression of the four miRNAs (bra-miR158a, bra-miR168a, bra-miR172b* and bra-miR172b) in the inflorescences and flower buds at the five developmental stages of *B. campestris* ssp. *chinensi*s. In summary, all previous works and our results suggested that an abundance of miRNAs exist in pollen.

miRNAs might play certain roles in the process of plant reproductive development

Previous studies demonstrated that many miRNAs exist in pollen, but they did not confirm the importance of miRNAs in pollen development. The question remained whether miRNAs play any role in the process of pollen development. In 2006, Wu *et al.* found that male sterility occurred in *Arabidopsis P35s::MIR167* plants or in miR167-immune *mARF6* plants [35]. These results indicated that overexpression of *MIR167* or loss of *MIR167* regulation both lead to male sterility. Although their studies could not directly prove the participation of *MIR167* in pollen development, the expression pattern of *MIR167* did indeed

influence the reproductive development of *Arabidopsis*. In 2011, Wei *et al.* conducted deep sequencing of rice on a genome-wide scale to explore the composition and expression patterns of miRNAs in developing pollen, including uninucleate microspores, bicellular and tricellular pollen, and sporophytic tissues, and 292 known miRNAs and 75 novel miRNAs were identified. Of the 292 known miRNAs, 103 were found in abundance in developing pollen, and more than half of the novel miRNAs displayed pollen- or stage-specific expression [36]. Their research revealed differences in the composition and expression profiles of miRNAs for developing pollen and sporophytes, which suggest the important roles of the miRNA pathway in pollen development.

In our study, the plant miRNA array screened 15 significant differently expressed miRNAs between the male sterile (*Bcajh97-01A*) and fertile (*Bcajh97-01B*) lines. bra-miR158a, bra-miR168a, bra-miR172b* and bra-miR172b were chosen for further qRT-PCR expression analysis in flower buds at five developmental stages of '*Bcajh97-01A/B*'. The qRT-PCR results concurred exactly with the array results for bra-miR158a and bra-miR168a. bra-miR172b did not appear in the array. The expression pattern for bra-miR172b* was not consistent with the array results. Therefore, we focused on bra-miR158 and bra-miR168 for further research.

In the qRT-PCR assay, the difference in the expression level of bra-miR158a was statistically significant from stage II to stage V. The fold change in relative expression was more obvious at stage IV and stage V, i.e. in the uninucleate and mature pollen stages. For bra-miR168a, the expression level was highly significantly different in flower buds at stage V (mature pollen grain stage) of '*Bcajh97-01A*' and '*Bcajh97-01B*', which suggested a potential role of bra-miR168 in the pollen development process. According to the results of unpaired *t*-test for bra-miR172b, significant differences in the relative expression abundances were also found at stages I and II, when microspore mother cells begin to form and enter meiosis. These results suggested that there might be a tissue- and developmental stage-specific manner for miRNAs to perform their functions. To verify this speculation, more direct evidence will be needed.

miRNA target genes may be conserved among various plant species

To determine the functions of miRNAs in plants, information about their target genes must first be obtained. In plants, miRNAs perfectly or almost perfectly bind to target mRNAs, so potential target genes can be easily predicted using computational methods. Many software packages are written for predicting target genes, such as Helper tool [37], Target-align [38] and TAPIR [39]. In recent years, high-throughput experimental approaches, such as the global sequencing of cleaved mRNAs, have become new methods of massively identifying target genes. In the current paper, the potential target genes were predicted in two ways. First, we speculated that miRNA target genes may be conserved in *A. thaliana* and *B. campestris*. The candidate target genes were obtained by searching homologous genes of *A. thaliana* target genes in the NCBI

and Brassica databases. Second, candidate target genes were predicted using the psRNA Target (http://plantgrn.noble.org/psRNATarget/).

Our speculation about the conservation of miRNA target genes in different species is reasonable on the basis of molecular evolution theory [40-41]. miRNAs are conserved in plant species. Furthermore, miRNA sequences are reverse complementary with their target genes sequences. Therefore, target genes in different plants may be homologous to some extent.

At present, the phenomenon of conservation of miRNA complementary sites among flowering plants has been observed by Rhoades *et al*., who found that many complementary sites in *A. thaliana* that are conserved in rice (*Oryza stativa*) [11]. For example, in *A. thaliana*, miR172 regulates flowering time and floral organ identity by downregulating *AP2*-like target genes [6, 42]. This regulatory relationship also exists in rice [43] and citrus (*Citrus sinensis*) [44]. These previous studies provide indirect evidence for our speculation that miRNA target genes may be conserved in various plant species.

In our research, by using 5' modified RACE, 2 target genes of miR168 and 5 target genes of miR172 were identified. All of them were respectively homologous to corresponding *A. thaliana* target genes. These results suggested that miRNA target genes in *B. campestris* might be homologous to *A. thaliana* target genes. Therefore, a homologous search for target genes can be another approach to target identification.

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