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Molecules and Cells

Identification of Potential microRNAs and Their Targets in *Brassica rapa* L.

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MicroRNAs (miRNAs) are recently discovered, noncoding, small regulatory RNA molecules that negatively regulate gene expression. Although many miRNAs are identified and validated in many plant species, they remain largely unknown in *Brassica rapa* (AA 2n =, 20). *B. rapa* is an important *Brassica* crop with wide genetic and morphological diversity resulting in several subspecies that are largely grown for vegetables, oilseeds, and fodder crop production. In this study, we identified 186 miRNAs belonging to 55 families in *B. rapa* by using comparative genomics. The lengths of identified mature and pre-miRNAs ranged from 18 to 22 and 66 to 305 nucleotides, respectively. Comparison of 4 nucleotides revealed that uracil is the predominant base in the first position of *B. rapa* miRNA, suggesting that it plays an important role in miRNA- mediated gene regulation. Overall, adenine and guanine were predominant in mature miRNAs, while adenine and uracil were predominant in pre-miRNA sequences. One DNA sequence producing both sense and antisense mature miRNAs belonging to the BrMiR 399 family, which differs by 1 nucleotide at the, 20th position, was identified. *In silico* analyses, using previously established methods, predicted 66 miRNA target mRNAs for 33 miRNA families. The majority of the target genes were transcription factors that regulate plant growth and development, followed by a few target genes that are involved in fatty acid metabolism, glycolysis, biotic and abiotic stresses, and other cellular processes. Northern blot and qRT-PCR analyses of RNA samples prepared from different *B. rapa* tissues for 17 miRNA families revealed that miRNAs are differentially expressed both quantitatively and qualitatively in different tissues of *B. rapa*.

INTRODUCTION

Gene expression of higher eukaryotes is regulated either at a transcriptional or posttranscriptional level. Various factors regulating gene expression at both the stages have been identified and demonstrated with experimental results (Depicker and Montagu, 1997; Vaucheret and Fagard, 2001). One class of such regulators at the posttranscriptional level is small, endogenous, noncoding RNA molecules known as microRNAs (miRNAs). miRNAs are generally 18-24 nucleotides (nt) long, and are abundant in plants and animals. miRNAs regulate target gene expression by degrading complementary mRNA sequences or by reducing the translation of the target gene (Bartel, 2004; Voinnet, 2009). miRNAs are transcribed by RNA polymerase II in plants, and the primary transcripts of mature miRNAs fold back into stable hairpin loop structures that form precursor miRNA (pre-miRNA). The processing of primary miRNA sequences, including capping, splicing, and polyadenylation, is completed in the nucleus by RNase III-like endonuclease and Dicer-like-1 enzyme (DCL1) in plants (Bartel, 2004; Dugas and Bartel, 2004). The *HASTY5* gene exports the processed methylated miRNA/miRNA* to the cytoplasm (Bollman et al., 2003; Park et al., 2005; Voinnet, 2009). The matured miRNAs are then incorporated into argonauts containing RNA-induced silencing complex; they interact with the complementary sites of the target gene transcripts, which then negatively regulate the target gene expression by degrading or repressing the mRNA transcripts (Carthew and Sontheimer, 2009; Voinnet, 2009).

At present, miRNAs are mainly identified using 2 methods: (a) computational identification and structure prediction because a majority of known mature miRNAs are conserved within and between different plant species, allowing for comparative analysis using presently available bioinformatics tools to search for putative miRNAs (Wang et al., 2004a; 2004b); (b) the construction and sequencing of a small RNA library (Griffiths-Jones, 2006; Zhang et al., 2006a). These 2 methods have been used to identify hundreds of miRNAs in several plant species, including the model plant *Arabidopsis thaliana* (Alves-Junior et al., 2009; Buhtz et al., 2008; Jagadeeswaran et al., 2009; Jin et al., 2008; Klevebring et al., 2009; Song et al., 2009; Sunkar et al., 2005; 2008; Szittya et al., 2008; Xie et al., 2007; Zhang et al., 2006b; 2007a; 2008). Recently, many miRNAs have been shown to regulate a wide range of biological functions of plants. A growing amount of experimental evidence shows that miRNAs are involved in the regulation of transcrip-

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tion factors and other genes that play important roles in developmental timing, including the transitions from juvenile to adult and from the vegetative to reproductive phase (Lauter et al., 2005; Schwab et al., 2005; Wu and Poethig, 2006), floral development (Chen, 2004; Rhoades et al., 2002), leaf formation (Palatnik et al., 2003), stem development (Mallory et al., 2004), root development (Guo et al., 2005), signal transduction (Rhoades et al., 2002), male and female reproductive development (Wu and Poethig, 2006), and many other cellular processes (Ambros and Chen, 2007; Carrington and Ambros, 2003; Zhang et al., 2007b). Changes in the expression levels of miRNAs in plant tissues subjected to biotic stresses such as viral, fungal, and bacterial infection are reported (Bazzini et al., 2007; Lu et al., 2007; Navarro et al., 2006). Recently, changes in the expression of miRNAs due to abiotic stresses such as cold, drought, salinity, oxidative stress, mechanical strain, and UV-B radiation have also been identified in plants (Lu et al., 2005; Sunkar and Zhu, 2004; Sunkar et al., 2006; Zhao et al., 2007; Zhou et al., 2007) apart from nutrient stresses, such as phosphate or sulfate starvation (Chiou et al., 2006; Fujii et al., 2005; Jones-Rhoades and Bartel, 2004), and other nutrient responses (Sunkar et al., 2007).

However, despite a growing number of informations regarding miRNAs for specific gene expression regulation, miRNAs in *Brassica* species - especially *B. rapa* - are largely unknown despite a previous study reporting few miRNAs in this species (He et al., 2008). The systematic identification and characterization of miRNAs in *B. rapa* would help to elucidate the background of a large amount of genetic and morphological diversity that might have resulted in part to the differential gene expression regulated by miRNAs within and between *B. rapa* subspecies. *B. rapa* is one of the economically important crop species and exhibits a wide range of morphological diversity, forming different subspecies grown mainly for their leafy vegetables (Chinese cabbage, Pakchoi), oilseeds (Yellow sarson, Brown sarson, and Toria) and fodder (Turnip). Although these subspecies have the same genome complement, they exhibit variations in leaf morphology, head formation, plant type, and other morphological traits. Due to the global economic importance of *B. rapa* and the fact that it is a diploid progenitor parent that contributes its genome to widely cultivated amphidiploid *Brassica* oilseed crops, such as *B. napus* (AACC) and *B. juncea* (AABB), the Multinational *B. rapa* Genome Sequencing Project was started in, 2003 (Yang et al., 2005). This project, which is near completion, has generated a large amount of genome survey sequences (GSS, including BAC sequences) in addition to a large number of expressed sequence tags (ESTs) and messenger mRNAs (mRNAs) taken from different tissues and developmental stages of *B. rapa*; these were deposited in the National Center for Biotechnology Information (NCBI) database. In this study, we comprehensively investigated the presence of miRNAs in *B. rapa* that could be involved in a variety of functions, using genome survey sequences (GSS), ESTs, mRNAs, and complementary DNA (cDNA) presently available in a public database (NCBI).

MATERIALS AND METHODS

Reference set of miRNAs and *B. rapa* sequences

Two thousand eighty-five previously known mature plant miRNA sequences from *A. thaliana*, *Brassica napus*, *Glycine max*, *Oryza sativa*, *Populus trichocarpa*, *Triticum aestivum*, *Vitis vinifera*, and *Zea mays* were downloaded from the miRBase (Griffiths-Jones, 2006; Griffiths-Jones et al., 2008). From these 1408 repeated miRNA sequences were removed using Perl script (http://www.perl.org/), and the remaining 977 miRNAs were used as final reference set. A total of 397,600 GSSs, ESTs, and mRNAs of *B. rapa* sequences were downloaded from the NCBI GenBank database (ftp://ftp.ncbi.nih.gov/genbank/, http://www.brassica-rapa.org, and http://www.brassica.info). In addition, we used 12,098 full-length cDNAs and 5× NGS whole-genome sequence data of the *B. rapa* cultivar "Chiifu," which is available in the laboratory. The 5× NGS whole-genome sequence data of the Chiifu cultivar were developed in our laboratory in collaboration with the Macrogen Company, Seoul, Korea (http://www.macrogen.com).

Software used to identify *B. rapa* miRNAs and their targets

We used BLAST-2.2.20 (Camacho et al., 2009) to identify conserved miRNAs from *B. rapa* sequences (GSSs, ESTs, mRNAs, and cDNAs), Mfold-3.1.2 (Zuker, 2003; Zuker et al., 1998) for predicting the secondary structure and stability of RNA, miRU (Zhang, 2005) to identify and confirm the targets genes of identified miRNA, and Perl scripts to remove overlapping and repeated mature miRNA sequences.

Computational identification of miRNAs

The computational method developed by Meyers et al. (2008) was used to search the conserved miRNAs in the *B. rapa* genome sequence. The nonredundant mature miRNAs were implemented as query sequence, using BLASTN to search for homologues from the downloaded *B. rapa* genome sequences, because they are highly conserved among plant species compared to precursor miRNA sequences. BLASTX was performed using the UniProt protein database (http://www.uniprot.org); putative *B. rapa* candidate miRNA nucleotide sequences with < 4 mismatches were compared to previously identified plant miRNAs; and all protein-coding sequences were removed. The hairpin structure for the remaining non-protein-coding putative *B. rapa* miRNA candidate sequences was predicted by the Mfold program (Zuker et al., 1998). The following criteria reported by Meyers et al. (2008) were used: (a) < 4 mismatches allowed for the identification of *B. rapa* mature miRNAs compared to previously known plant miRNAs; (b) precursor miRNAs folded into stem-loop hairpin secondary structures; (c) mature miRNAs fall on the stem part of the hairpin secondary structure; (d) mature miRNAs allowed < 6 mismatches with the other arm of the structure; and (e) predicted precursor miRNAs secondary structures have high negative minimal folding free energy (MFE), adjusted minimum folding free energy index (AMFE), and higher minimal folding free energy indexes (MFEIs). MFE, MFEI, and AFME values were determined using the methods outline by Zuker (2003) and Zhang et al. (2006c; 2008), respectively. To avoid redundancy and to confirm different members of the gene, we compared the predicted whole and precursor miRNA sequence-containing gene by using *B. rapa* unigenes and *A. thaliana* genes (http://www.arabidopsis. org).

Expression analyses of putative *B. rapa* miRNAs

Northern blot analyses

Total RNA was isolated from, 20-day-old *B. rapa* cultivar Kenshin seedling plants by using Trizol reagent (Invitrogen, USA) according to the procedure previously described by Kim et al. (2005). Total RNA was separated in 15% urea-polyacrylamide gel and electrically transferred to Hybond-N+ membranes (Amersham Bioscience, UK). Membranes were UV cross-linked and baked for 30 min at 80°C. Blots were hybridized with the ULTRA-Hyb Oligo solution (Ambion, USA) and oligonucleotide probes that were end-labeled using ^{32}P-γATP

Table 1. Primer sequences used for qRT-PCR analyses

Name	Sequence (5′→ 3′)
BrMiR 158b	TCCCAAATGTAGACAAAGCA
BrMiR 162b	TCGATAAACCTGTGCATCCAG
BrMiR 164c	TGGAGAAGCAGGGCACGTGCA
BrMiR 170b	TGATTGAGCCGTGCCAATATC
BrMiR 171f	TGATTGAGCCGCGCCAATATC
BrMiR 172g	GAATCTTGATGATGCTGCAT
BrMiR 396b	TTCCACAGCTTTCTTGAACTG
BrMiR 400b	TATGAGAGTATTATACGTCAC
BrMiR 837	ATCAGTTTCTTGTTCTTTTC
BrMiR 842	ATGGTCAGATCGGTCATC
BrMiR 1132b	ATTATGAAACGGAAGGAG
BrMiR 1521b	CTGTTGATGGAAAATGTT
Poly(T) adaptor	GCGAGCACAGAATTAATACGACTCACT ATAGG$(T)_{12}V^aN^b$
Reverse primer	GCGAGCACAGAATTAATACGAC
BrACT Forward primer	GAACCGGGTGCTCCTCAGGA
BrACT Reverse primer	ATGGTACCGGAATGGTCAAGGC

[a]V = A,G,C
[b]N = A,T,G,C

and T4 polynucleotide kinase (New England Biolabs). After hybridization, the blots were washed with washing solution (2× SSC + 0.5% SDS) and briefly air dried. Images were obtained by exposing the blots to X-ray film. EtBr-stained 5S rRNA was used as a loading control. The 5 miRNA probes used to test for their expression analysis in *B. rapa* were as follows: (5′-3′, antisense) BrMiR 159 TAGAGCTCCCTTCAATCCAAA; BrMiR 160, TGGCATACAGGGAGCCAGGCA; BrMiR 167, TAGAT CATGCTGGCAGCTTCA; BrMiR 398, AGGGGTGACCTGAG AACACA; and BrMiR 408, GCCAGGGAAGAGGCAGTGCAT.

Quantitative real-time polymerase chain reaction

Total RNA was isolated from *B. rapa* cultivar Chiifu plants as described in previous paragraph. Shoot apex (unopened floral buds and primary shoot apex), stems, old leaves (source, green tissues of old leaves larger than 13 cm length and 8 cm width), young leaves (smaller than 3 cm length and 2.5 cm Width), midribs (sink, white tissue of old leaves larger than 13 cm length and 8 cm width) and roots were collected for RNA extraction. Real time PCR was performed six times for each miRNAs following Shi and Chiang (2005). One microgram of total RNA polyadenylated with ATP by poly(A) polymerase according to the manufacturer's protocol. After phenol-chloroform extraction, the RNAs were reverse-transcribed using Reverse Transcription Kit (Promega, USA) with poly(T) adaptor primer (Table 1). For real-time PCR, 1 ul cDNA was mixed with 12.5 ul of 2× SYBR Green Mix (Qiagen, USA) and 5 pmol each of the miRNA specific primer and reverse primers in a final volume of 25 ul. PCR runs were 45 cycles each cycles at 95°C for 10 s, 65°C for 15 s and 72°C for 20 s. The relative microRNA expression was quantified using comparative ΔΔCT method (Livak and Schmittgen, 2001). All expression profiles are normalized with expression levels at shoot apex. *B. rapa* Actin gene (BrACT) was used as an internal control and each primer sequences were described in Table 1.

Prediction of identified miRNA targets

miRNAs bind to targets with perfect or near-perfect complementary; this nature motivates the prediction of potential targets through a computational comparative approach. To find the target genes, we compared the complementary sequences of the predicted miRNAs with all the available *B. rapa* sequences downloaded from the NCBI database. Less than 4 mismatches without gaps were allowed between predicted complementary miRNAs and their target sequences. We used miRU (http://bioinfo3.noble.org/psRNATarget; Zhang, 2005) and TAIR BLAST (http://www.arabidopsis.org/Blast/index.jsp) software to identify the targets in *A. thaliana.* To validate the identified targets, we compared miRNA as well as whole-target gene sequences with those of *A. thaliana* genes and identified the corresponding target genes in *A. thaliana.*

RESULTS

Identification of *B. rapa* miRNAs

miRNAs are conserved between plant species due to their important regulatory mechanisms. Their highly conserved nature allows the identification of miRNAs in different plant species for which genome sequence information is partially or fully available, or previously unidentified. The summary of steps

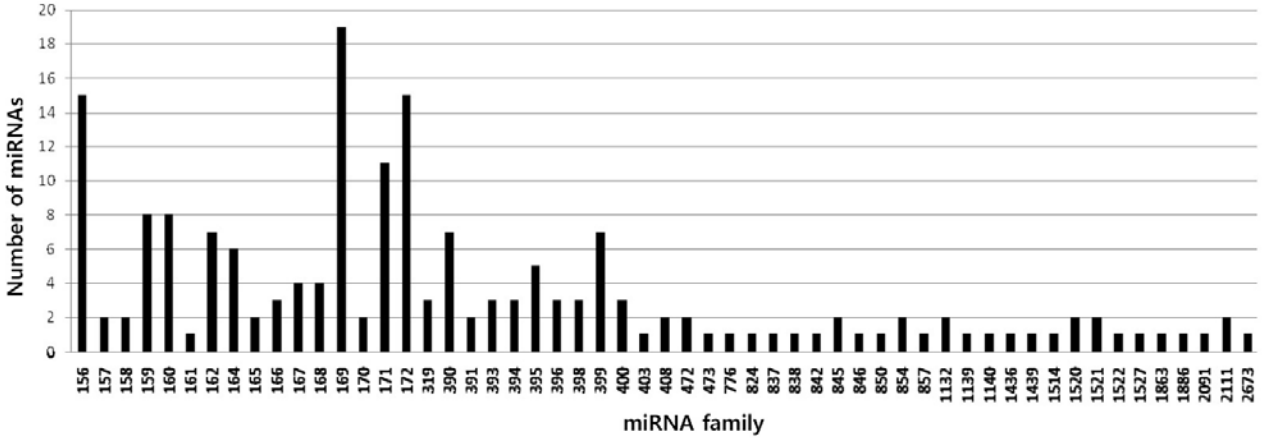

Fig. 1. Number of miRNAs identified in 55 miRNA families in *Brassica rapa*

Table 2. Details of identified *Brassica rapa* (Br) miRNAs, their structure information, and corresponding *Arabidopsis thaliana* (At) genes

miRNA family	Br-GSS/EST	At-gene	Mature miRNA	ML	MAS	Len(Pre)	GC%	MFE	AMFE	MFEI
BrMiR 156a	ED524353	AT5G55835.1	UUGACAGAAGAAAGAGAGCAC	21	5′	109	48.62	42.9	39.36	0.809
BrMiR 156b	AJ858639	AT4G30972.1	UGACAGAAGAGAGUGAGCAC	20	5′	151	46.36	64.8	42.91	0.925
BrMiR 156c	DU829396	AT4G30972.1	UGACAGAAGAGAGUGAGCAC	20	5′	92	47.83	30.9	33.59	0.702
BrMiR 156d	DU831758	AT4G30972.1	UGACAGAAGAGAGUGAGCAC	20	5′	98	45.92	39	39.8	0.866
BrMiR 156e	DU121870	AT5G10945.1	UGACAGAAGAGAGUGAGCAC	20	5′	111	36.94	39.5	35.59	0.963
BrMiR 156f	AJ859176		GACAGAACAGAGUGAGCAC	19	3′	211	33.65	50.8	24.08	0.715
BrMiR 156g	ED529145		GACAGAAUAGAGUGAGCAC	19	3′	209	32.5	47.7	22.82	0.701
BrMiR 156h	DX011343		UGACAGAAGAGAGAGAGC	18	5′	241	47.72	75.4	31.29	0.655
BrMiR 156i	contig182998	AT4G31877.1	UGACAGAAGAGAGUGAGCAC	20	3′	118	40.68	48.1	40.76	1.002
BrMiR 156j	CV432746.1	AT4G31877.1	UGACAGAAGAGAGUGAGCAC	20	5′	151	45.03	53.2	35.23	0.782
BrMiR 156k	contig156839	AT5G26147.1	UGACAGAAGAGAGUGAGCAC	20	3′	158	41.77	54.5	34.49	0.825
BrMiR 156l	contig067399	AT5G10945.1	UGACAGAAGAGAGUGAGCAC	20	5′	144	38.89	51.4	35.69	0.917
BrMiR 156m	contig024357	AT4G31877.1	UGACAGAAGAGAGUGAGCAC	20	3′	95	40	15.9	16.74	0.418
BrMiR 156n	contig162829	AT2G19425.1	CGACAGAAGAGAGUGAGCAC	20	5′	104	49.04	45.1	43.37	0.884
BrMiR 156o	contig010293	AT5G10945.1	UGACAGAAGAGAGCGAGCACA	21	5′	114	38.6	43.8	38.42	0.995
BrMiR 157a	contig117388	AT1G66783.1	UUGACAGAAGAUAGAGAGCAC	21	3′	136	38.97	45.5	33.46	0.858
BrMiR 157b	contig126773		UUGACAGAAGAAAGAGAGCAC	21	5′	126	46.83	44.4	35.24	0.752
BrMiR 158a	CT015463	AT3G10745.1	CCAAAUGUAGACAAAGCA	18	3′	88	37.5	25.8	29.32	0.781
BrMiR 158b	contig145627	AT3G10745.1	UCCCAAAUGUAGACAAAGCA	20	5′	120	41.67	37.1	30.92	0.741
BrMiR 159a	ED518706	AT1G73687.1	UUUGGAUUGAAGGGAGCUCUA	21	3′	71	36.62	15.1	21.27	0.58
BrMiR 159b	EX050542.1	AT1G73687.1	UUUGGAUUGAAGGGAGCUCUA	21	3′	71	36.62	15.1	21.27	0.58
BrMiR 159c	EX048967.1	AT1G73687.1	UUUGGAUUGAAGGGAGCUCUA	21	3′	71	36.62	15.1	21.27	0.58
BrMiR 159d	EX047628.1	AT1G73687.1	UUUGGAUUGAAGGGAGCUCUA	21	3′	71	36.62	15.1	21.27	0.58
BrMiR 159e	ED525625	AT1G18075.1	UUGGAUUGAAGGGAGCUC	18	3′	73	35.62	15.6	21.37	0.6
BrMiR 159f	CT016164	AT5G61730.1	UUGCAUGCCCCAGGAGCU	18	3′	70	48.57	16.6	23.71	0.488
BrMiR 159g	EX039355.1	AT1G73687.1	UUUGGAUUGAAGGGAGCUCUA	21	3′	194	36.08	58.9	30.36	0.841
BrMiR 159h	contig176646	AT1G73687.1	UUUGGAUUGAAGGGAGCUCUA	21	3′	206	38.35	65.8	31.94	0.832
BrMiR 160a	DX045892	AT2G39175.1	UGCCUGGCUCCCUGUAUGCCA	21	5′	94	51.06	39.9	42.45	0.831
BrMiR 160b	EX044968.1	AT2G39175.1	UGCCUGGCUCCCUGUAUGCCA	21	5′	94	51.06	39.9	42.45	0.831
BrMiR 160c	DX016569	AT1G77850.1	UGCCUGGCUCCCUGCAUGCCA	21	3′	186	51.61	54	29.03	0.562
BrMiR 160d	contig053638	AT4G17788.1	UGCCUGGCUCCCUGUAUGCCA	21	5′	126	46.03	48.7	38.65	0.839
BrMiR 160e	contig136837	AT5G46845.1	UGCCUGGCUCCCUGUAUGCCA	21	5′	134	41.79	49.6	37.01	0.885
BrMiR 160f	contig152673	AT4G17788.1	UGCCUGGCUCCCUGUAUGCCA	21	3′	121	41.32	48.2	39.83	0.964
BrMiR 160g	contig133622	AT5G46845.1	UGCCUGGCUCCCUGUAUGCCA	21	5′	105	43.81	36	34.29	0.782
BrMiR 160h	EX025484.1	AT2G39175.1	UGCCUGGCUCCCUGUAUGCCA	21	5′	126	43.65	44.7	35.48	0.812
BrMiR 161	contig045367		UCAACGCAUUGAAAGUGACUA	21	5′	128	35.94	56	43.75	1.21
BrMiR 162a	ED527680	AT5G08185.3	UCGAUAAACCUGUGCAUCCAG	21	3′	108	42.59	31.1	28.8	0.676
BrMiR 162b	EX133401.1	AT5G08185.3	UCGAUAAACCUGUGCAUCCAG	21	3′	108	42.59	31.1	28.8	0.676
BrMiR 162c	EX071919.1	AT5G08185.3	UCGAUAAACCUGUGCAUCCAG	21	3′	107	42.99	33.3	31.12	0.723
BrMiR 162d	EX071254.1	AT5G08185.3	UCGAUAAACCUGUGCAUCCAG	21	3′	108	42.59	31.1	28.8	0.676
BrMiR 162e	EX069816.1	AT5G08185.3	UCGAUAAACCUGUGCAUCCAG	21	3′	108	42.59	31.1	28.8	0.676
BrMiR 162f	AC189332.2	AT5G08185.3	UCGAUAAACCUGUGCAUCCAG	21	3′	99	43.43	30.4	30.71	0.707
BrMiR 162g	contig177394	AT5G23065.1	UCGAUAAACCUGUGCAUCCAG	21	3′	118	44.92	43.5	36.86	0.82
BrMiR 164a	CW984396	AT2G47585.1	UGGAGAAGCAGGGCACGUGCA	21	5′	108	50	51.1	47.31	0.944
BrMiR 164b	DX051151	AT2G47585.1	UGGAGAAGCAGGGCACGUGCA	21	5′	81	48.15	30.8	38.02	0.789
BrMiR 164c	contig142689	AT5G01747.1	UGGAGAAGCAGGGCACGUGCA	21	3′	151	44.37	52.6	34.83	0.785
BrMiR 164d	contig167289	AT2G47585.1	UGGAGAAGCAGGGCACGUGCA	21	5′	109	49.54	39.1	35.87	0.724
BrMiR 164e	contig016289	AT2G47585.1	UGGAGAAGCAGGGCACGUGCA	21	3′	110	51.82	57.5	52.27	1.008
BrMiR 164f	contig181636	AT5G27807.1	UGGAGAAGCAGGGCACGUGC	20	3′	112	42.86	25.9	23.13	0.539

(continued)

miRNA family	Br-GSS/EST	At-gene	Mature miRNA	ML	MAS	Len(Pre)	GC%	MFE	AMFE	MFEI
BrMiR 165	contig026374		UCGGACCAGGCUUCAUCCCCC	21	3′	122	41.8	38.5	31.56	0.754
BrMiR 166a	DX911364	AT3G61897.1	UCGGACCAGGCUUCAUUCCCC	21	3′	140	41.43	33.9	24.21	0.584
BrMiR 166b	EX063968.1	AT2G46685.1	UCGGACCAGGCUUCAUUCCCC	21	3′	140	41.43	33.9	24.21	0.584
BrMiR 166c	EX063242.1	AT2G46685.1	UCGGACCAGGCUUCAUUCCCC	21	3′	140	41.43	33.9	24.21	0.584
BrMiR 166d	contig073929		UCGGACCAGGCUUCAUUCCCC	21	3′	136	42.65	43.7	32.13	0.753
BrMiR 167a	CT022223	AT3G22886.1	UGAAGCUGCCAGCAUGAUCUA	21	5′	93	41.94	23.1	24.84	0.592
BrMiR 167b	DX028242	AT3G22886.1	UGAAGCUGCCAGCAUGAUCUA	21	5′	124	39.52	30.8	24.84	0.628
BrMiR 167c	DX025819	AT1G31173.1	UGAAGCUGCCAGCAUGAUCU	20	5′	268	32.46	56.8	21.19	0.652
	EX041799.1									
BrMiR 167d	contig181922	AT3G22886.1	UGAAGCUGCCAGCAUGAUCU	20	5′	116	37.93	43.1	37.16	0.979
BrMiR 168a	DU984956	AT5G45307.1	UCGCUUGGUGCAGGUCGGGAA	21	5′	111	57.66	31	27.93	0.484
BrMiR 168b	contig164758	AT4G19395.1	UCGCUUGGUGCAGGUCGGGAA	21	5′	136	50.74	53.6	39.41	0.776
BrMiR 168c	contig127394	AT5G45307.1	UCGCUUGGUGCAGGUCGGGAA	21	5′	167	52.12	58.3	34.91	0.677
BrMiR 168d	contig043838	AT4G19395.1	UCGCUUGGUGCAGGUCGGGA	20	3′	134	52.99	33.5	25	0.471
BrMiR 169a	DX901870	AT3G13405.1	CAGCCAAGGAUGACUUGCCGA	21	5′	186	33.87	51.4	27.63	0.815
BrMiR 169b	DU982154	AT1G53687.1	CAGCCAAGGAUGACUUGCCGA	21	5′	109	33.03	40.6	37.25	1.128
BrMiR 169c	DU988169	AT3G13405.1	CAGCCAAGGAUGACUUGCCGA	21	5′	112	33.04	41.1	36.7	1.111
BrMiR 169d	ED535040	AT3G26818.1	AGCCAAGGAUGACUUGCC	18	5′	139	36.69	27.2	19.57	0.533
BrMiR 169e	KFRT-048E03	AT5G12840.3	AGCCAAGAAUGAUUUGCCGG	20	5′	271	43.91	63.5	23.43	0.534
BrMiR 169f	ED527257	AT3G26812.1	UAGCCAAGGAGACUGCCUA	19	5′	161	36.02	43	26.71	0.741
BrMiR 169g	contig153738	AT3G13405.1	CAGCCAAGGAUGACUUGCCGA	21	3′	181	32.6	46	25.41	0.779
BrMiR 169h	contig132829	AT3G13405.1	CAGCCAAGGAUGACUUGCCGA	21	5′	134	36.57	47.9	35.75	0.977
BrMiR 169i	contig122748	AT3G13405.1	CAGCCAAGGAUGACUUGCCGA	21	3′	195	31.28	39.9	20.46	0.654
BrMiR 169j	contig062782	AT4G21595.1	AGCCAAGGAUGACUUGCCGA	20	5′	129	39.53	44.1	34.19	0.864
BrMiR 169k	contig176484	AT5G24825.1	CAGCCAAGGAUGACUUGCCG	20	5′	148	41.89	50.8	34.32	0.819
BrMiR 169l	contig139483	AT3G14385.1	AGCCAAGGAUGACUUGCCGG	20	3′	149	44.97	35.2	23.62	0.525
BrMiR 169m	contig063427	AT4G21595.1	UGAGCCAAGGAUGACUUGCC	20	5′	108	46.3	31	28.7	0.619
BrMiR 169n	contig172893	AT4G21595.1	UGAGCCAAAGAUGACUUGCCG	21	5′	122	40.98	43.1	35.33	0.862
BrMiR 169o	contig131930	AT1G53687.1	UGAGCCAAAGAUGACUUGCCG	21	5′	110	38.18	40.4	36.73	0.961
BrMiR 169p	contig023436	AT1G19371.1	UAGCCAAGGAUGACUUGCCUG	21	5′	131	47.33	46.4	35.42	0.748
BrMiR 169q	contig155478	AT1G19360.1	UAGCCAAGGAUGACUUGCCUG	21	5′	155	41.29	44.5	28.71	0.695
BrMiR 169r	contig000299	AT3G26813.1	UAGCCAAGGAUGACUUGCCUG	21	3′	193	38.86	51.4	26.63	0.685
BrMiR 169s	contig000299	AT3G26813.1	UAGCCAAGGAUGACUUGCCU	20	3′	162	34.57	38	23.46	0.678
BrMiR 170a	contig152783	AT5G66045.1	UGAUUGAGCCGCGUCAAUAUC	21	3′	108	47.22	26.3	24.35	0.515
BrMiR 170b	contig043748		UGAUUGAGCCGUGCCAAUAUC	21	5′	111	36.94	24.2	21.8	0.59
BrMiR 171a	DX044654	AT1G11735.1	UUGAGCCGUGCCAAUAUCACG	21	3′	97	44.33	39	40.21	0.907
BrMiR 171b	ED531830	AT1G62035.1	UUGAGCCGUGCCAAUAUCACG	21	3′	89	38.2	32.8	36.85	0.964
BrMiR 171c	ED514994	AT1G11720.1	UUGAGCCGUGCCAAUAUCACG	21	3′	91	39.56	37.2	40.88	1.033
BrMiR 171d	ED515731	AT1G11735.1	UUGAGCCGUGCCAAUAUCACG	21	3′	93	37.63	32.8	35.27	0.937
BrMiR 171e	DU980843	AT1G62035.1	UUGAGCCGUGCCAAUAUCACG	21	3′	102	40.2	27.8	27.25	0.678
BrMiR 171f	DU827625	AT3G51375.1	UGAUUGAGCCGCGCCAAUAUC	21	3′	126	44.44	38.4	30.48	0.685
BrMiR 171g	DY013547.1	AT1G11735.1	UUGAGCCGUGCCAAUAUCACG	21	3′	89	38.2	32.8	36.85	0.964
BrMiR 171h	contig183747	AT3G51375.1	UGAUUGAGCCGCGCCAAUAUC	21	3′	127	43.31	32.9	25.91	0.598
BrMiR 171i	contig179438	AT3G51375.1	UGAUUGAGCCGCGCCAAUAUC	21	5′	111	44.14	34.8	31.35	0.71
BrMiR 171j	contig134903	AT1G11735.1	UUGAGCCGUGCCAAUAUCACG	21	5′	116	42.24	31.2	26.9	0.636
BrMiR 171k	contig128488	AT1G11735.1	UUGAGCCGUGCCAAUAUCACG	21	3′	86	39.53	25.9	30.12	0.761
BrMiR 172a	AJ860723	AT2G39730.3	GAAUCUUGAUGAUGUUACA	19	5′	111	48.65	29.7	26.76	0.55
BrMiR 172b	DX011558	AT5G59505.1	GAAUCUUGAUGAUGCUGCAU	20	3′	90	42.22	36	40	0.947
BrMiR 172c	DX034851	AT2G28056.1	GAAUCUUGAUGAUGCUGCAU	20	3′	99	41.41	35.6	35.96	0.868
BrMiR 172d	DX078594	AT5G59505.1	GAAUCUUGAUGAUGCUGCAU	20	3′	89	42.7	39.1	43.93	1.029

(continued)

miRNA family	Br-GSS/EST	At-gene	Mature miRNA	ML	MAS	Len(Pre)	GC%	MFE	AMFE	MFEI
BrMiR 172e	ED520082	AT5G59505.1	GAAUCUUGAUGAUGCUGCAU	20	3′	86	41.86	36	41.86	1
BrMiR 172f	ED525000	AT5G59505.1	GAAUCUUGAUGAUGCUGCAU	20		116	44.83	46	39.66	0.884
BrMiR 172g	ED516998	AT2G28056.1	GAAUCUUGAUGAUGCUGCAU	20	3′	109	45.87	30	27.52	0.6
BrMiR 172h	ED526567	AT5G59505.1	GAAUCUUGAUGAUGCUGCAU	20	3′	91	41.76	40.6	44.62	1.068
BrMiR 172i	EX060396.1	AT2G39730.3	GAAUCUUGAUGAUGUUACA	19	5′	111	48.65	29.7	26.76	0.55
BrMiR 172j	EX059018.1	AT2G39730.3	GAAUCUUGAUGAUGUUACA	19	5′	111	48.65	29.7	26.76	0.55
BrMiR 172k	EX057674.1	AT2G39730.3	GAAUCUUGAUGAUGUUACA	19	5′	111	48.65	29.7	26.76	0.55
BrMiR 172l	EX019450.1	AT2G39730.3	GAAUCUUGAUGAUGUUACA	19	5′	111	48.65	29.7	26.76	0.55
BrMiR 172m	contig138929	AT2G28056.1	AGAAUCUUGAUGAUGCUGCAU	21	5′	122	50	40.2	32.95	0.659
BrMiR 172n	contig084904	AT3G55512.1	AGAAUCUUGAUGAUGCUGCA	20	3′	126	35.71	41.7	33.1	0.926
BrMiR 172o	contig164892	AT3G11435.1	AGAAUCUUGAUGAUGCUGCA	20	5′	133	39.1	41.7	31.35	0.801
BrMiR 319a	DX903478	AT4G23713.1	UUGGACUGAAGGGAACUCCCU	21	5′	176	40.91	66.9	38.01	0.929
BrMiR 319b	AJ856769	AT2G40805.1	UUGGACUGAAGGGAGCUC	18	3′	202	40.1	68.7	34.01	0.848
BrMiR 319c	contig173636	AT5G41663.1	UUGGACUGAAGGGAGCUCCUU	21	3′	200	38.5	68.8	34.4	0.893
BrMiR 390a	DU830650	AT5G58465.1	AAGCUCAGGAGGGAUAGCGCC	21	5′	96	43.75	30.3	31.56	0.721
BrMiR 390b	DX059137	AT2G38325.1	AAGCUCAGGAGGGAUAGCGCC	21	5′	97	43.3	31.3	32.27	0.745
BrMiR 390c	DX890076	AT5G58465.1	AAGCUCAGGAGGGAUAGCGCC	21	5′	139	41.73	58.3	41.94	1.005
BrMiR 390d	contig070593	AT2G38325.1	AAGCUCAGGAGGGAUAGCGCC	21	5′	96	44.79	37	38.54	0.86
BrMiR 390e	contig054389	AT5G58465.1	AAGCUCAGGAGGGAUAGCGCC	21	3′	94	42.55	32.6	34.68	0.815
BrMiR 390f	contig039203		AAGCUCAGGAGGGAUAGCGCC	21	3′	120	40	40.2	33.5	0.837
BrMiR 390g	contig110394		AAGCUCAGGAGGGAUAGCGCC	21	3′	119	44.54	34.1	28.66	0.643
BrMiR 391a	contig125684	AT5G60408.1	UUCGCAGGAGAGAUAGCGCCA	21	5′	112	41.96	36.6	32.68	0.778
BrMiR 391b	contig083747	AT5G60408.1	UUCGCAGGAGAGAUAGCGCCA	21	5′	111	43.24	37.2	33.51	0.775
BrMiR 393a	contig164532	AT3G55734.1	UCCAAAGGGAUCGCAUUGAUCC	22	3′	156	35.9	37.7	24.17	0.673
BrMiR 393b	contig009383	AT3G55734.1	UCCAAAGGGAUCGCAUUGAUCC	22	5′	171	34.5	47.8	27.95	0.81
BrMiR 393c	contig053732	AT2G39885.1	UCCAAAGGGAUCGCAUUGAUCC	22	3′	129	33.33	33.2	25.74	0.772
BrMiR 394a	DU120003	AT1G20375.1	UUGGCAUUCUGUCCACCUCC	20	5′	100	39	26.7	26.7	0.684
BrMiR 394b	contig137399	AT1G20375.1	UUGGCAUUCUGUCCACCUCC	20	5′	107	40.19	31.4	29.35	0.73
BrMiR 394c	contig133329	AT1G20375.1	UUGGCAUUCUGUCCACCUCC	20	5′	131	38.17	40.1	30.61	0.801
BrMiR 395a	DX020424	AT1G26973.1	CUGAAGUGUUUGGGGGAACUC	21	3′	88	44.32	32.8	37.27	0.841
BrMiR 395b	DU832403	AT1G26975.1	CUGAAGUGUUUGGGGGGACUC	21	3′	115	40	39.8	34.61	0.865
BrMiR 395c	contig143422	AT1G69795.1	CUGAAGUGUUUGGGGGAACUC	21	3′	111	38.74	41.4	37.3	0.962
BrMiR 395d	contig124372	AT1G69797.1	CUGAAGUGUUUGGGGGGACUC	21	3′	130	43.08	50.1	38.54	0.894
BrMiR 395e	contig153647		CUGAAGUGUUUGGAGGAACUC	21	5′	123	34.96	32.8	26.67	0.762
BrMiR 396a	DX897816	AT3G52910.1	UCCACAGGCUUUCUUGAAC	19	5′	85	51.76	21	24.71	0.477
BrMiR 396b	contig029367	AT2G10606.1	UUCCACAGCUUUCUUGAACUG	21	5′	161	33.54	44	27.33	0.814
BrMiR 396c	contig159446	AT5G35407.1	UUCCACAGCUUUCUUGAACU	20	3′	164	31.1	50.9	31.04	0.997
BrMiR 398a	AJ861920	AT5G14565.1	UGUGUUCUCAGGUCACCCCU	20	3′	121	47.11	32.7	27.02	0.573
BrMiR 398b	EX049578.1	AT5G14545.1	UGUGUUCUCAGGUCACCCCU	20	3′	126	46.83	41.5	32.94	0.703
BrMiR 398c	contig015561	AT2G03445.1	UGUGUUCUCAGGUCACCCCUU	21	3′	96	39.58	31.8	33.13	0.836
BrMiR 399a	contig182431	AT1G29265.1	UGCCAAAGGAGAUUUGCCCUG	21	3′	179	35.2	53.2	29.72	0.844
BrMiR 399b	contig182431	AT1G29265.1	UGCCAAAGGAGAUUUGCCCGG	21	3′	160	38.75	67.34	42.09	1.086
BrMiR 399c	contig152434	AT5G62162.1	UGCCAAAGGAGAGUUGCCCUG	21	5′	128	46.88	40.5	31.64	0.674
BrMiR 399d	contig153243	AT2G34202.1	UGCCAAAGGAGAUUUGCCCCG	21	5′	135	33.33	48.2	35.7	1.071
BrMiR 399e	contig135242	AT2G34208.1	UGCCAAAGGAGAUUUGCCCGG	21	3′	111	39.64	42.1	37.93	0.956
BrMiR 399f	contig122533	AT1G29265.1	UGCCAAAGGAGAUUUGCCCGG	21	3′	105	41.9	46.6	44.38	1.059
BrMiR 399g	contig176382	AT2G34208.1	UGCCAAAGGAGAUUUGUCCGG	21	5′	113	42.48	32	28.32	0.666
BrMiR 400a	ED535204	AT1G32582.1	UAUGAGAGUAUUAUAAGUCAC	21	5′	134	32.84	46.6	34.78	1.059
BrMiR 400b	DX912125	AT1G64580.1	UAUGAGAGUAUUAUACGUCAC	21	3′	221	46.61	60.4	27.33	0.586
BrMiR 400c	DU827877	AT1G62680.1	AUGAGAGUAUUAUAACUCAC	20	3′	124	45.16	29.1	23.47	0.519

(continued)

miRNA family	Br-GSS/EST	At-gene	Mature miRNA	ML	MAS	Len(Pre)	GC%	MFE	AMFE	MFEI
BrMiR 403	contig102674	AT2G47275.1	UUAGAUUCACGCACAAACUCG	21	5′	91	35.16	27	29.67	0.843
BrMiR 408a	DX037057	AT2G47015.1	AUGCACUGCCUCUUCCCUGGC	21	3′	149	42.95	38.2	25.64	0.596
BrMiR 408b	EX141550.1	AT2G02850.1	AUGCACUGCCUCUUCCCU	18	3′	151	54.3	43.9	29.07	0.535
BrMiR 472a	ED514754	AT1G14850.1	UCCCUACUCCACUCAUCCC	19	5′	241	43.15	63.9	26.51	0.614
BrMiR 472b	CX271339.1	AT1G14850.1	UCCCUACUCCACUCAUCCC	19	5′	241	43.15	63.9	26.51	0.614
BrMiR 473	DU120348		UCUCCCUCAAGGUUUCCA	18	5′	179	34.64	29.6	16.54	0.477
BrMiR 776	DX019504		UCUAAGUCUUCUUUUGAU	18	5′	251	22.31	29.2	11.63	0.521
BrMiR 824	contig141281	AT4G24415.2	UAGACCAUUUGUGAGAAGGGA	21	5′	66	40.91	11.1	16.82	0.411
BrMiR 837	DX902366	AT4G16143.2	AUCAGUUUCUUGUUCUUUUC	20	5′	202	29.21	25.9	12.82	0.439
BrMiR 838	DU127124	AT1G65590.1	UUUUCUUAUACUUCUUGCA	19	3′	280	43.57	59.4	21.21	0.486
BrMiR 842	DX012634	AT2G14370.1	AUGGUCAGAUCGGUCAUC	18	5′	196	53.57	52.6	26.84	0.5
BrMiR 845a	CT021891	AT5G04290.1	GGCUCUGAUACCAAUUGA	18	5′	139	53.24	40.2	28.92	0.543
BrMiR 845b	DX897026	AT4G31200.1	UGGCUCUGAUACCAACUGAUG	21	3′	141	32.62	50.3	35.67	1.093
BrMiR 845c	contig151543		UGGCUCUGAUACCAACUGAUG	21	3′	103	34.95	40.6	39.42	1.127
BrMiR 846	ED536287	AT1G52110.1	UUGAACUGAAGUGCUUGAAU	20	5′	195	32.82	42.9	22	0.67
BrMiR 850	DU832285	AT1G32990.1	GAUCCGGACUAAAACAAAG	19	5′	83	43.37	17.3	20.84	0.48
BrMiR 854a	DX017286	AT2G44710.1	UGAGGAGAGGGAGGAGGAG	19	3′	305	40	62.9	20.62	0.515
BrMiR 854b	DX899834	AT5G58490.1	GAGGAGAGGGAGGAGGAG	18	3′	98	63.27	32	32.65	0.516
BrMiR 857	DX077868	AT1G30400.2	UUUUGCAUGUUGAAGGUGU	19	5′	109	37.61	22.6	20.73	0.551
BrMiR 1132a	DX047066		CAUUAAGGAACGGAAGGAG	19	3′	271	19.93	42.3	15.61	0.783
BrMiR 1132b	DX910272	AT3G26570.2	AUUAUGAAACGGAAGGAG	18	3′	255	22.35	46.3	18.16	0.812
BrMiR 1139	AJ855061		AGAGUAAAAUACACUAGUA	19	3′	85	29.4	10.1	11.88	0.404
BrMiR 1140	contig123251		ACAGCCUAAACCAAUCGGAGC	21	3′	151	37.09	61.4	40.66	1.096
BrMiR 1436	DX082315	AT5G49680.2	UUAUGGGACGGAAGGAGU	18	3′	214	22.43	30.7	14.35	0.639
BrMiR 1439	contig149192		UUUUGGAACGGAGAGAGUAUU	21	3′	271	23.62	43.9	16.2	0.685
BrMiR 1514	DX899429	AT2G45560.1	UUCAUUUUUAUAAAUAGACAUU	22	3′	183	18.03	19.8	10.82	0.6
BrMiR 1520a	DX016618		AGAACUUGACACGUGACAA	19	5′	164	31.1	25.7	15.67	0.504
BrMiR 1520b	ED528582		AGAACUUGACACGUGACAA	19	5′	153	28.1	23.6	15.42	0.548
BrMiR 1521a	CW982899	AT1G79580.3	CUGUUAAUGGAAAAAGUUG	19	5′	136	36.03	17.1	12.57	0.349
BrMiR 1521b	CT022839	AT5G39862.1	CUGUUGAUGGAAAAUGUU	18	5′	268	41.33	69.2	25.82	0.624
BrMiR 1522	AJ858836	ATMG00110.1	UUUAUUUCUUAAAAUGAAA	19	3′	166	32.53	32	19.28	0.592
BrMiR 1527	DX023754		AACUCAACCUUACAAAAC	18	3′	167	34.73	34.5	20.66	0.605
BrMiR 1863	DX895479	AT2G36990.1	AGCUCUGAUACCAUGUUAGAUU	22	5′	236	30.08	40.5	17.16	0.57
BrMiR 1886	KBFL-140B03	AT5G53120.1	UGAGAGAAGUGAGAAGAAA	19	5′	124	44.88	24.6	19.84	0.439
BrMiR 2091	DX017320	AT5G65683.1	AACCGAGCCGAAGAGGAG	18	5′	96	52.08	33.1	34.48	0.662
BrMiR 2111a	contig014252		UAAUCUGCAUCCUGAGGUUUA	21	3′	113	42.11	36.1	31.95	0.644
BrMiR 2111b	contig148483		UAAUCUGCAUCCUGGGGUUUA	21	3′	135	40.74	27.4	20.3	0.498
BrMiR 2673	contig154626		CCUCUUCCUCUUCCUCUUCC	20	5′	119	47.06	32	26.89	0.571

Br, *Brassica rapa*; At, *Arabidopsis thaliana*; ML, mature miRNA length; MAS, mature miRNA arm side in hairpin secondary structure; MFE, minimum folding free energy; AMFE, adjusted minimum folding free energy; MFEI, minimum folding free energy index.

followed in the present study is provided in Supplementary Fig. 1. Initially 977 known mature miRNA sequences were analyzed against *B. rapa* sequences (GSS, EST, and mRNA) in BLASTN, of which 780 probable conserved sequences appeared to contain miRNAs. Further screening removing the repeats and protein-coding sequences narrowed down the probable miRNA candidates to 234. Structure prediction using the Mfold program finally gave 186 mature miRNAs with reasonable stem-loop formation. The 186 identified *B. rapa* miRNAs belong to 55 families, and, 20 miRNAs belonging to 9 miRNA families were derived from EST sequences. Varying numbers of miRNAs from the 55 families were identified (See Table 2 and Fig. 1 for details). A maximum of 19 miRNAs and minimum of 1 miRNA were detected in a single family, and the length of matured ranged from 18 to 22 nt long (Table 2 and Fig. 1), consistent with the findings in *B. napus* (Xie et al., 2007) and cotton (Qiu et al., 2007). The majority of miRNAs were 21 nt long (54.84%) followed by, 20 (21.51%), 19 (10.22%) 18 (9.14%), and 22 (2.69%) (Fig. 2). It is suggested that the difference in size of the identified miRNAs within different families might offer unique functions for the regulation of miRNA biogenesis or gene expression in plants. It is reported that uracil is more represented

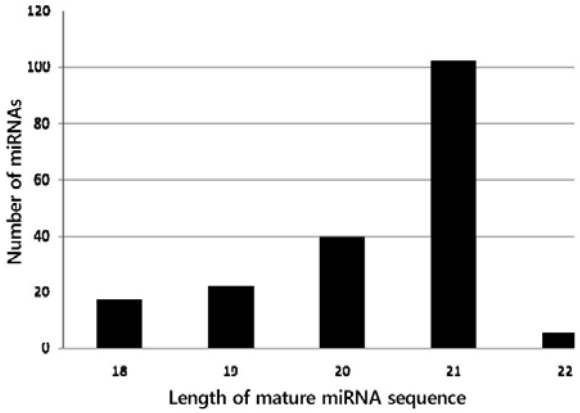

Fig. 2. Number of *Brassica rapa* miRNAs against different lengths of mature miRNAs

at the 5′ side of mature miRNAs; the percentage of representation of mature miRNAs having U at the 5′ side of the stem loop is 66.67%, followed by A (14.52%), C (9.68%), and G (9.14%); this is consistent with previous findings (Mi et al., 2008; Montgomery et al., 2008; Takeda et al., 2008; Zhang et al., 2008). It is speculated that the strong bias of uracil in the first 5′ nucleotide position is due to its important role in the recognition of the miRNA by *argonaute1* (Zhang et al., 2006b). The analyses of nucleotide contents in the mature miRNAs show a majority of G [26.96% (10.13%)], followed by A [26.61% (10.07%)], U [23.71% (10.87%)], and C [22.72% (10.61%)] nucleotides. The magnitude of diversity of the identified miRNAs was also found in the location of mature miRNAs sequences (Fig. 3). Of the 186 newly identified mature miRNAs, 93 were located toward the 5′ side of the stem loop of the precursor sequences, while the other 93 toward the 3′ side.

Precursor sequence characteristics

The 186 *B. rapa* miRNAs were derived from varying sizes of precursor sequences. The precursor miRNAs (pre-miRNA) ranged from 66 to 305 nucleotides with an average of 135.62 (47.79) nucleotides (Supplementary Table 1). The majority (77.69%) of the precursor miRNAs were 81-202 nt in length (Supplementary Table 1 and Fig. 4). The findings of pre-miRNA sequences ranging from 66 to 305 nt in the present study shows that *B. rapa* also contains miRNAs with varying pre-miRNA lengths as is the case in other plant species (Song et al., 2009; Zhang et al., 2007a; 2008). The distributions of A, U, G, and C nucleotides are different and uneven. A [28.89% (5.51%)] and U [30.23% (5.80%)] nucleotides were predominant compared to G [20.30% (4.39%)] and C [20.58% (4.43%)]. The percentage of GC content ranged from 18.03% to 63.27% with an average of 40.88% (6.86%) (Supplementary Table 1). Lower minimal folding free energy (MFE) is important for high thermodynamic stability of the sequences to form stable secondary loop structures (Zhang et al., 2008). In the present study, the

> BrMiR 156c

```
   C           U  UU   -  UUU  ---   AG  AA          -     -       AU       CU      U
AGA AGAGAAGGAGG GA  GAG GA   GC   AAC  AG  AACUGACAGAA GAGAG UGAGCAC  GCAGGCA  GUUAUG G
UCU UCUCUUUCUCU CU  CUC CU   CG   UUG  UC  UUGACUGUCUU CUCUC ACUCGUG  UGUUCGU  CAAUAC U
   C           -  CU   A  UU-  CUC   AG  CG           U     C       CG       UU      C
```

> BrMiR 162a

```
A--   -      G   GC    G        C  UG  G

   GAU GCUGGA GCA  GGUU AUCGAUCU UU  UG       UUUUG  \
   CUG CGACCU CGU  CCAA UAGCUAGA AA  AC       AAAAC  A
CCG   G      A   GU    A        C  GU  GAAAAUU     AA
```

> BrMiR 164a

```
-        G   -            UG A             ------        U
 GGUGAGGA CUC AUGUUGGAGGAG  G GCACGUGCAAAUU      ACAUGAGA A
 CUACUCUU GAG UGCAACCUCUUC  C CGUGCACGUUUGG      UGUGCUCU G
C        G   G            GU C             U
```

> BrMiR 169c

```
 ---------------        CU      U
GUG AGCCAAGGA  ACUUGCCGAUU              UAAAAUAU  GAUAAG \
CAU UCGGUUCCU  UGAACGGCUAG              AUUUUAUG  UUAUUU A
-   A         GU           UACUAAAAAAA
```

> BrMiR 171f

```
-AAAGAG   G         CA  GA--        GC  C   A      AA-  G   GA----

      AGU ACGAGAGAG  CU    GAUAUUGGC  GG UCA UCAGAU   CU AGA      AUGUA  A
      UCG UGCUUUCUC  GA    CUAUAACCG  CC AGU AGUCUA   GG UCU      UGCAU  U
 ------   -         AG  GAAA         GA  A   G      GAA  -   AAUGUG     GA
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(continued)

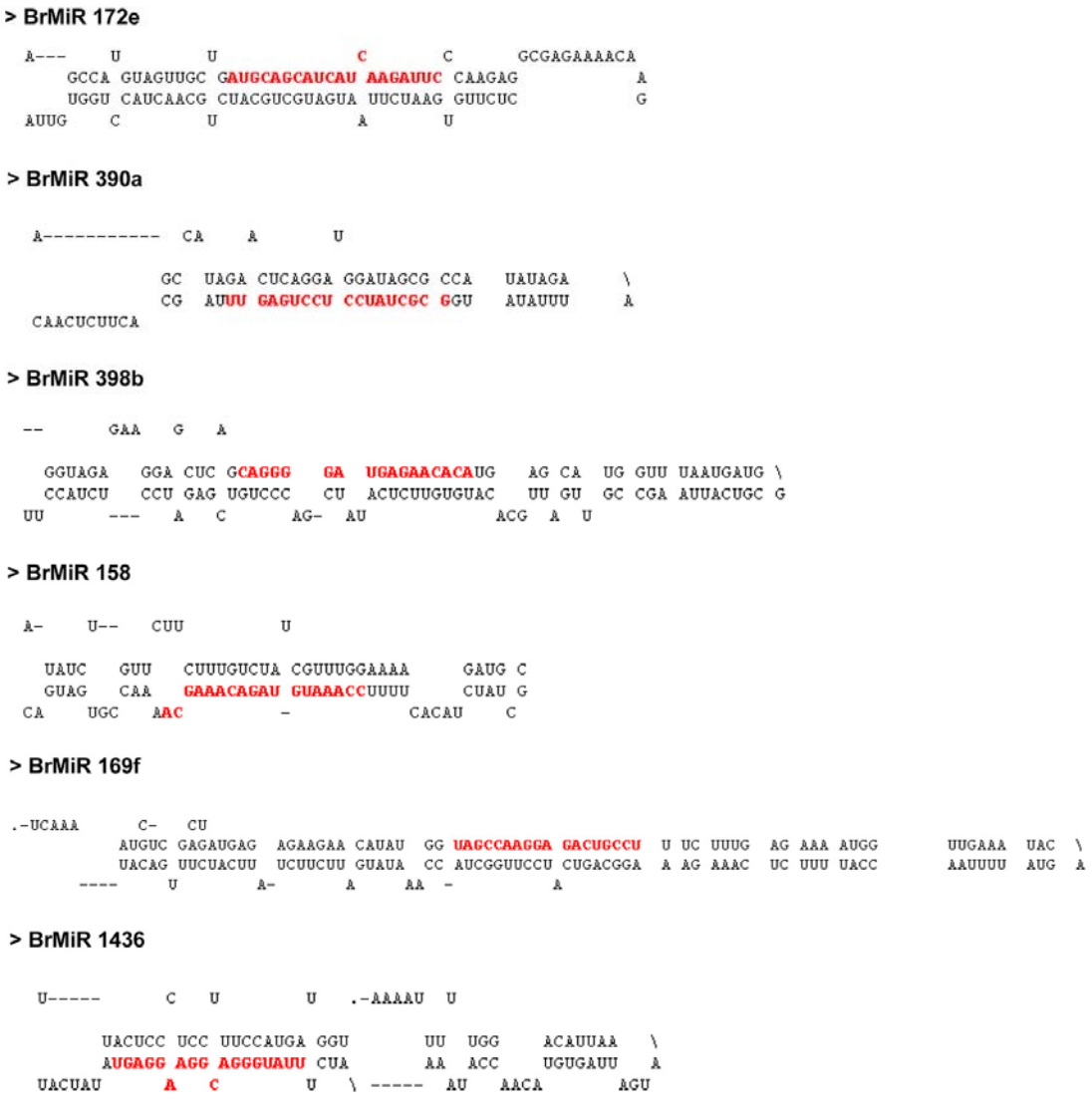

Fig. 3. Stem-loop hairpin structures of representative miRNA families. Red indicates matured miRNA sequences. The actual size of precursor sequences may be slightly shorter or longer than presented.

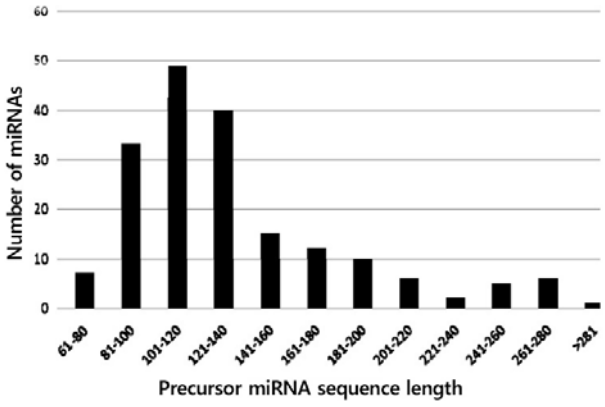

Fig. 4. Number of matured miRNAs against different lengths of precursor miRNAs

MFE of 186 identified *B. rapa* miRNAs ranged from -10.1 to -75.4 kcal/mol with an average of -39.22 (12.52) kcal/mol. However, due to the strong positive influence of DNA/RNA length in the MFE calculations, a new method, adjusted minimal folding free energy (AMFE), was adopted to normalize the potential effects of nucleotide sequence length (Zhang et al., 2008). AMFE ranged from -10.82 to -52.28 kcal/mol with an average of -29.96 (7.84) kcal/mol (Table 2). The observation of high MFE and AMFE of pre-miRNAs in the present study is consistent with previous reports (Bonnet et al., 2004b; Zhang et al., 2006a; 2008). Recently, the minimum folding free energy index (MFEI), which is calculated on the basis of MFE, sequence length, and nucleotide GC content, was widely adopted for distinguishing miRNAs from other RNAs (Zhang et al., 2006a; 2006b). The MFEI in our study for precursor miRNA sequences ranged from 0.349 to 1.210 with an average of 0.737 (0.178). Although it is suggested that MFEI values ≥ 0.85 are strongly indicative of actual miRNAs, lower values cannot be ruled out as potential miRNAs (Zhang, 2007a; Zhang et al., 2006a; 2006b) (Table 2).

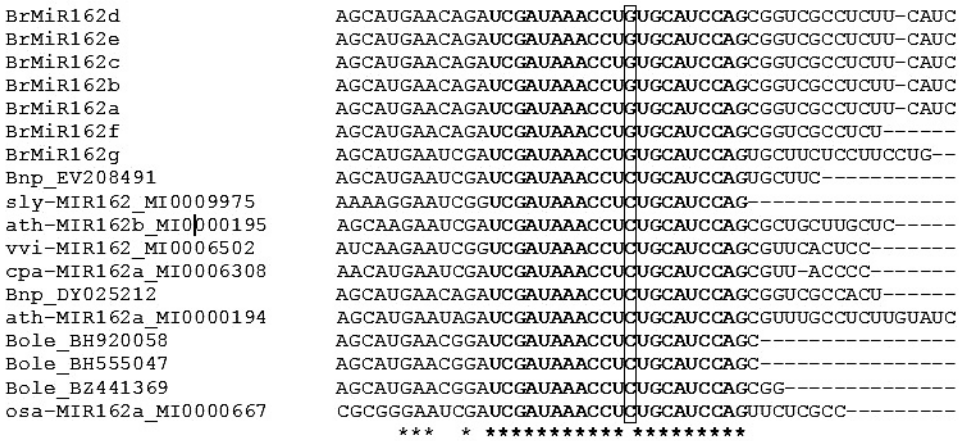

Fig. 5. *Brassica rapa* miRNA162 family showing a single nucleotide difference at the 12th nucleotide position of mature miRNA in comparison to the miRNA 162 family of other plants; BrMiR, *Brassica rapa* miRNA; Bnp, *Brassica napus;* Bol, *Brassica oleracea;* Sly, *Solanum lycopersicum*; Vvi, *Vitis vinifera*; Osa, *Oryza sativa*; Ath, *Arabidopsis thaliana*; Cpa, *Carica papaya.*

In the present study, only 48 (25.81%) of 186 identified *B. rapa* miRNAs had MFEI values ≥ 0.85, in contrast to that of soybean miRNAs having values ≥ 0.85 (Zhang et al., 2008).

Identification of novel *B. rapa* and *Brassica* lineage-specific miRNAs

Of the 186 miRNAs belonging to 55 families identified in the present study 15 miRNAs belonging to 10 families were reported previously (He et al., 2008); thus, the remaining 40 miRNA families in our study are newly identified. Furthermore, we identified more members belonging to those previously reported *B. rapa* miRNA families. He et al. (2008) identified 1 member each for miRNA families 157, 159, 164, 167, 390, and 1885 and 2 members each for miRNA families 160, 172 and 398; only miRNA family 171 had 3 identified members (He et al., 2008). In the present study, we identified 15 members each for BrMiR 159 and 172; 6 for miRNA 164; 8 for miRNA 160; 4 for miRNA 167; 7 for miRNA 390; and 3 for miRNA 398 (Table 2). Further analysis of the miRNA families with previously identified miRNAs present in the miRBase revealed that of 186 miRNAs belonging to 55 families, 48 miRNAs belonging to 30 miRNA families were not identical and had 1-2 mismatches with the previously reported miRNAs from one or more plant species; this suggests that these miRNA nucleotide mismatches are specific to *B. rapa*. One such example is the BrMiR 162 family, which has a guanine in *B. rapa* instead of a conserved cytosine in other plant species at the 12th nucleotide position of the mature miRNA. The single nucleotide difference is also observed between *B. rapa* and other *Brassica* species, such as *B. napus* and *B. oleracea*, apart from *Arabidopsis*, although these 4 species belongs to same family of Brassicaceae (Fig. 5). The conserved and nonconserved regions of miRNA family 162 of different plant species such as *B. napus, B. oleracea, Solanum lycopersicum*, *Vitis vinifera*, *O. sativa*, *A. thaliana,* and *Carica papaya* are shown in Fig. 5. Further analyses could identify 5 *Arabidopsis-Brassica* lineage-specific miRNA families (BrMiR 158, 391, 400, 824, and 1140) in *B. rapa*. Sunkar and Jagdeeswaran (2008) identified miRNA family 158 in *B. rapa* and *B. oleracea*, and family 391 in *B. oleracea*. In the present study, through computational analysis, we identified BrMiR 391 in *B. rapa.* Of the remaining 3 newly identified *Brassica* lineage-specific miRNAs identified in *B. rapa*, BrMiR 400 is only reported in *A. thaliana*; BrMiR 824 in *B. oleracea*, *B. napus*, and *A. thaliana*; and BrMiR 1140 only in *B. napus*.

Detection of sense, antisense, and clusters of *B. rapa* miRNAs

Although many animals studies found that miRNAs are transcribed and processed from the sense and antisense strands of the same genomic locus, this was reported only recently in plants (Xie et al., 2010; Zhang et al., 2008). In our study, we found only a DNA sequence producing identical matured BrMiR 399 from both sense and antisense strands. Although sense and antisense miRNAs are transcribed from the same genomic locus, they are not identical with respect to the, 20th nucleotide position; U and G nucleotides were present in sense and antisense BrMiR 399, respectively. Previous studies also report the occurrence of miRNA clusters in plants, especially in cotton and soybean (Xie et al., 2010; Zhang et al., 2008). miRNAs in these clusters are produced from the same precursors or from different precursor sequences separated by a few nucleotides on the same genomic locus (Xie et al., 2010; Zhang et al., 2008). In this study, we identified only 1 miRNA cluster in *B. rapa*. This cluster includes 2 miRNAs belonging to the BrMiR family 169 (BrMiR 169r and 169s), which are 353 nucleotides apart. These 2 BrMiRs differ with respect to a single nucleotide difference of their mature miRNA sequences (Table 2 and Fig. 6).

Expression analyses of identified *B. rapa* miRNAs

The expression of *B. rapa* miRNAs was identified in 2 ways. First, we examined the original tissues of the EST sequences obtained from public databases from which miRNAs were derived. A total of, 20 miRNAs belonging to 9 BrMiR families were found to be expressed in different tissues in *B. rapa*. The tissues where miRNAs were expressed include floral buds (BrMiR 159, 160, 167, and 398), roots (BrMiR 162), callus (BrMiR 160), seedlings (BrMiR 162), primary leaves (BrMiR 408), etiolated mature leaves (BrMiR 166), and salt-treated whole plant (BrMiR 172) (Table 3). This observation suggests that some of these identified *B. rapa* miRNAs exhibit tissue-specific expression whereas some are expressed throughout the plant.

Second, the expression of identified miRNAs in various tissues was further examined using microarray data from the tissues of various stages of *B. rapa* development from another experiment. The 50K *B. rapa* DNA chip contains 12 identified miRNA-producing genes belonging to 8 miRNA families; these

were confirmed by computational identification and secondary structure prediction by using the sequences found in microarray DNA chip (data not shown). Microarray analysis was performed to analyze gene expression at different stages of floral buds, stamens, carpels, siliques, and different parts of leaves (e.g., inner and outer parts). The expression of 12 miRNA genes was observed in microarray data taken at different stages of bud development from male sterile and fertile plants, photosynthetic (source) and mid-leaves (sink), and young shoots including the white and yellow portions of leaves. The microarray data showed very low to high expression of 12 miRNA genes in different tissues (data not shown).

To confirm the results of the microarray data analysis, we performed a subsequent Northern blot analysis of 5 miRNA families from 3 tissues, namely the leaves, stem, and roots (Fig. 7). Among these 5 miRNA families, BrMiR 159 was highly expressed in all analyzed tissues (Fig. 7). Furthermore, we observed different sizes of miRNAs in stem tissues from the northern blot analysis using these miRNAs; this convincingly supports the computational analysis identification that the matured miRNAs are 18-22 nt long (Table 2 and Fig. 7). BrMiR 160 exhibited a low level of expression in the 3 tissues; BrMiR 167 was highly expressed in leaves and roots, but very little expression was observed in the stem; BrMiR 398 was highly expressed in leaves, whereas expression was low in the stem and no expression was detected in the roots; BrMiR 408 exhibited medium expression in leaves but low expression in the stem and roots (Fig. 7).

Further, we performed quantitative real time polymerase chain reaction (qRT-PCR) for 12 miRNAs (Table 1) in six different tissues of *B. rapa* cultivar Chiifu. The qRT-PCR analyses

Table 3. Identified *Brassica rapa* expressed sequence tags (ESTs) producing miRNA and their expressing tissues

Brassica rapa miRNA	*Brassica rapa* EST	Expressing tissue
BrMiR 156j	CV432746.1	Root
BrMiR 159b	EX050542.1	Floral buds
BrMiR 159c	EX048967.1	Floral buds
BrMiR 159d	EX047628.1	Floral buds
BrMiR 159g	EX039355.1	Floral buds
BrMiR 160b	EX044968.1	Floral buds
BrMiR 160h	EX025484.1	Callus
BrMiR 162b	EX133401.1	Root
BrMiR 162c	EX071919.1	Seedling
BrMiR 162d	EX071254.1	Seedling
BrMiR 162e	EX069816.1	Seedling
BrMiR 166b	EX063968.1	Etiolated mature leaf
BrMiR 166c	EX063242.1	Etiolated mature leaf
BrMiR 167c	EX041799.1	Floral buds
BrMiR 172i	EX060396.1	Salt-treated whole plant
BrMiR 172j	EX059018.1	Salt-treated whole plant
BrMiR 172k	EX057674.1	Salt-treated whole plant
BrMiR 172l	EX019450.1	Light-chilled whole plant
BrMiR 398b	EX049578.1	Floral buds
BrMiR 408b	EX141550.1	Primary leaf

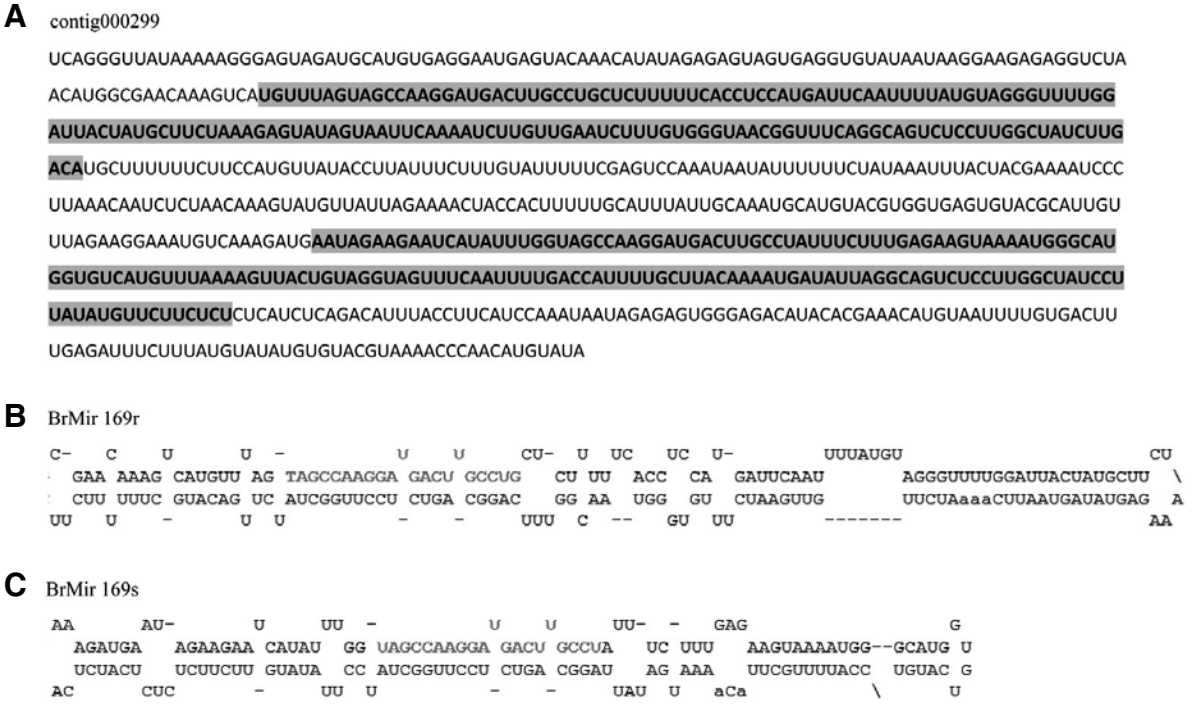

Fig. 6. BrMiR 169r-BrMiR 169s cluster in *B. rapa* contig000299. (A) *Brassica rapa* genomic contig000299 showing 2 miRNA clusters. Shaded sequences indicate pre-miRNAs and red sequences indicate mature miRNA. (B) Predicted miRNA secondary structure of BrMir 169r. (C) Predicted miRNA secondary structure of BrMir 169s.

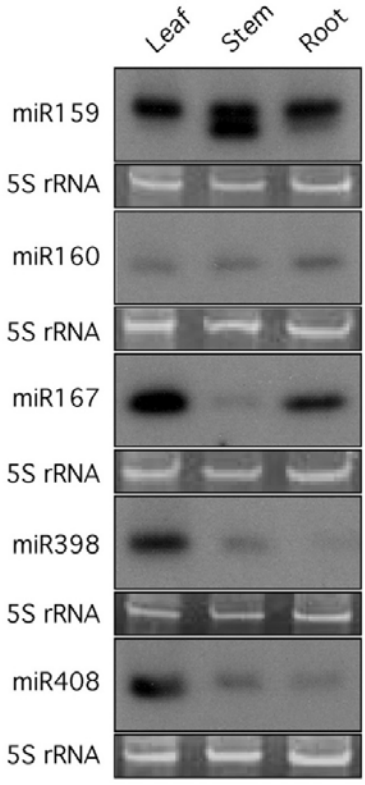

Fig. 7. Northern blot analysis of leaf, stem, and root tissues of *Brassica rapa* seedlings. Low-molecular-weight RNA (10 μg) was used for Northern analysis. Antisense oligonucleotide probes were designed, radiolabeled, and used as probes for the detection of the *B. rapa* miRNAs. 5s rRNA was used as a loading control.

demonstrated that all miRNAs were expressed in all the six tissues tested. However, while analyzing the results from qRT-PCR, we observed that the expression level of miRNAs differ from each other in the six *B. rapa* tissues tested (Fig. 8). qRT-PCR result showed that *BrMir158b* expression levels was not significantly different in all tested tissues except for in midrib tissue where there was decreased expression level of this miRNA was detected. BrMir162b showed almost equal expression levels in all tested tissues except the increased expression in midrib tissues. BrMir164c, BrMir396b, BrMir396b and BrMir 1132b showed very high expression in root tissues, BrMir171f in shoot apex, BrMir172g and BrMir1521b in stem, respectively. BrMir170b, BrMir837 and BrMir842 did not show any significant expression differences in the six tissues of *B. rapa*.

Identification of target genes

Due to the perfect or near-perfect complementarities of mature miRNAs and target mRNAs, it is possible to find target genes that are regulated by specific miRNA. Several studies utilized this fact and identified many target genes in different plant species while allowing 1-4 nucleotide mismatches between target mRNA and miRNAs (Song et al., 2009; Zhang et al., 2008). In this study, to identify miRNA targets, we searched for *B. rapa* mRNA sequences available in the laboratory and public databases showing complementarity with < 3 mismatches. Gaps, G:U pairs, and other noncanonical pairs were not allowed and considered mismatches (Xie et al., 2007). By using this criterion, the target genes of 33 out of 55 *B. rapa* miRNA families were identified (Table 4). We could not identify the target genes for the following 22 miRNA families: BrMiR 157, 161, 165, 166, 169, 170, 319, 391, 393, 396, 399, 403, 408, 824, 842, 850, 1140, 1436, 1439, 1514, 2111, and 2673. A total of 66 target genes that are involved in different biological functions were identified for 33 miRNA families. The number of potential target genes identified per miRNA family ranged from 1 to 10. All target genes share high homology with *Arabidopsis* orthologues. The majority of these potential target genes are transcription factor/regulator genes (Table 4). The BrMiR 156 family largely targets squamosa promoter-binding protein like (SPL) transcription factor gene families, which are important in leaf development and phase change. Targeting of the SBP genes by miRNA 156 is reported in other plant species (Song et al., 2009; Zhang et al., 2008). The other miRNA families that target transcription factors are as follows: BrMiR 160, 164, 167, 171, 172, 394, 473, 845, 1132, 1139, 1521, 1527, 1886, and, 2091 (Table 4). The conserved targets between *Arabidopsis* and *B. rapa* confirm the conserved function of miRNAs in both species. *Apetala 2* is a transcription factor that establishes floral meristem identity and ovule and seed coat development (Chen, 2004); BrMiR 172 targets *AP2*.

Many studies report that miRNAs also target genes that are

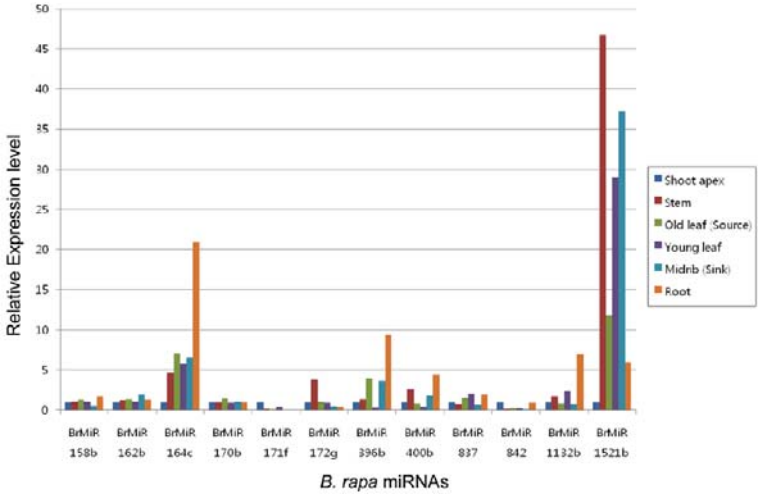

Fig. 8. The characterization of *Brassica rapa* miRNA expression patterns by real-time PCR analysis. Relative expression levels of BrMiR 158b, BrMiR 162b, BrMiR 164c, BrMiR 170b, BrMiR 171f, BrMiR 172g, BrMiR 396b, BrMiR 400b, BrMiR 837, BrMiR 842, BrMiR 1132b, and BrMiR 1521b were analyzed in shoot apex, stem, old leaf, young leaf, midrib, and in root tissues of *B. rapa* plants. The expression levels of all miRNAs by real-time PCR were normalized to the expression of *BrACT*.

Table 4. Potential targets of identified *Brassica rapa* miRNAs and similar *Arabidopsis thaliana* genes

miRNA family	*Brassica rapa* genes	Target protein	Target function	*Arabidopsis thaliana* gene
BrMiR 156	AC155337.1, DX032440	Squamosa promoter-binding protein-like 9	Transcriptional factor (TF)	AT2G42200.1
	AC189445.2	Squamosa promoter-binding protein	TF	AT3G57920.1
	AC189413.1	Squamosa promoter-binding protein-like 2	TF	AT5G43270.1
	AC189458.2, AC189298.1	Squamosa promoter-binding protein-like 6	TF	AT1G69170.1
	AC189298.1	Squamosa promoter-binding protein-like 3	TF	AT2G33810.1
	AC189649.2	Squamosa promoter-binding protein-like 10	TF	AT1G27370.4
	AC232495.1	TOC1 (TIMING OF CAB1 1)	Transcription regulator	AT5G61380.1
BrMiR 158	AC189530.2	Alpha-Dioxygenase	Oxidoreductase	AT1G73680.1
BrMiR 159	CW988048	Unknown		
BrMiR 160	AC189370.2	ARF17 (Auxin Response Factor 17)	TF	AT1G77850.1
BrMiR 162	DX038960	Unknown		
BrMiR 164	AC232542.1	NAC100 (NAC domain containing protein 100)	TF	AT5G61430.1
	AC189572.1	VIM1 (Variant In Methylation 1)	TF	AT1G57820.1
BrMiR 167	AC189484.2	ARF8 (Auxin Response Factor 8)	TF	AT5G37020.2
BrMiR 168	AC189554.2	Pyruvate kinase	Catalytic activity	AT5G56350.1
BrMiR 171	AC189445.2, DX039307, DX039875, DX050715, DX079200, DX080294	Scarecrow transcription factor family protein	TF	AT2G45160.1
BrMiR 172	AC189306.2	RCA (RUBISCO ACTIVASE)	ATP binding	AT2G39730.3
	AC234737.1	TOE2 (Target Of Eat 2)	TF	AT5G60120.1
	AC172870.1	SMZ (SCHLAFMUTZE)	TF	AT3G54990.1
	AC189433.2	CDT1A	Protein binding	AT2G31270.1
	AC232512.1	AP2 (APETALA 2)	TF	AT4G36920.1
	AC189432.2	TOE1 (Target Of Eat 1)	TF	AT2G28550.2
BrMiR 390	DX043165	Unknown		
BrMiR 394	AC189649.2	F-box family protein	TF	AT1G27340.1
BrMiR 395	AC189402.2, DU984698	APS4	ATP activity	AT5G43780.1
	DX024391, DX075472	APS1 (ATP sulfurylase 3)		AT3G22890.1
BrMiR 398	AC189227.2	Unknown protein		AT5G14550.1
BrMiR 400	AC189437.2	Unknown protein		AT1G63300.1
BrMiR 472	DX049611, DX038688	Unknown		
BrMiR 473	AC189542.2	Agenet domain-containing protein/bromo-adjacent homology (BAH) domain-containing protein	DNA binding	AT5G55600.1
	AC189298.1	Zinc finger (C3HC4-type RING finger) family protein	Protein binding, zinc ion binding	AT1G69330.1
BrMiR 776	AC189308.2	CUT1 (CUTICULAR 1)	Acyltransferase	AT1G68530.2
BrMiR 837	L31937.1	LCR69/PDF2.2 (Low-molecular-weight cysteine-rich 69)	Peptidase inhibitor activity	AT2G02100.1
BrMiR 838	EU117118.1	SPT1	Serine C-palmitoyltransferase	AT3G48780.1
BrMiR 845	AC232527.1	KOW domain-containing transcription factor family protein	TF	AT5G04290.1
	AC232513.1, AC189578.2, EU180578.1	Unknown		
BrMiR 846	AC189248.2	Jacalin lectin family protein		AT5G38550.1
	AC189524.2	Jacalin lectin family protein		AT1G57570.1
BrMiR 854	AC189355.2	Glycosyl hydrolase family 17 protein	Cation binding	AT5G58480.1
	AC189591.2, AC189402.2	Beta-ketoacyl-CoA synthase	Fatty acid elongase activity	AT5G43760.1
BrMiR 857	CW981541	Unknown		
BrMiR 1132	AC189577.2	Unknown protein		AT5G48610.1

(continued)

miRNA family	*Brassica rapa* genes	Target protein	Target function	*Arabidopsis thaliana* gene
BrMiR 1132	AC189568.2	Nucleoporin interacting component family protein	Protein binding	AT2G41620.1
BrMiR 1139	AC189599.2	Zinc finger (CCCH-type) family protein	Zinc-ion binding	AT5G12440.1
BrMiR 1520	AC189550.1	UDP-glucose:sterol glucosyltransferase (UGT80A2)	Transferase activity	AT3G07020.2
BrMiR 1521	AC189564.2	Transducin family protein/WD-40 repeat family protein	Nucleotide binding	AT5G14050.1
BrMiR 1522	AC232493.1	UBX domain-containing protein	Unknown	AT4G10790.1
BrMiR 1527	AC189385.2	Cullin 3A	Protein binding	AT1G26830.1
BrMiR 1863	AC189561.3	ACO1 (ACC OXIDASE 1)	Unknown	AT2G19590.1
	AC189318.1, AC189464.2			
BrMiR 1886	AC189592.2	UB2 (HISTONE MONO-UBIQUITINATION 2)	Protein binding	AT1G55255.1
BrMiR 2091	AC189359.2	Zinc finger (C3HC4-type RING finger) family protein	Zinc ion binding	AT3G19950.1

involved in signaling pathways. In the present study, BrMiR 167 and 160 were found to target Auxin response factor (ARF) 8 and 17, respectively; these mediate auxin response via the expression of auxin-regulated genes, and control stamen and flower maturation. *B. rapa* miRNAs were also found to target genes that control intracellular processes such as enzymes involved in metabolism, protein degradation, and transporters genes. BrMiR 168 targets the pyruvate kinase gene involved in glycolysis. BrMiR 854 targets glycosyl hydrolase family proteins, which have functions such as cation binding and hydrolase activity, including hydrolyzing *O*-glycosyl compounds mainly in carbohydrate metabolism. BrMiR 1520 targets the UDP-glucose:sterol glucosyltransferase (UGT80A2) genes, which are involved in the transfer of glycosyl groups in carbohydrate and lipid metabolism. BrMiR 838 targets the *SPT1* gene, which is involved in sphingolipid biosynthesis. BrMiR 776 and 854 target the cuticular 1 (*CUT1*) and beta-ketoacyl-CoA synthase genes, respectively, which are both involved in the biosynthesis of very-long-chain fatty acids (VLCFA). BrMiR 395 targets *APS1* and *APS4*, which are involved in sulfate assimilation in *Arabidopsis*; this shows that the functions of different miRNA families are conserved in plant species. BrMiR 1132 targets nucleoporin-interacting component family proteins, which are involved in protein binding/transport. Many studies report that miRNAs also target genes that are involved in biotic and abiotic stress. In the present study, BrMiR 837 was found to target LCR69/PDF2.2 (low molecular weight cysteine-rich 69), which is a family of pathogenesis-related and plant defensin proteins that have peptidase activity.

DISCUSSION

Since the discovery of plant miRNAs in, 2002 (Llave et al., 2002b), miRNAs have become an important and integral topic in functional genomic research; consequently, several studies show that miRNAs are important regulatory elements involved in plant development, metabolism, biotic and abiotic stresses, and many other functions (Lu et al., 2005; Pulido and Laufs, 2010; Ruiz-Ferrer and Voinnet, 2009; Zhang et al., 2006a; 2007c). *B. rapa* is an important crop with varying leaf and plant morphophytes. It has a comparatively smaller genome size compared to other *Brassica* species, and is recently considered a model plant for *Brassica* A genome sequencing (http://www.brassica-rapa.org, http://www.brassica.info). Although a previous study reports the identification of few miRNAs in *B. rapa* (He et al., 2008), their presence remains largely unknown in this species. An increasing number of genomic/DNA sequences in the form of GSS, EST, mRNA, and cDNA are being deposited into the NCBI database every day. Comparative genome-based *in silico* screening of *B. rapa* EST, mRNA, cDNA, and GSS sequences, using previously reported miRNAs from different plant species, would allow us to systematically and comprehensively identify 186 miRNAs belonging to 55 families in the present study. Although earlier studies identified a few miRNAs in *B. rapa*, they could not find all of the miRNAs reported in the present study. Of the 55 miRNA families, 38 are reported for the first time; in addition, more members of previously identified *B. rapa* miRNA families were found. Furthermore, we identified 5 *Arabidopsis-Brassica* lineage-specific miRNA families in *B. rapa* by using computational analysis; of these, BrMiR 391, 400, 824, and 1140 are newly identified, and BrMiR 158 was previously reported (Sunkar and Jagdeeswaran, 2008). These findings suggest that with the continuous availability of genome sequences, we could identify many more miRNAs that regulate important genes in *B. rapa*.

The transcription of miRNAs from sense and antisense strands is recently reported in insects, human, and plants (Bender, 2008; Stark et al., 2008; Tyler et al., 2008; Zhang et al., 2008). In the present study, we found only 1 DNA sequence from which both sense and antisense mature miRNAs were produced compared to 5 miRNAs belonging to 3 families identified in soybean (Zhang et al., 2008); the sense and antisense miRNAs differ by 1-3 nt in that study. We also observed a single-nucleotide difference between the sense and antisense miRNAs belonging to the BrMiR 399 family. The identification of only 1 DNA sequence producing both sense and antisense miRNAs may be due to the limited availability of whole-genome sequence information for *B. rapa*. Several miRNA clusters, including miRNA family 166, 169, 171, and 2118, have been identified in plants (Xie et al., 2010; Zhang et al., 2007b; 2008). However, we only observed one miRNA cluster for BrMiR 169, which suggests that miRNA clusters are conserved among plant species. We believe that increased availability of *B. rapa* EST and complete genome sequences will help identify more miRNAs that are produced from either sense or antisense, or in clusters from the same genomic locus.

Computationally identified miRNAs need to be validated through expression analysis. First, we analyzed *B. rapa* ESTs derived from different tissues obtained from public databases and determined the expression of 9 miRNA families in different tissues such as floral buds, roots, callus, seedlings, primary and etiolated mature leaves, and in the entire plant (Table 3). Second we did Northern blot analyses of 5 miRNA families and qRT-PCR analyses of 12 different miRNA families which revealed both differential and tissue-specific expression in leaf, stem, shoot apex, midrib and root tissues. Northern blot analyses revealed that BrMiR 159 was highly expressed in all the analyzed tissues, BrMiR 167 in leaves and roots, and BrMiR 398 in leaves. On the other hand, BrMiR 408 was moderately expressed in leaves, but showed low expression in the other 2 tissues (Fig. 4). qRT-PCR analyses revealed that BrMir164c, BrMir 396b, BrMir 400b and BrMir 1132b showed significantly high expression in root tissues, BrMir172g and 1521b showed stem specific expression, BrMir171f showed shoot specific expression while BrMir162b showed increased expression in midrib tissues, respectively. This observation suggests that some of the identified miRNAs are expressed in tissue-specific manner, whereas some are expressed in whole plants as indicated by previous findings in other plant species (Song et al., 2009; Zhang et al., 2008). The comprehensive characterization of all the remaining identified *B. rapa* miRNAs in the different tissues would be helpful for understanding the tissue-specific expression of all the miRNAs as well as their regulatory roles with respect to different tissues, organs, and conditions.

The identification of target genes for miRNAs is an important step in understanding the regulation of miRNA by structural genes. It is well understood that miRNAs and their counterpart target genes have perfect or near-perfect complementarities; computational identification coupled with experimental results have been successful in proving this in plants (Llave et al., 2002a; Park et al., 2002; Reinhart et al., 2002; Rhoades et al., 2002; Song et al., 2009). BLAST search analysis allowing for 1-4 nt mismatches without gaps for 186 miRNA could identify a total of 66 candidate target genes in *B. rapa* for 33 miRNA families. Since *Brassica* and *Arabidopsis* are the closest relatives belonging to the same family and have nucleotide sequence identities of 80-90%, the target genes can be confirmed by aligning them with their orthologues in *Arabidopsis*. Our results show that most of the predicted targets have conserved functions with those of the miRNA targets in *Arabidopsis*. The highly conserved nature of miRNA target sequences among a wide variety of plant species has been reported by many researchers (Floyd and Bowman, 2004). In the present study, the majority of target genes were transcription factors such as SBPs, *Apetala* 2, and many other DNA and RNA binding proteins. The targets of BrMiR 156 are mainly SBP proteins, as reported by many studies in different plant species, suggesting the conserved function of this miRNA family (Zhang et al., 2006b; 2007a; 2008). The other notable transcription factor genes that were found to be miRNA targets were NAC domain-containing protein 100, scarecrow transcription factor family protein, and zinc finger protein genes in addition to above-mentioned genes. The genes of transcription factors that are involved in hormone signaling, such as ARF8 and ARF17, were also found to be targets of BrMiR 167 and 160, respectively. Several studies also report these genes as targets of miRNAs in different plant species including *A. thaliana* (Chen, 2004; Jones-Rhoades et al., 2004; Mallory et al., 2004; Song et al., 2009; Zhang et al., 2006b; 2007a; 2008). Apart from transcription factors, we observed genes involved in various functions, such as fatty acid metabolism, glycolysis, and other cellular functions, as targets of miRNAs in *B. rapa*. The targets of BrMiR 837 were pathogenesis-related protein genes such as low-molecular-weight cysteine-rich 69 (LCR69). Similar observations were made in several studies of other plants (Bonnet et al., 2004a; Rhoades et al., 2002; Zhang et al., 2006b). Our result supports the previous reports of extensive evolutionary and functional conservation of miRNAs in plant species.

This is the first comprehensive study that identifies and characterizes miRNAs in a diploid *Brassica* species, *B. rapa*. However, the number of identified miRNAs is substantially less compared to the, 199 miRNAs belonging to 121 miRNA families identified in *Arabidopsis* deposited in the miRNA database (http://www.mirbase.org). Many comparative mapping studies of *A. thaliana* show the presence of an average of 3 copies of an *Arabidopsis* chromosome segment in the *Brassica* genome (Panjabi et al., 2008; Parkin et al., 2005; Teutonica and Osborn, 1994; Truco et al., 1996), although this might not hold true for miRNA genes. Therefore, with the availability of complete genome sequences in the near future, the identification of more members of miRNA belonging to previously identified or new miRNA families is expected. Furthermore, direct cloning and sequencing of miRNAs from different tissues, organs, and developmental timing would speed up the identification of more unidentified miRNAs. The identification of 186 *B. rapa* miRNAs belonging to 48 families in the present study will aid in the study of the involvement of miRNAs in various plant development, cellular, metabolic, and other functions including biotic and abiotic stresses, which might be a part of the driving force behind expression variation, reflecting the phenotypic plasticity and numerous subspecies of *B. rapa* with diverse morphophytes.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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