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RESEARCH PAPER

Identification of conserved and novel microRNAs that are responsive to heat stress in *Brassica rapa*

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Abstract

The species *Brassica rapa* includes various vegetable crops. Production of these vegetable crops is usually impaired by heat stress. Some microRNAs (miRNAs) in *Arabidopsis* have been considered to mediate gene silencing in plant response to abiotic stress. However, it remains unknown whether or what miRNAs play a role in heat resistance of *B. rapa*. To identify genomewide conserved and novel miRNAs that are responsive to heat stress in *B. rapa*, we defined temperature thresholds of non-heading Chinese cabbage (*B. rapa* ssp. *chinensis*) and constructed small RNA libraries from the seedlings that had been exposed to high temperature (46 °C) for 1 h. By deep sequencing and data analysis, we selected a series of conserved and novel miRNAs that responded to heat stress. In total, Chinese cabbage shares at least 35 conserved miRNA families with *Arabidopsis thaliana*. Among them, five miRNA families were responsive to heat stress. Northern hybridization and real-time PCR showed that the conserved miRNAs bra-miR398a and bra-miR398b were heat-inhibitive and guided heat response of their target gene, *BracCSD1*; and bra-miR156h and bra-miR156g were heat-induced and its putative target *BracSPL2* was down-regulated. According to the criteria of miRNA and miRNA* that form a duplex, 21 novel miRNAs belonging to 19 miRNA families were predicted. Of these, four were identified to be heat-responsive by Northern blotting and/or expression analysis of the putative targets. The two novel miRNAs bra-miR1885b.3 and bra-miR5718 negatively regulated their putative target genes. 5′-Rapid amplification of cDNA ends PCR indicated that three novel miRNAs cleaved the transcripts of their target genes where their precursors may have evolved from. These results broaden our perspective on the important role of miRNA in plant responses to heat.

Key words: *Brassica rapa*, heat response, miRNA, small RNA.

Introduction

The species *Brassica rapa* includes various vegetable crops. Comparative genomic study reveals the conserved linkage arrangements and collinear chromosome segments between *B. rapa* and *Arabidopsis thaliana*, which diverged from a common ancestor approximately 13–17 million years ago (Mun *et al.*, 2009). The *B. rapa* genome contains triplicated homologous counterparts of corresponding segments of the *A. thaliana* genome due to triplication of the entire genome (whole-genome triplication). Production of these vegetable crops is usually impaired by heat stress in many regions. Worldwide, extensive agricultural losses are attributed to heat, often in combination with drought or other stresses (Mittler, 2006). Although heat-resistant molecular breeding has been possible, genetic improvement of crops is hindered by a lack of gene resources relating to heat resistance.

Abbreviations: miRNA, microRNA; RACE, rapid amplification of cDNA ends; siRNA, small interfering RNA; ta-siRNA, *trans*-acting small interfering RNA. TE, transposable element; EST, expressed sequence tag; SSC, 0.15 M NaCl/0.015 M sodium citrate; RT, reverse transcriptase; nt, nucleotide; NT, normal temperature; HT, high temperature.

Plant endogenous small non-coding RNAs are involved in the plant response to abiotic stress, and are divided into four categories: microRNAs (miRNAs), *trans*-acting small interfering RNAs (ta-siRNAs), natural antisense transcripts siRNAs, and repeat associated siRNAs (Jamalkandi and Masoudi-Nejad, 2009). Plant canonical miRNAs are ≈21-nucleotide (nt) small RNAs that are processed by the DCL1/HYL1 complex from stem-loop precursors (Kurihara *et al.*, 2006), and are loaded to the RISC complex for regulating plant development and stress response (Shukla *et al.*, 2008; Chen, 2009). Recently, a more complex mechanism of miRNA processing in plants and animals was identified. Most known miRNAs are derived from intergenic regions, while some of them are produced from introns (called mirtrons) (Babiarz *et al.*, 2008; Zhu *et al.*, 2008), exons (Li *et al.*, 2010*a*), transposable elements (TEs) (Piriyapongsa *et al.*, 2008; Devor *et al.*, 2009), and even tRNA (Miyoshi *et al.*, 2010). Meanwhile, more evidence for crosstalk between the miRNA pathways and other siRNA pathways has also been reported. For example, the secondary siRNAs are triggered by 22-nt miRNA rather than by canonical 21-nt miRNA (Axtell *et al.*, 2006; Cuperus *et al.*, 2010; Chen *et al.*, 2010). The miRNA hairpins are also processed by the DCL3/RDR2 pathway to produce 24-nt siRNA (Vazquez *et al.*, 2008; Chellappan *et al.*, 2010). miRNAs are known to function in cleaving target genes and inhibiting translation (Chen, 2004; Brodersen *et al.*, 2008; Lanet *et al.*, 2009), whereas 24-nt siRNAs from the miRNA precursors are involved in the methylation of the target genes at the transcriptional level (Chellappan *et al.*, 2010; Wu *et al.*, 2010). On the other hand, miRNA-targeted genes have been identified by high-throughput degradome sequencing in *Arabidopsis* and rice (Addo-Quaye *et al.*, 2008; German *et al.*, 2008; Li *et al.*, 2010*b*). miRNAs and their targets play a very important role in the vegetative phase and floral transition (Wang *et al.*, 2009; Wu *et al.*, 2009), hormone biosynthesis and signaling (Mallory *et al.*, 2005; Reyes and Chua, 2007), and polarity formation and morphogenesis (Sieber *et al.*, 2007; Zhou *et al.*, 2007; Liu *et al.*, 2010; Liu *et al.*, 2011). Others function in stress resistance such as drought (Li *et al.*, 2008), over-oxidation (Sunkar *et al.*, 2006), and phosphate starvation (Fujii *et al.*, 2005). One of the miRNAs associated with stress tolerance is miR398, the expression of which is transcriptionally down-regulated by oxidative stress. In *Arabidopsis*, miR398 was found to target the two closely related Cu/Zn superoxide dismutase-coding genes *CSD1* and *CSD2*, and to regulate plant tolerance to oxidative stress conditions (Sunkar *et al.*, 2006). Recently, the genome-wide analysis of miRNAs under heat stress conditions in wheat has been reported (Xin *et al.*, 2010). In general, identification of conserved and novel heat-responsive miRNAs in *B. rapa* could advance our understanding of their functions in plant heat resistance.

Heat stress disturbs cellular homeostasis and can lead to leaf etiolation, severe retardation in growth and development, and even death. The accumulation of heat-shock proteins under the control of heat stress transcription factors is assumed to play a central role in the heat stress response and in acquired thermotolerance in plants (Kotak *et al.*, 2007). Several proteins, such as DREB2A (Sakuma *et al.*, 2006), MBF1C (Suzuki *et al.*, 2005), and CTL1 (Kwon *et al.*, 2007), have been shown to improve the heat resistance of plants when over-accumulated. Some signal pathways such as ethylene, abscissic acid (Larkindale *et al.*, 2005), hydrogen peroxide, and inositol trisphosphate (Liu *et al.*, 2006) are involved in crosstalk with pathways of plant thermotolerance. How these proteins protect specific critical targets and whether small RNAs regulate these proteins are major open questions.

With the technological development of small RNA deep sequencing, many miRNAs have been discovered in various crops (Zhu *et al.*, 2008; Lelandais-Briere *et al.*, 2009; Pantaleo *et al.*, 2010). The species *B. rapa* is one of the crops that is mostly closely related to the model plant *Arabidopsis thaliana* (Snowdon, 2007). Vegetable crops belonging to *B. rapa* are very sensitive to heat stress. Losses in the yield and quality of these crops occur especially in summer and in warm regions. Recently, great progress has been made in the sequencing and annotating of *B. rapa* genomes, making it possible to conduct a genome-wide survey of miRNAs in *B. rapa*. Identification of heat-responsive miRNAs offers the opportunity to understand mechanisms of plant response to heat stress.

Materials and methods

Small RNA deep sequencing

Wu-11, an inbred line belonging to non-heading Chinese cabbage (*B. rapa.* ssp. *chinensis*), was used for constructing small RNA libraries. All plants were grown under a 16/8 h light/dark photoperiod at 22 °C for 3 weeks, and some of the samples were treated with heat shock at 46 °C for 1 h. RNA samples from aboveground parts of the seedlings were prepared using the mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) and the Alternative v1.5 Protocol (Illumina, San Diego, CA, USA), and small RNA sequencing was performed using Illumina GAII sequencer, according to the manufacturer's protocol.

Analysis of conserved miRNAs

We aligned our small RNA deep-sequencing data with the mature sequences of *Arabidopsis* miRNA families in miRBase, allowing two mismatches. We then matched all potential miRNAs with *Brassica* genomic Scaffold sequence (http://brassicadb.org) or expressed sequence tag (EST) databases from the Brassica Genome Gateway (http://brassica.bbsrc.ac.uk/) to export 600-bp flanking sequences from their genomic loci or whole EST sequences. Both of these types of sequence were used to predict secondary structures using RNAfold software. Finally, we identified whether these precursors satisfied the standard of miRNA stem-loop structure (Meyers *et al.*, 2008).

Identification of novel miRNAs in B. rapa

All of the small RNAs we obtained from small RNA deep sequencing were blasted with the *Brassica* genomic Scaffold database and *Brassica* ESTs (see above). Small RNAs that had fewer than 10 hits in the genome were retained. From these databases, 600-bp flanking sequences of small RNAs were used to predict their secondary structures using RNAfold software.

Accordingly, a judgement was made by determining whether the small RNAs and their reverse sequences were on the stem with fewer than four bulges (more than three continuous bulges also not been allowed), the standard miRNA stem-loop described in the literature (Meyers *et al.*, 2008). To investigate the origins of the proper precursors of the stem-loop, the small RNAs were aligned with the *Arabidopsis* TAIR9 cDNA database by local BLASTN. Finally, all siRNAs in our small RNA database were aligned with the precursors and the map of siRNA distribution in the precursors was displayed. The precursors that were homologous to ribosomal RNA and TE genes were mostly excluded. Novel miRNAs were selected according to the criteria of miRNA and miRNA* that form a duplex, and small RNA distribution without many smear sequences in the precursors.

Differential expression analysis of miRNAs

The total number of reads and the number of unique sequences of small RNAs derived from normal-temperature (NT) and high-temperature (HT) treatments in experiment 1 were recorded as a pair of data sets, and those from experiment 2 were recorded as another pair of data sets. The abundance of each data set was normalized to 10 million. In each experiment, the fold change of miRNA reads was calculated as the ratio of HT to NT. Since the data derived from two experiments were not enough to calculate *P* values, we chose the small RNAs up- and down-regulated by more than 1.5-fold in both experiments as the criterion for a heat response. Small RNAs with fewer than 20 reads were removed.

Target prediction of novel miRNAs

Complementary EST sequences were searched with fewer than four differences with novel miRNAs in the *Brassica* EST database. Matches were given a ranking score, with complementary base pairing assigned 1 point, G–U bonds between the miRNA and target assigned 0.5 point, a mismatch assigned −1 point, and mismatches at position 10 or 11 assigned −2 points. Matches were dismissed if they contained consecutive mismatches, more than two mismatches in the first 10 bases, or more than three mismatches in the remaining bases. Finally, the EST sequences of potential targets were aligned with the *Arabidopsis* TAIR9 cDNA database to identify ESTs which are homologous with *Arabidopsis* genes as targets.

Northern blotting

RNA (30–50 μg) was separated on 19% polyacrylamide denaturing gels. RNAs were transformed to Hybond membrane (Amersham Biosciences/GE Healthcare, Little Chalfont, Bucks., UK) for 2 h at 200 mA. After crosslinking for 3 min with UV irradiation, the Hybond membrane was hybridized with the biotin-marked DNA probes complementary to predicted miRNA sequences at 42 °C overnight, and the Hybond membrane was washed at 42 °C twice with 2×0.15 M NaCl/0.015 M sodium citrate (SSC)/0.1% SDS, followed by two higher-stringency washes of 0.1×SSC/0.1% SDS at 42 °C. Next, the membrane was incubated with stabilized streptavidin–horseradish peroxidase conjugate (Thermo, Franklin, MA, USA) in nucleic acid detection blocking buffer, and then washed five times with 1×wash buffer. Finally, after washing with substrate equilibration buffer and adding stable peroxide solution and enhancer solution, blots were imaged using an FLA-5000 PhosphoImager (Fujifilm, Tokyo, Japan). Blots were also probed with a DNA probe complementary to U6 to confirm uniform loading. The sequences of DNA probes for small RNA Northern blotting were synthesized with biotin modification by Invitrogen (Carlsbad, CA, USA) using the 3′-end DNA labelling method.

5′-RACE PCR

The 5′ Full Race PCR kit (Takara, Tokyo, Japan) was used without the alkaline phosphatase and tobacco acid pyrophosphatase steps. RNA was ligated directly to the 5′ adaptor to detect the cleavage sites of the miRNA-targeted genes. In addition, oligo-dT primers were used for reverse transcription rather than random primers. Two gene-specific primers were used for each rapid amplification of cDNA ends (RACE; Supplementary Table S1). The PCR products from a positive 5′-RACE reaction were gel purified and cloned (PMD18T vector; Takara) for sequencing.

Real-time PCR and RT-PCR

Total RNA was extracted with TRIzol reagent (Invitrogen) and treated with DNase I (Takara) to remove DNA contamination. Appoximately 4 μg of RNA was used for reverse transcription with oligo-dT primers. Real-time PCR and reverse transcriptase (RT)-PCR were performed using specific pairs of primers (Supplementary Table S1). The comparative threshold cycle (C_t) method was used to determine relative transcript levels in real-time PCR (MyiQ2, two-color Real-time PCR Detection System; Bio-Rad, Hercules, CA, USA), while the relative transcript levels were determined by running a gel following RT-PCR. *BrcACT4* of *B. rapa*, which is homologous to *Arabidopsis ACT4* and encodes actin, was used as an internal control. Three biological replicates and three technological replicates were performed.

Results

Genome-wide analysis of small RNAs responsive to heat stress

High temperature inhibits plant growth and causes leaf etiolation and even death. Severity of leaf etiolation is correlated with the level and duration of temperature treatment. To define temperature thresholds such as base temperature and thermal time requirement in changing environments during seedling growth, we incubated the seedlings of non-heading Chinese cabbage at 44, 45, 46, 47, and 48 °C for durations of 0.5, 1, and 2 h, respectively (Wang *et al.*, 2011). We found that the temperature threshold for the genotype Wu-11 was 46 °C for 1 h since the plants in this treatment stopped growing when transplanted to normal temperature, and were etiolated 12 days after heat treatment. We expected that this kind of short-term heat treatment affected biogenesis of early-responsive small RNAs but would have fewer secondary consequences in terms of morphological and physiological changes.

To examine heat-responsive small RNAs, we performed heat treatment on the 3-week-old seedlings of non-heading Chinese cabbage in two separate experiments. In the first experiment, the seedlings were exposed to 46 °C (high temperature, HT1) and 22 °C (normal temperature, NT1), respectively, for 1 h. After heat treatment, the aboveground parts of the seedlings were harvested immediately. The small RNA fraction ranging from 9 to 36 nt in size was isolated from the plant samples. The small RNA libraries constructed from HT and NT treatments were designated the HT1 and NT1 libraries. To confirm the quality of the HT1 and NT1 libraries, we performed a second experiment to construct the sister small RNA libraries (HT2 and NT2) using the same methods as in the first experiment.

The NT1 and HT1 treatments generated 14.67 million and 12.77 million small RNA reads in the first experiment; and NT2 and HT2 treatments generated 11.25 million and 14.61 million small RNA reads in the second experiment (Table 1). Because of the difference in abundance of total small RNAs between NT and HT data sets in each experiment, we normalized the abundance of each data set to 10 million [units of transcripts per 10 million (TP10M)].

Some small RNAs were seen in the HT data sets rather than in the NT data sets and hence designated as HT1- or HT2-enriched, while others were seen in the NT data sets rather than in the HT data sets and regarded as NT1- or NT2-enriched. HT-enriched small RNAs (28 and 29% for HT1 and HT2) were more abundant than NT-enriched ones (22 and 24% for NT1 and NT2), meaning that heat stress induces some small RNAs and represses others, and that more unique small RNAs are induced than repressed. Nevertheless, the majority of small RNAs were shared by the HT and NT data sets as they were seen in common for the two populations.

Small RNA sequences of Chinese cabbage exhibited a wide variation in length, from 9 to 36 nt (Fig. 1). Among them, small RNAs of 24-nt were the most abundant. Total reads of 24-nt small RNAs in each HT data set were increased compared to those in the NT data set, suggesting that 24-nt small RNAs are predominant in *B. rapa* and that their biogenesis is sensitive to heat stress. Small RNAs of 21 nt, including miRNAs, were fewer than those of 24 nt but more than numerous than any other length. Under heat stress, total reads of 21-nt small RNAs were higher than in the NT treatment, indicating that more miRNAs are produced under heat stress than at normal temperature. Surprisingly, there was a small peak at 36 nt. Nevertheless, these small RNAs were mainly derived from nuclear or chloroplast rRNA and tRNA. In general, the length distribution of unique small RNAs was consistent with that of total reads.

Table 1. Number of total reads in the small RNA databases of Chinese cabbage. NT1 and HT1 are the two small-RNA data sets derived from the NT and HT treatments in experiment 1; NT2 and HT2 are the respective libraries from experiment 2. 'Enriched' small RNAs are those detected in either the HT or NT library but not the other. 'Shared' small RNAs are present in both types of data set.

Experiment 1	Small-RNA data set	Total reads	%
NT1	Whole	14 665 848	100
	Enriched	3 174 419	22
	Shared with HT	11 491 429	78
HT1	Whole	12 767 792	100
	Enriched	3 638 641	28
	Shared with NT	9 129 151	72
Experiment 2			
NT2	Whole	11 250 443	100
	Enriched	2 653 762	24
	Shared with HT	8 596 681	76
HT2	Whole	14 607 504	100
	Enriched	4 196 326	29
	Shared with NT	10 411 178	71

Identification of conserved miRNAs that are responsive to heat stress

In *B. rapa*, 19 miRNAs belonging to 10 miRNA families have been separately reported in three previous studies (He *et al.*, 2008; Hsieh *et al.*, 2009; Kutter *et al.*, 2007). Of these, 18 were identical to miRNAs from *Arabidopsis*. All of these miRNAs, including one miRNA known only in *B. rapa*, were present in the NT and HT treatment data sets.

To select the conserved miRNAs from the entire population of our data sets, small RNAs were aligned with the known miRNAs in miRBase, allowing for one or two mismatches. Then the selected small RNAs were matched with DNA sequences of the *Brassica* scaffold database and ESTs. In total, 62 small RNAs were identical (perfect match) or similar (one or two mismatches) to the 35 known miRNA families of *Arabidopsis* and were therefore designated as the conserved miRNAs. The 158 small flanking sequences of these miRNA families were defined as miRNA precursors according to the miRNA/miRNA* standard (Table 2, Supplementary Tables S2 and S3). We aligned all of these *Brassica* miRNA precursors with the *Arabidopsis* miRNA precursors using local BLASTN. There were two or three homologous copies in the *Brassica* genome that corresponded to most of the *Arabidopsis MIRNA* genes. These findings are consistent with the report that the diploid Chinese cabbage has characteristics of whole-genome triplication compared to *Arabidopsis* (Mun *et al.*, 2009). We named the conserved *MIRNA* genes of *B. rapa* according to their homologous *Arabidopsis* genes (Supplementary Table S2). Of these 158 *MIRNA* genes of Chinese cabbage, 136 had identical and 22 had similar miRNA sequences (Table 2).

We investigated how many miRNAs among these 35 miRNA families of *B. rapa* were conserved beyond the Brassicaceae family. In miRBase, 38 miRNA families in *Arabidopsis* have homologues in species beyond the Brassicaceae family. Among these, miR413–miR420 and miR426 have been questioned (Jones-Rhoades *et al.*, 2006). None of these questioned miRNAs were found in our small RNA database or matched with genomic sequences in the *Brassica* genome database. On the contrary, miR783 is conserved between *A. thaliana* and *Pinus taeda*, but was not found in *Arabidopsis lyrata* (Fahlgren *et al.*, 2010) or *B. rapa*. Therefore there were 28 miRNA families in *B. rapa* that are highly conserved between the Brassicaceae and species beyond the Brassicaceae, among which at least 22 were conserved between dicotyledonous and monocotyledonous plants, and nine miRNA families were conserved even in moss (Supplementary Fig. S1). The other seven miRNA families including miR165 of *B. rapa* are likely to be Brassicaceae-specific.

miR173 is involved in the biogenesis of ta-siRNAs by processing the precursors of *TAS1* and *TAS2*. Surprisingly,

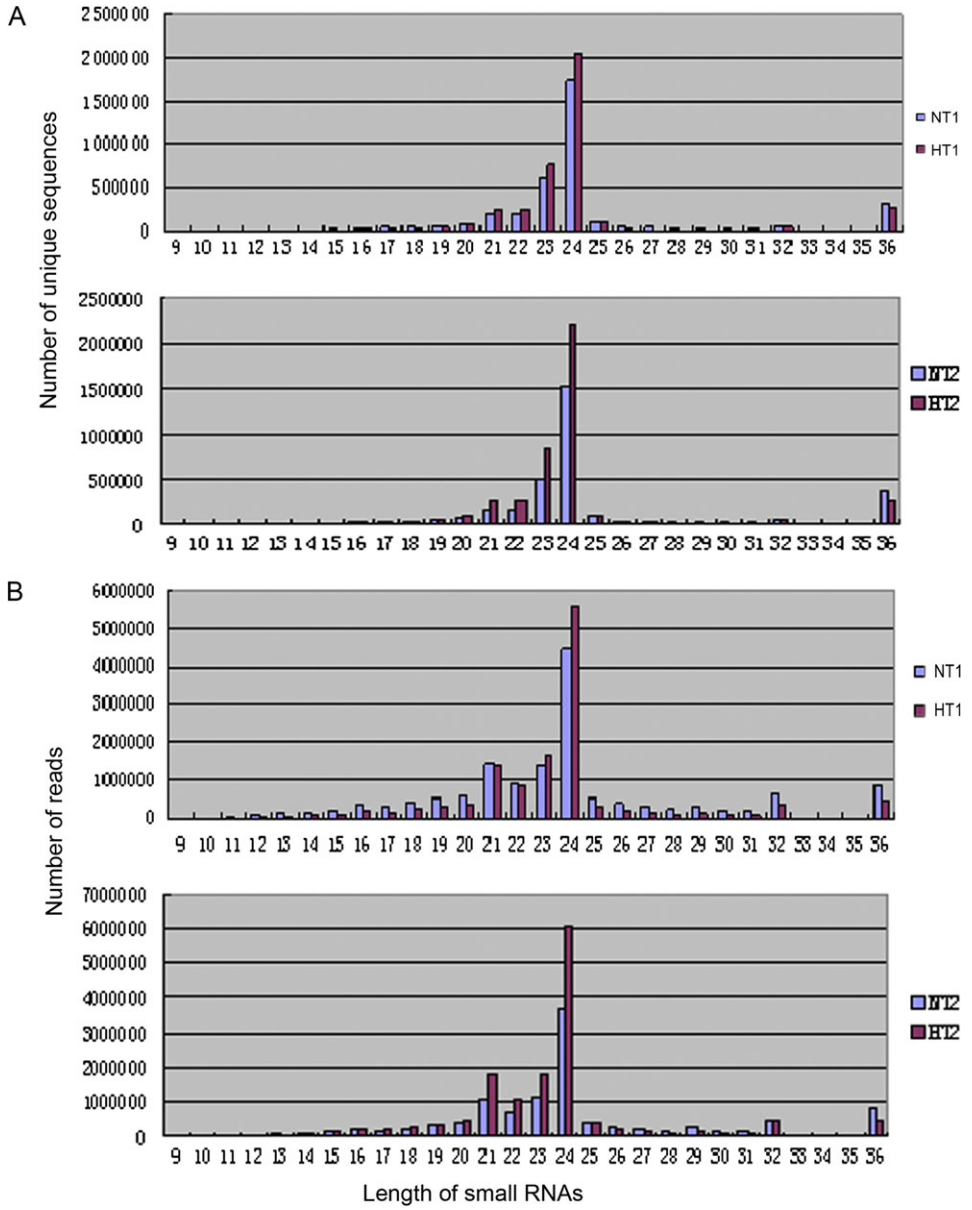

Fig. 1. Length distribution of small RNAs in two databases of non-heading Chinese cabbage after HT and NT treatments (NT1 and HT1, and NT2 and HT2, from experiments 1 and 2, respectively). (A) Length distribution of unique sequences in NT and HT small-RNA data sets. (B) Length distribution of total reads in NT and HT small-RNA data sets.

we did not find miR173 and miR173-derived ta-siRNA sequences or their homologues in all of the four small-RNA data sets. To examine whether there are any homologous genes of *MIR173* in Chinese cabbage, we searched the *Brassica* genomic Scaffold database. No genomic sequences of Chinese cabbage were homologous to the *MIR173*, *TAS1*, or *TAS2* genes in *Arabidopsis*. Probably, the genome of Chinese cabbage is lacking the *MIR173*, *TAS1*, and *TAS2* genes. In contrast, miR390 and the corresponding ta-siRNA sequences were present in our small-RNA data set and were matched to the *Brassica* scaffold database, indicating that the *MIR390* and *TAS3* genes in Chinese cabbage are functional. In addition, the bra-miR828 that targets at *TAS4* was detected in Chinese cabbage but an siRNA generated from *TAS4* was not found. We deduce that the ta-siRNAs from the *TAS1, TAS2*, and *TAS4* genes may not exist in Chinese cabbage. Nevertheless, neither miR390 nor ta-siRNAs from *TAS3* were affected by high temperature.

After annotating the conserved miRNA, we were able to examine which miRNAs are responsive to heat stress according to the differential expression analysis of miRNAs. The 35 conserved miRNA families in Chinese cabbage include 62 sequence-specific miRNAs. Among heat-responsive miRNAs, four were up-regulated and three were down-regulated with a more than 1.5-fold change (Table 3). Remarkably, bra-miR398a and bra-miR398b were down-regulated with more than 10- and 3-fold changes, respectively. bra-miR398a and bra-miR398b are derived from two homologous precursors of miR398, and have one mismatch

Table 2. Number of the miRNAs conserved between Chinese cabbage and *Arabidopsis*. Identical members (IM) represent the sequences of Chinese cabbage bra-miRNA identical to *Arabidopsis* ath-miRNA; similar members (SM) represent the sequences of bra-miRNA similar to ath-miRNA with one or two mismatches. (H) represents the miRNA families that are highly conserved between the Brassicaceae family and other species beyond the Brassicaceae family. (L) represents the Brassicaceae-specific miRNA families.

miRNA family	Number of IM	Number of SM	Total
bra-miR156 (H)	16	1	17
bra-miR157 (H)	4	0	4
bra-miR158 (L)	1	1	2
bra-miR159 (H)	4	0	4
bra-miR160 (H)	6	1	7
bra-miR161 (L)	0	1	1
bra-miR162 (H)	2	0	2
bra-miR164 (H)	6	0	6
bra-miR165 (L)	2	0	2
bra-miR166 (H)	6	0	6
bra-miR167 (H)	3	2	5
bra-miR168 (H)	3	2	5
bra-miR169 (H)	13	3	16
bra-miR171 (H)	2	3	5
bra-miR172 (H)	11	0	11
bra-miR319 (H)	6	1	7
bra-miR390 (H)	6	0	6
bra-miR391 (L)	3	0	3
bra-miR393 (H)	4	0	4
bra-miR394 (H)	5	0	5
bra-miR395 (H)	9	1	10
bra-miR396 (H)	2	0	2
bra-miR397 (H)	1	0	1
bra-miR398 (H)	4	0	4
bra-miR399 (H)	5	2	7
bra-miR400 (L)	1	0	1
bra-miR403 (H)	1	0	1
bra-miR408 (H)	1	0	1
bra-miR472 (H)	0	1	1
bra-miR824 (L)	3	0	3
bra-miR827 (H)	0	1	1
bra-miR828 (H)	2	1	3
bra-miR838 (L)	0	1	1
bra-miR845 (H)	1	0	1
bra-miR2111 (H)	3	0	3
Total	136	22	158

Table 3. Reads of the miRNAs that were down- or up-regulated by more than 1.5-fold under HT treatment. NT1 and HT1 are the two small-RNA data sets derived from the NT and HT treatments in experiment 1; NT2 and HT2 are the respective libraries from experiment 2.

miRNA	Experiment 1			Experiment 2		
	NT1	HT1	HT1/NT1	NT2	HT2	HT2/NT2
bra-miR156h	15	68	4.53	11	57	5.18
bra-miR398a	894	70	0.08	933	62	0.07
bra-miR398b	1297	336	0.26	1031	352	0.34
bra-miR399b	35	17	0.49	31	10	0.32
bra-miR827	5	2	0.4	4	2	0.5
bra-miR5714	30	103	3.43	36	104	2.89
bra-miR5716	87	27	0.31	89	17	0.19
bra-miR1885b.3	3229	140	0.04	2872	202	0.07
bra-miR5718	600	982	1.64	596	1169	1.96
bra-miR5726	7	27	3.86	4	25	6.25

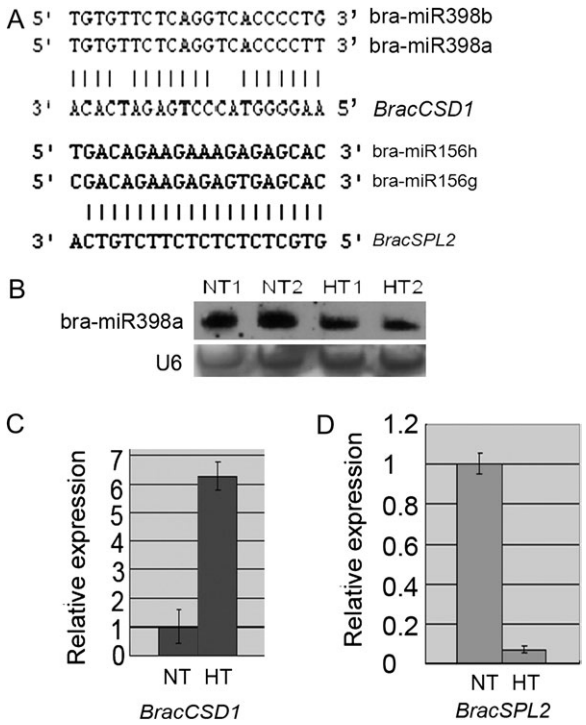

Fig. 2. Accumulation of bra-miR398a and expression of miR398 and miR156 targets under heat stress. (A) Alignment of the miRNAs with the targets: bra-miR398 with *BracCSD1* and miR156 with *BracSPL2*. (B) Northern blotting of bra-miR398a in the seedlings of Chinese cabbage, using two biological replicates of NT and HT, respectively. (C) Real-time PCR showing the expression of *BracCSD1*, with three biological replicates. (D) Real-time PCR showing the expression of *BracSPL2*, with three biological replicates.

in sequence. The homologue of bra-miR398 in *Arabidopsis* responds to oxidative stress by regulating its target *CSD1*, a Cu/Zn superoxide dismutase (Sunkar *et al.*, 2006). To confirm the heat response of bra-miR398, we performed Northern blotting of miR398 and real-time PCR of the target gene. The accumulation of both bra-miR398a and bra-miR398b in the HT seedlings was much lower than in the NT seedlings (Fig. 2A and B). On the contrary, the expression of *BracCSD1*, as a target gene of bra-miR398 (Supplementary Table S4), was much higher in the HT seedlings than in the NT seedlings (Fig. 2C). This result indicates that *BracCSD1* regulates heat response of *B. rapa* and that bra-miR398 guides the *BracCSD1* gene by silencing it. miR156h and miR156g were especially up-regulated in contrast to miR156a–f. We validated that

BracSPL2 was sharply down-regulated under heat stress (Fig. 2D), and therefore designated it as the proper target of miR156h and miR156g in heat response. miR399 is repressed and miR167 is induced by heat stress, consistent with a report in wheat (Xin *et al.*, 2010).

Analysis of heat-responsive small RNAs originating from miRNA precursors

Many small RNAs are generated from the miRNA precursors in *Arabidopsis*. Some of them, called miRNA-sibling RNAs, also play a post-transcriptional role by cleaving target genes (Zhang *et al.*, 2010). According to the distribution of small RNAs in miRNA precursors, small RNAs are classified into three types: miRNA variants overlapped with miRNA, miRNA* variants overlapped with miRNA*, and miRNA-sibling RNAs in the flanking sequences of miRNAs or miRNA*. Consistent with the heat-responsive miRNAs, some small siRNAs from the same precursor display the same pattern of heat response. For example, both the miRNA variants and miRNA* variants of bra-miR156h-2 were up-regulated under heat stress (Fig. 3A). However, the heat-responses of a few miRNA* variants is different from that of their own miRNA variants.

Accumulation of the miRNA* variants of bra-miR167a and bra-miR400 was repressed by heat stress whereas their miRNA variants were slightly up-regulated or unchanged (Fig. 3B and C). These results suggest that high temperature affects the processing or stability of miRNAs and/or their miRNA* variants in different ways.

Identification of novel miRNAs responsive to heat stress

To predict novel miRNAs in *Brassica*, we matched all of the small RNAs (20–22 bp) of Chinese cabbage with the *Brassica* scaffold database and EST. The 600-bp flanking fragments of small RNAs were used to draw their secondary structure using RNAfold. Candidate precursors were selected according to whether the small RNAs and their reverse sequences were on the stem, with fewer than four bulges, according to one of the miRNA standards (Meyers *et al.*, 2008). Then the candidate precursors were aligned with the *Arabidopsis* cDNA database using local BLASTN. Lastly, all small RNAs in our database were matched to the candidate precursors and the distribution of the small RNAs was mapped along the candidate precursors. We excluded the precursors homologous to rRNA, most TE genes, and those with many smear sequences. According to the miRNA/miRNA* criteria, 21 novel miRNAs belonging to 19 miRNA families were selected (Table 4, Supplementary Table S5). Potential targets of all of the novel miRNA were predicted according to the complementarity between miRNAs and EST sequences (Supplementary Table S6).

Among the novel miRNAs, bra-miR5714 and bra-miR5726 were up-regulated and bra-miR5716 and bra-miR1885b.3 were down-regulated by more than 2-fold under heat stress (Table 3). bra-miR1885b.3 precursors were able to produce three pairs of miRNA/miRNA* (Fig. 4A and C). Under heat stress, the accumulations of bra-miR1885b.3, bra-miR1885b.3*, and bra-miR1885b.2* were repressed severely (Fig. 4B). To confirm the heat response of these novel miRNAs, we performed Northern blotting of small RNA. The accumulation of bra-miR1885b.3 in HT seedlings was reduced sharply by heat stress (Fig 4D). In contrast, its putative target gene was up-regulated under the same condition (Fig. 4E). We also tested the expression of the putative targets of bra-miR5714 and bra-miR5726, and found that they were repressed under heat-stress, corresponding to the two miRNAs that were induced by heat stress (Fig. 4F and G).

Another potential heat-responsive novel miRNA is bra-miR5718 (Fig. 5A and B). Under heat stress, the accumulation of bra-miR5718 was increased by more than 1.5-fold. In contrast, *BracPAP10*, the putative target gene of bra-miR5718, was remarkably down-regulated (Fig. 5C). We noticed that most of the small RNAs originated from the bra-miR5718 precursor were also heat-induced. These results reveal that bra-miR1885b.3 and bra-miR5718 are two novel miRNAs that function in the *B. rapa* heat response.

Evolutionary relationship between novel miRNAs and their targets

By comparing the sequence similarity of the putative miRNA precursors against the *Arabidopsis* cDNA database by BLASTN, we found that the putative precursors of 10 novel miRNAs were homologous to certain fragments of the protein-coding genes. Among these novel miRNAs, three cleaved their targets, from which they may have evolved. The precursor of bra-miR5713 may evolve from the gene *BracDRL1* (*DEFORMED ROOTS AND LEAVES 1*) (Fig. 6A). As indicated in Fig. 6B, bra-miR5713-3p and bra-miR5713-5p are two small RNAs that are derived from the same precursor. The low-abundance bra-miR5713-3p was predicted to target *BracDRL1* while the high-abundance bra-miR5713-5p was predicted to target *BracVEL1*. To define the cleavage sites of these miRNA-targeted genes, we performed 5′-RACE experiments. As expected, the transcripts of *BracDRL1* and *BracVEL1* were cleaved in the regions complimentary with bra-miR5713-3p and bra-miR5713-5p, respectively. Similarly, bra-miR1885b.1 and bra-miR1885b.3 are two small RNAs from the same precursor. They may have evolved from the gene *BracTAO1* (Fig. 6C). 5′-RACE experiments showed that bra-miR1885b.1 rather than bra-miR1885b.3 cleaves *BracTAO1* (Fig. 6D). We suggest that miRNAs derived from the same miRNA precursors regulate different targets at the post-transcriptional level. The third miRNA that may have evolved from its target was bra-miR5718 (Fig. 5A). Our 5′-RACE PCR confirmed that the transcripts of *BracPAP10* were cleaved by bra-miR5718 (Fig. 5B).

We noticed that the major cleavage sites in the transcripts of several miRNA targets were not in the center of the binding sites. This indicated the broad variance of cleavage

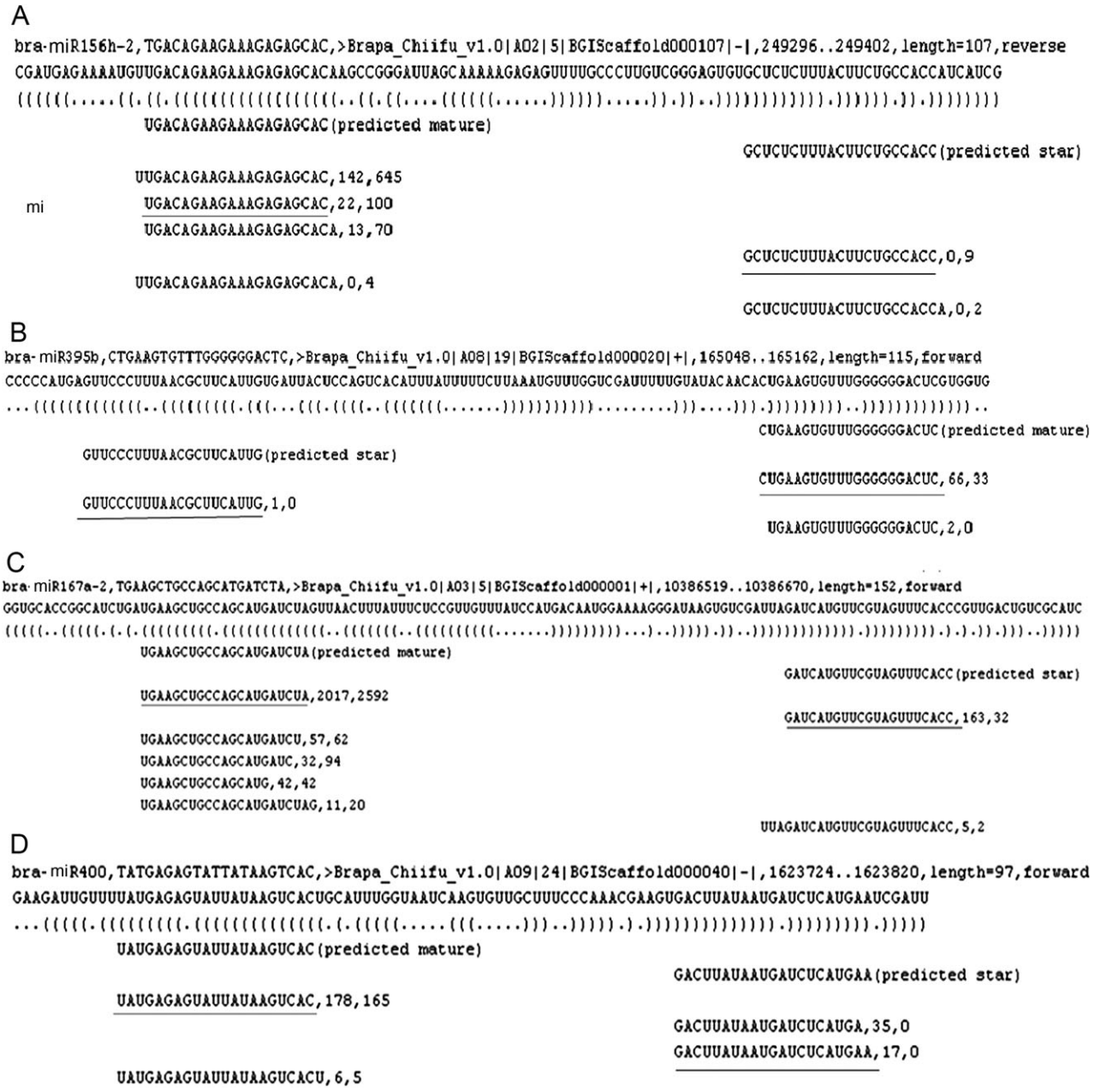

Fig. 3. Heat-responsive small RNAs originated from miRNA precursors. The mature miRNAs and miRNA* are underlined. Arabic numbers following the sequences are the normalized reads of small RNAs in the NT1 and HT1 libraries. (A–D) Position and abundances of small RNAs originated from the (A) bra-miR156h-2 precursor; (B) bra-miR395b; (C) bra-miR167a-2 precursor, and (D) bra-miR400 precursor.

sites of the targets in *B. rapa*. Addo-Quaye *et al.* (2008) pointed out that the unexpected sites adjacent to the tenth position are also considered as evidence for miRNA-mediated cleavage. In fact, the occasional positional heterogeneity was seen for the targets of some canonical miRNAs.

Some novel miRNAs without similarity to the genes are thought to have acquired their features through random mutation of the original region (Chen and Rajewsky, 2007), and a few plant miRNA genes are known to be derived from TEs (de Felippes *et al.*, 2008). We noticed that bra-miR5712 is a unique TE-like precursor (Supplementary Table S5). In Chinese cabbage seedlings both bra-miR5712 and bra-miR5712* were detected, while no bra-miR5712-sibling RNA was found, revealing that the DCL1/HYL1 complex recognizes this hairpin structure. miRNA precursors such as bra-miR5712 may have evolved from TE sequences.

Discussion

B. rapa *has diverse miRNAs*

A few miRNAs have been reported in Chinese cabbage. A genome-wide profiling of small RNAs in *B. rapa* is necessary for the genome-wide annotation of *MIRNA*

Table 4. The sequences of novel miRNAs in *B. rapa*.

Novel miRNA	Sequence	Length (nt)	Locus number
bra-miR1140	ACAGCCTAAACCAATCGGAGC	21	1
bra-miR1885.2	TACATCTTCTCCGCGGAAGCTC	22	2
bra-miR1885b.3	ATTGTGGACAAAGAAGGAAG	20	1
bra-miR5654	ATAAATCCCAAGCATCATCCA	21	2
bra-miR5711	TGTTTTGTGGGTTTCTACCGA	22	1
bra-miR5712	AATATTAATATAATTGGTGAG	21	1
bra-miR5713-3p	TTCTAAGCCTACCTAGCTCCGG	22	1
bra-miR5713-5p	AGGCTTAGAAGAACGTTTGTT	21	1
bra-miR5714	AGACTCTACGACATCAAGAAAC	22	1
bra-miR5715	ACGTGATAAGCCTCTGAAGAA	21	1
bra-miR5716	TTGGATAATTGAAGATATAAA	21	1
bra-miR5717	GTTTGGATTGTTTGCCTTGGC	21	1
bra-miR5718	TCAGAACCAAACACAGAACAAG	22	1
bra-miR5719	TTGTGATGATAATACGACTTC	21	1
bra-miR5720	TTGTGATTTGGTTGGAATATC	21	1
bra-miR5721	AAAAATGGAGTGAGAAATGGA	21	1
bra-miR5722	TGAAATAGAGTCATGTGGAACG	22	1
bra-miR5723	AATGTGCTGCAATATCTCTGC	21	1
bra-miR5724	AACCGCCGGTTTGATAATAGC	21	1
bra-miR5725	ATTTGGCACAATCTGATCTGC	21	1
bra-miR5726	CAAAGGTTGCTTGAATAAGGT	21	1

genes. By comparing the known miRNAs and the miRNA precursors, we identified 35 miRNA families conserved between Chinese cabbage and *Arabidopsis*. The conserved miRNAs in Chinese cabbage are identical or similar (with one or two mismatches) to their counterparts in *Arabidopsis*. *Brassica* species are closely related to the model plant *A. thaliana*. The gene order within their genomes has remained extensively collinear and remarkably conserved over a long evolutionary period. Although Chinese cabbage is diploid, its genome shows characteristics of triplication (Town *et al.*, 2006; Mun *et al.*, 2009). Many *MIRNA* genes in *B. rapa* show far more copies than in *Arabidopsis*. In *B. rapa*, mature miRNA sequences are more diverse than those of *Arabidopsis*. For example, the precursor of bra-miR156d-2 shares the most homology with ath-miR156d among all members of the miR156 family, but the mature miRNA contains one nucleotide substitution compared with ath-miR156d (Supplementary Table S2). In total, 22 miRNAs from the conserved miRNA families in *Brassica* show one or two nucleotide substitutions compared with those of *Arabidopsis* (Table 2).

Recently, many types of miRNAs have been identified in the genome of *A. lyrata*, in which 13% are species-specific, because they are not found in its close relative *A. thaliana* (Zhang *et al.*, 2010). Only one *Brassica*-specific miRNA has been reported (He *et al.*, 2008). Using the almost complete genomic sequences of *B. rapa*, we selected 19 novel miRNA families according to the strict miRNA/miRNA* criterion. Interestingly, some novel miRNA precursors have evolved from their target genes. For example, bra-miR5713, bra-miR1885, and bra-miR5718 are evolved from their targets *BracDRL1*, *BracTAO1*, and *BracPAP10*, respectively. Kutter *et al.* (2007) reported that miR824 was duplicated from its target *AGL16*. We suggest that co-evolution between miRNA genes and their target genes is partly responsible for the diversity of gene silencing.

miRNA is involved in gene-regulation pathways of the plant heat response

A miRNA directs cleavage of a highly complementary target mRNA. Upon heat stress, any change in the type or amount of miRNAs in plant tissues may alter the expression levels of target genes. In this study, the conserved miRNA miR398a is heat-inhibitive while *BracCSD1* gene is heat-sensitive, showing an inverse relationship between miRNA and target. Recently, some components in thermotolerance are found to have the functions in protection from oxidative damage. For example, genetic engineering of the biosynthesis of glycinebetaine enhances thermotolerance of photosystem II in tobacco plants (Yang *et al.*, 2007). Chloroplast NAD(P)H dehydrogenase in tobacco leaves functions in alleviation of oxidative damage caused by temperature stress (Wang *et al.*, 2006). In this study, we found that bra-miR398 and its target genes *BracCSD1* and *BracCSD2* showed an inverse response to heat stress in *B. rapa*. *CSD1* and *CSD2* genes that are the targets of miR398 regulate the plant tolerance to oxidative stress conditions (Sunkar *et al.*, 2006). We wonder whether bra-miR398-mediated *BracCSD1* and *BracCSD2* genes play roles in thermotolerance through protection from oxidative damage.

The miR399 is responsive to phosphate starvation (Fuji *et al.*, 2005). Both of their targets encode putative ubiquitin-conjugating enzyme (UBCs). Under heat stress plant would consume more nitrogen and phosphate; repressing these two miRNAs would accumulate their target genes to adapt the nutrient stress. The miR156s were found to be up-regulated under heat stress in wheat recently (Xin

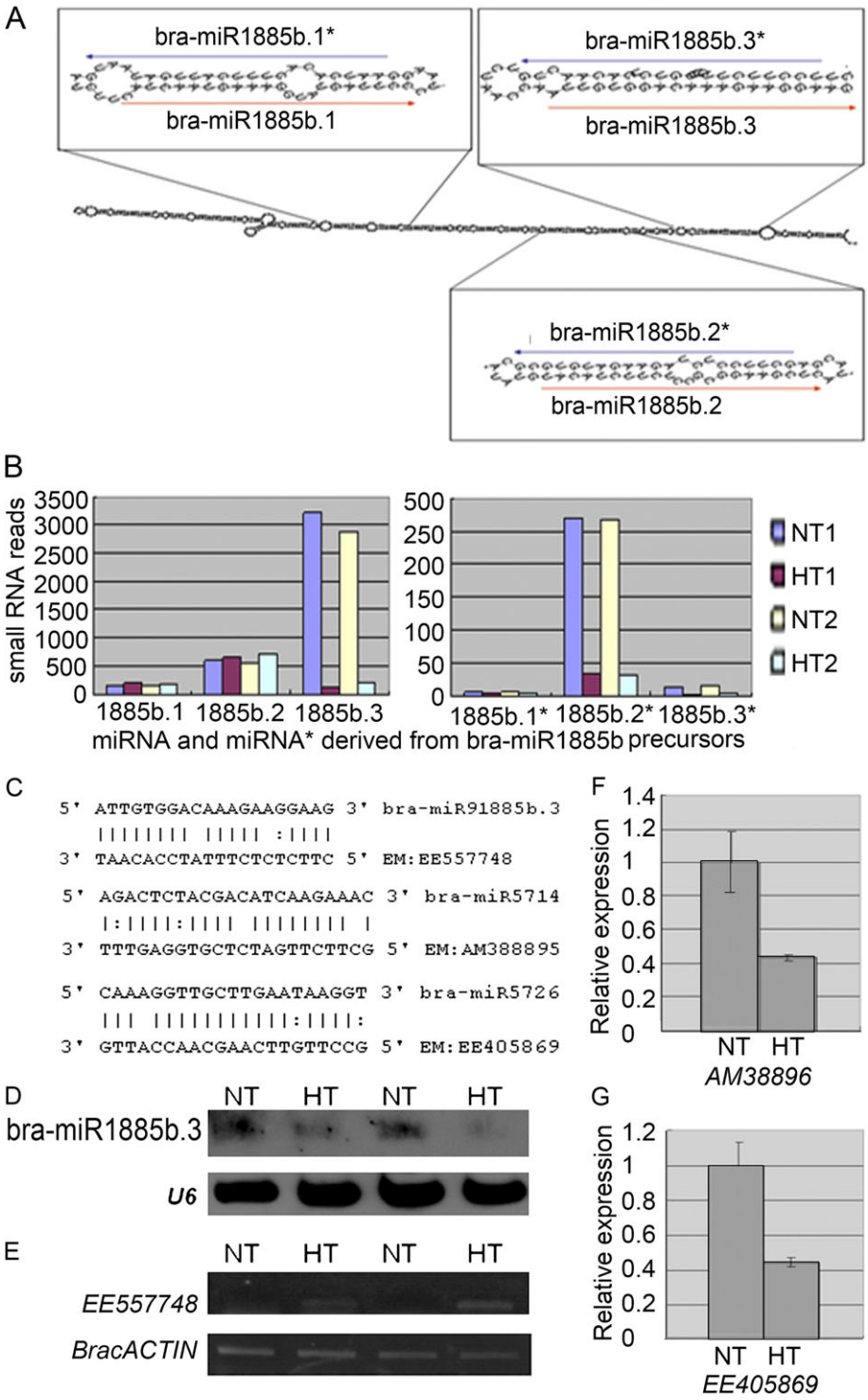

Fig. 4. The inverse expression pattern between bra-miR1885b and the putative target genes under heat stress. (A) Three pairs of miRNA/miRNA* in the loop hairpin of the bra-miR1885b precursor. (B) Abundance of the miRNAs and miRNA* derived from the bra-miR1885b precursor under heat stress in the four small-RNA data sets. (C) Alignment of three novel miRNAs with the binding sites of their predicted targets. (D) Northern blotting of miR1885b.3 under heat stress. (E) RT-PCR showing relative expression of the putative target genes under heat stress. (F) Real-time PCR showing relative expression of miR5714-targeted AM38895 (*AM38896*). (G) Real-time PCR showing relative expression of miR5726-targeted *EE405869*.

et al., 2010), and we found that miR156h and miR156g were specifically induced among the miR156 families. The targets of miR156 are the SPL transcription factor families, which take part in the vegetative phase and floral transition (Wang *et al.*, 2009; Wu *et al.*, 2009). Among the miR156 targets, *SPL2* were specifically down-regulated under heat stress. The negative correlation between miR156h/miR156g and *SPL2* may suggest that miR156h and miR156g may

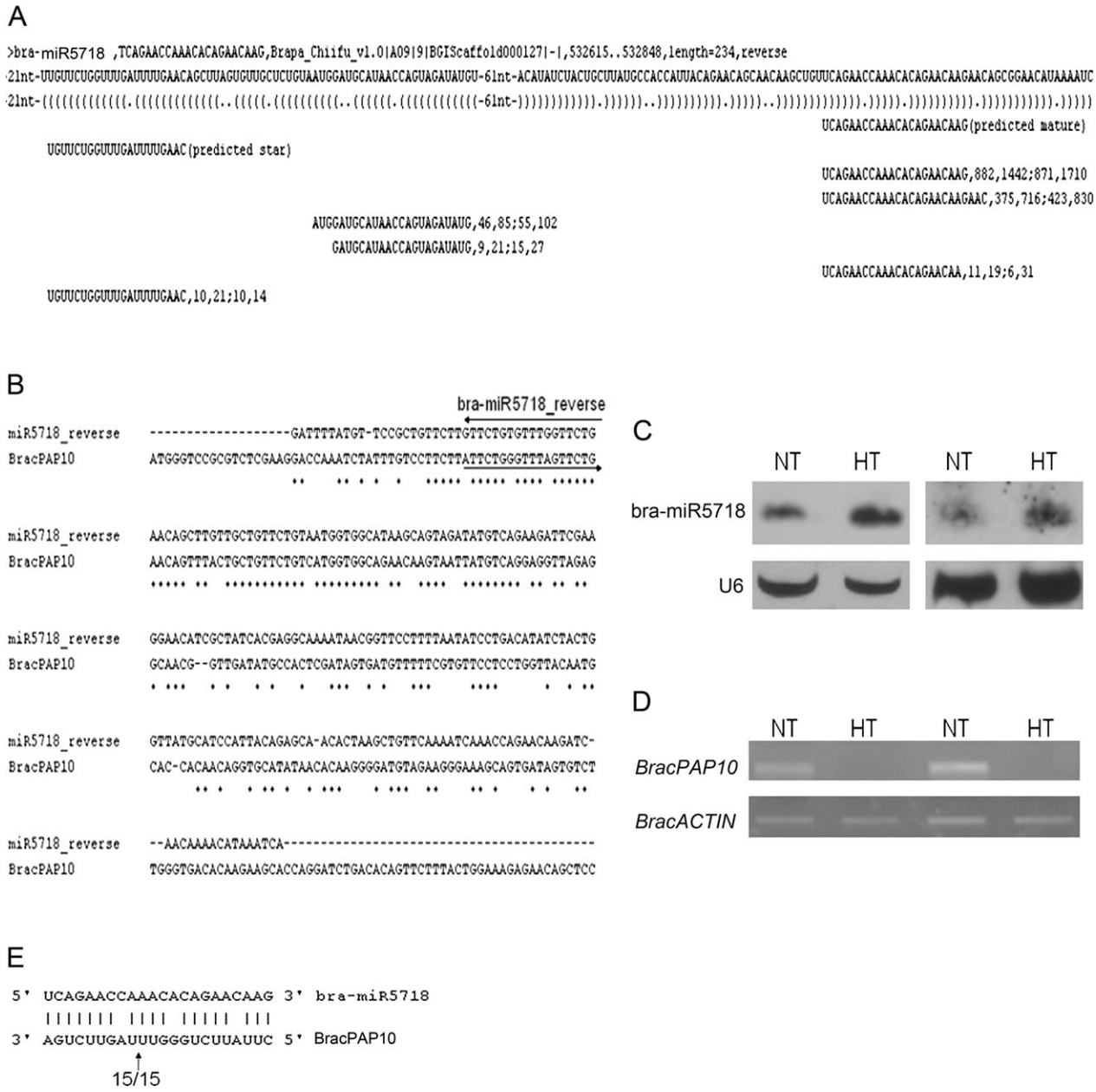

Fig. 5. Evolutionary relationship and heat-response of bra-MIR5718 and its target *BracPAP10*. (A) Heat-responsive small RNAs originated from bra-miR5718 precursors. Arabic numbers after the sequences are the normalized reads of small RNAs in the NT1, HT1, NT2, and HT2 data sets, respectively. (B) Alignment of bra-miR5718 precursors with *BracPAP10*. The reverse sequence and direction of bra-miR5718 are indicated with a reverse arrow, while the DNA sequence of *BracPAP10* is underlined with a forward arrow. (C) Northern blotting bra-miR5718 under heat stress. (D) RT-PCR showing the expression of the target *BracPAP10* under heat stress. (E) The position of dominant 5′-RACE products of *BracPAP10* mRNA is indicated by a vertical arrow in the expanded region.

target *SPL2* effectively rather than *SPL3, SPL9,* and *SPL10*, which are regulated by miR156a. However, the downstream targets of *SPL2* are still unknown.

The novel miRNAs bra-miR1885b.3 and bra-miR5718 are responsive to heat stress, in contrast to their targets. To identify function of bra-miR398, bra-miR1885b.3, and bra-miR5718 in terms of thermotolerance, an attempt has been made in our laboratory to construct transgenic plants overexpressing these miRNAs and their target genes. Identification of the conserved and novel miRNAs involved in the heat response of *B. rapa* will facilitate our understanding of the molecular mechanisms governing the plant heat response and thermotolerance.

Supplementary material

Supplementary material is available at *JXB* online.

Supplementary Fig. S1. Number of miRNAs belonging to 33 families conserved among 14 plant species the whole-genome sequences of which are available in miRBase.

Supplementary Table S1. Primers used in 5′-RACE PCR and real-time PCR.

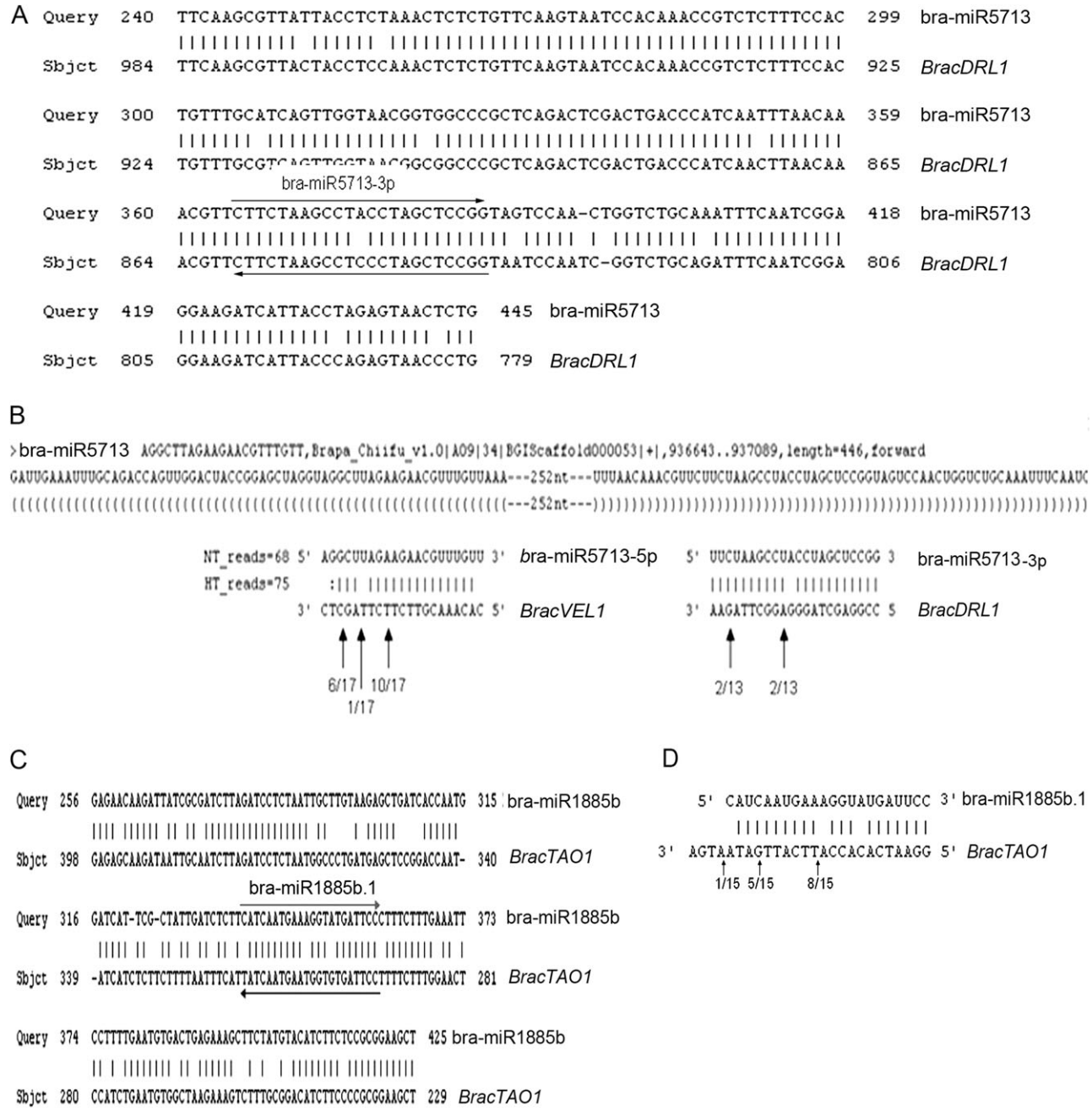

Fig. 6. Evolutionary relationship between two novel miRNAs and their validated targets. (A) Alignment of bra-miR5713 precursors with *BracDRL1*; the DNA sequence and direction of bra-miR5713-3p are indicated with a forward arrow, while the reverse complementary DNA sequence of *BracDRL1* is underlined with a reverse arrow. (B) Positions of dominant 5′-RACE products of *BraVEL1* mRNA and *BraDRL1* mRNA are indicated by vertical arrows in the expanded regions. (C) Alignment of bra-miR1885b precursor with *BracTAO1*. The forward arrow indicates bra-miR1885b.1 while the reverse arrow shows the reverse complementary DNA sequence of *BracTAO1*. (D) Positions of dominant 5′-RACE products of *BracTAO1* mRNA are indicated by vertical arrows in the expanded regions.

Supplementary Table S2. Information about conserved miRNA families in *B.rapa*. Name: the conserved miRNA families in *B.rapa* were named according to their homologues in *Arabidopsis*, and the last number represents the serial number of homologous copies. Mismatch: the number of mismatches between bra-miRNA with the first ath-miRNA gene in one miRNA family was calculated.

Supplementary Table S3. Precursors of the conserved miRNA. Most sequences of miRNA precursors were deduced from the *Brassica* genomic Scaffold database, except for the precursor of bra-miR408, which was deduced from the EST database.

Supplementary Table S4. Putative targets of the miRNAs in *B. rapa*.

Supplementary Table S5. Precursors of novel miRNAs.

Supplementary Table S6. The binding sites of miRNA to the predicted targets (EST). Complementary score were showed, and the homologous genes in *Arabidopsis* were also revealed.

Acknowledgements

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