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Characterization of conserved and novel microRNAs and their targets, including a TuMV-induced TIR–NBS–LRR class *R* gene-derived novel miRNA in Brassica

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Abstract **Nine conserved miRNA families and three potential novel miRNAs in *Brassica rapa* were identified from a small RNA library. The expression patterns of some conserved miRNAs had different tissue specificity in Brassica and Arabidopsis. One of the three potential miRNAs, named bra-miR1885, was verified as a true functional miRNA. It could be induced specifically by *Turnip mosaic virus* (TuMV) infection, and target TIR–NBS–LRR class disease-resistant transcripts for cleavage. Based on the hypothesis for de novo generation of new miRNA genes and the sequence similarity between *bra-MIR1885* precursor loci and target transcript sequences, we suggest that bra-*MIR1885* is a new miRNA gene that originated through inverted duplication events from TIR–NBS–LRR class disease-resistant protein-coding gene sequences, which became bra-miR1885 targets.**

Keywords: miRNA; TIR–NBS–LRR resistance gene

1. Introduction

MicroRNAs (miRNAs), a conserved class of small RNAs approximately 21–24 nucleotides (nt) in length, are processed as duplexes (miRNA:miRNA*) from primary miRNA transcripts (pri-miRNA) containing imperfect intramolecular stem loops in the nucleus. The mature guide-stranded miRNAs mediate sequence-specific, post-transcriptional repression of mRNA targets through the miRNA-induced silencing complex (miRISC) [1]. The miRNA*s (passenger strand) are normally labile and degraded [1].

Plant miRNAs typically direct cleavage of their targets [2–4] and have functions in a range of developmental processes, including proper embryonic, vegetative, and floral development [1,5,6] as well as non-developmental processes, such as stress-responses [7–9]. One miRNA can potentially target several members of a gene family [3], and the precise physiological functions of miRNAs are determined by their temporal and spatial patterns of expression. Only a small number of miRNAs in Arabidopsis are expressed ubiquitously, whereas many miRNAs are differentially expressed at different developmental stages and in different tissues and cell types [1,2].

About 320 plant miRNA families have been identified so far, based on the Browse miRBase database (http://microrna.sanger.ac.uk/cgi-bin/sequences/browse.pl), and more than half of these are found in the fully sequenced genomes of Arabidopsis, Populus, and rice. Many plant miRNAs are well conserved in multiple species [1,10–13], indicating that the miRNA-directed gene regulation process is very ancient. Recently, by deep sequencing Arabidopsis miRNAs, many newly emergent and diverse miRNAs have been discovered [11,14]. Analyses examining the sequence similarities of miRNA precursor foldback arms have revealed evidence for some recent evolutionary *MIRNA* loci through inverted duplication events from protein-coding gene sequences. These sequences could potentially become new miRNA targets [14]. Many newly emergent non-conserved miRNAs are expressed in specialized tissues or are present at relatively low abundances in plants [11,14].

Brassica is one of the most economically important crops in China, Europe and other Asian countries. *B. rapa* subsp. *oleifera* is widely cultivated in Asia as a source of seed oil. The haploid genome equivalent of *B. rapa* is closely related to *Arabidopsis*. Comparative genetic mapping has revealed collinear chromosome segments [15] in the *Brassicacea* family and conserved linkage arrangements between *Arabidopsis* and *Brassica*. In this study, we have cloned a small RNA library of *B. rapa* and identified nine conserved miRNA families and three candidates for novel miRNAs. Analyses of the expression patterns of conserved miRNAs in *Brassica* and *Arabidopsis* revealed that the tissue specificity of some miRNAs differed between two species. Informatics search and cleavage site mapping by 5′RACE revealed potential target transcripts for one of the novel miRNA candidates (bra-2010), which are members of the "TIR–NBS–LRR" family of disease resistance proteins. Our data also suggested that the novel miRNA precursor was probably a transitional loci derived from the "TIR–NBS–LRR" *R* genes through the recently proposed "inverted duplication" mechanism. Transient assays further confirmed that bra-2010 was a true functional young miRNA, named bra-miR1885, which could be greatly induced by infection with the *Turnip mosaic virus* (TuMV) but not with the *Cucumber mosaic virus* (CMV), the *Tobacco mosaic virus* (TMV), or a necrotrophic fungal pathogen called *Sclerotinia sclerotinorium*. The increased accumulation of the bra-miR1885 caused by TuMV infection through an unknown mechanism, or simply as a result of HC-Pro silencing suppressor activity, is discussed.

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2. Materials and methods

2.1. Plant materials, growth condition and pathogenic infection

Seeds of the *Arabidopsis thaliana* ecotype Columbia (Col) were surface sterilized and plated on a Murashige and Skoog (MS) medium. After germination, seedlings were grown in a greenhouse. Leaves, stems, flowers and siliques were collected for RNA extraction after growing for 40 days in a greenhouse. Seeds of *Brassica napus* cv. Westar and *B. rapa* subsp. *oleifera* were grown in soil in a greenhouse at 22 degree Celsius (°C). Leaves, flowers and siliques of *B. napus* were collected 90 days after germination for RNA extraction.

Twenty-one-day-old *A. thaliana* and 14-day-old *B. rapa* were mock-inoculated with 5 mM phosphate buffer (pH 7.2) or a fresh sap prepared from TuMV-infected *Brassica* leaves or CMV-infected or TMV-infected *Nicotiana tobacco* leaves (1 g of ground leaf material diluted into 2 ml of phosphate buffer). Plant materials were collected for RNA extraction 21 days after inoculation. Non-infected wild-type plant materials were also collected for control purposes.

The open end of a 1000-μl pipette tip was pushed into the margin of a 3-day-old colony of *S. sclerotiorum* to acquire an 8-mm-thick plug of PDA and mycelium. Petioles of the third fully expanded leaf of 21-day-old *B. napus* and *B. rapa* were severed 2.5 cm from the main stem. The tapered end of the petiole was forced through the agar plug of PDA and mycelium within the pipette tip. Plant materials were harvested at 4 days post-inoculation for RNA extraction.

2.2. Small RNA cloning and sequencing

Small RNA were extracted from *B. rapa* 21 days after germination and separated by electrophoresis in 17% denaturing polyacrylamide gel (PAGE). RNA fractions 18–26 nt in size, which corresponded to the small RNA population, were purified and cloned as described [16]. The PCR product was directly ligated into a pCR2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen). Colonies were screened using PCR. Candidate single colonies were submitted for custom sequencing (Sequence Company Sunbio).

2.3. Mapping of miRNA-guided cleavage site and amplification of upstream sequences of mapped target sequences

The FirstChoice RLM-RACE kit (Ambion) was used for RLM-RACE assays according to the manufacturer's instructions. For analysis of miRNA-guided cleavage sites, the RNA Oligo adaptor was directly ligated to total RNA without calf intestinal phosphatase treatment. For the first round of nested PCR, the 5′RACE Outer Primer together with gene-specific outer primers, as described below, were used

(Brn-NAC1 Po 5′-CAATGTCGGTCAAGAGGCTCT-3′,
Brn-TOE1 Po 5′-CCTCCTCTGTAAGTTAAGGTTGT-3′,
Brn-TOE2 Po 5′-GCAAGTACGCTTATGGTGGTGGTT-3′
Brn-AC189193 Po 5′-CCTCCATGCCCTCTTCACCTCTT-3′ and
Brn-2010 target upstream Po 5′-CCTCCATGCCCTCTTCACCTCTT-3′).

The 5′RACE Inner Primer and gene-specific inner primers
(Brn-NAC1 Pi 5′-GCCTCCATCGATCTTAGTGAGCT-3′,
Brn-TOE1 Pi 5′-CTCTGTAAGTTAAGGTTGTGGA-3′,
Brn-TOE2 Pi 5′-GAAGCGTAATCAGATGGGTGGAT-3′
Brn-AC189193 Pi 5′-TTCCGAAATCTCCGGTCTGCTT-3′ and
Brn-2010 target upstream Pi 5′-TTCCGAAATCTCCGGTCTGCTT-3′). Gel-purified PCR products were then cloned into pGM-T vector (TIANGEN) for sequencing.

2.4. Plasmid construction

A 377 bp sequence of mapped target sequence (target2), including the bra-2010 cleavage site, was used to construct GFP-sensor. A 309 bp sequence from AC189642 containing bra-2010, bra-158a/b and bra-178 was used as a precursor sequence (Fig. 4A). These two sequences were amplified from *B. napus* through a one-step RT-PCR using two pairs of primers, respectively:

target2 5P (5′-TCTAGACATTTTCCTCGTGATGTTATCCAT-3′) and
target2 3P (5′-TCTAGATCCGGTCTGCTTCCTCACAT-3′).
Pre-2010 5P (5′-TCTAGAGTCTTCACGAGCTTCCTCTA-3′) and
Pre-2010 3P (5′-ACTAGTTTGTTTTCATGAGCTTCCGCGGAGA-3′). The sequences were then cloned into pGM-T Vector (TIANGEN), producing pT-target2 and pT-Pre-2010, which were verified by sequencing. The GFP sequence was amplified from pSK-mGFP by PCR using the following primers:

GFP 5P(5′-TCTAGAATGAGTAAAGGAGAAGAACTTT-3′) and
GFP 3P(5′-TTATTTGTATAGTTCATCCATGCCA-3′). This amplified sequence was cloned into the pGM-T vector, producing pT-GFP. An XbaI–GFP-SacI fragment of pT-GFP was inserted into XbaI/SacI-digested pCAMBIA1300-221 in which the GUS sequence was between XbaI and SacI in pCAMBIA1300-221, producing pCAMBIA1300-221-GFP. For constructing GFP-sensor, an XbaI fragment from pT-target2 was cloned into XbaI-digested pCAMBIA1300-221-GFP, giving pCAMBIA-1300-221-target2-GFP (named GFP-sensor).

For constructing 35S-Pre-2010, an XbaI–SpeI fragment from pT-Pre-2010 was cloned into XbaI–SpeI-digested pCAMBIA1300-221-GFP.

2.5. Agrobacterium tumefaciens infiltration in Nicotiana benthamiana

The constructs GFP-sensor, 35S-Pre-2010 and pCAMBIA1300 (vector control) were transformed into an *A. tumefaciens* strain EHA105 by electroporation and selected on a Luria-Bertani medium containing rifampicin at 10 μg/mL and kanamycin at 50 μg/mL. Agrobacterial cells were infiltrated into leaves of *N. benthamiana*. For coinfiltration experiments, equal volumes of an Agrobacterium culture containing 35S-Pre-2010 or pCAMBIA1300 (OD600 = 3) with GFP-sensor (OD600 = 1) were mixed before infiltration into *N. benthamiana* leaves.

2.6. Analysis of nucleic acids

Total RNA was extracted from leaves, flowers and siliques of *B. napus* and *Arabidopsis* using hot-phenol extraction. High molecular weight RNA was selectively precipitated from the total RNA by the addition of one volume of 4 M LiCl. The low molecular weight RNA was precipitated with three volumes of ethanol and dissolved in 50% deionized formamide. The resulting low-molecular-weight-enriched RNA was separated by electrophoresis on denaturing 17% polyacrylamide gels and electrically transferred to Hybond-N+ membranes. Blots were hybridized with oligonucleotide and LNA-modified oligonucleotide probes that were end-labeled with γ-^{32}P-ATP using T4 kinase (NEB). Hybridization signal intensity was measured using a PhosphorImager (GE Healthcare).

3. Results and discussion

3.1. Cloning and identification of miRNAs in B. rapa

Small RNAs were purified from four-week-old seedlings of *B. rapa* (subsp. *oleifera*) and were then cloned and sequenced. Those with recognizable flanking adaptor sequences and with lengths between 18 and 26 nt were compared with the *Brassica* genome. Of the 400 small RNAs, 237 matched the *Brassica* genome sequence databases, including expressed sequence tags (EST), genomic survey sequence (GSS) and CoreNucleotide. The 21–24 nt RNAs represent the predominant species with more than 77.6%, among which the 21 nt small RNA is the most abundant (Fig. 1).

Sixty-five percent (154/237) of the cloned small RNAs have sequences that perfectly or near-perfectly match the *Arabidopsis* genome by BLASTN search in the www.arabidopsis.org transcripts database. Nineteen cloned *B. rapa* small RNAs matched the conserved, previously identified plant miRNAs perfectly, with another two showing, respectively, one and two nucleotide mismatches with *Arabidopsis* miRNAs. The miRNAs belong to seven conserved families, namely, bra-miR157, bra-miR159, bra-miR164, bra-miR167, bra-miR171, bra-miR172, and bra-miR390. Three miRNA*s: bra-miR160*, bra-miR172* and bra-miR398* were also cloned (Table 1). All cloned *B. rapa* miRNA sequences were 21 nt in length, and the first nucleotide in the majority of the bra-miRNAs was uridine, which was consistent with the characteristics of miRNAs [1,16].

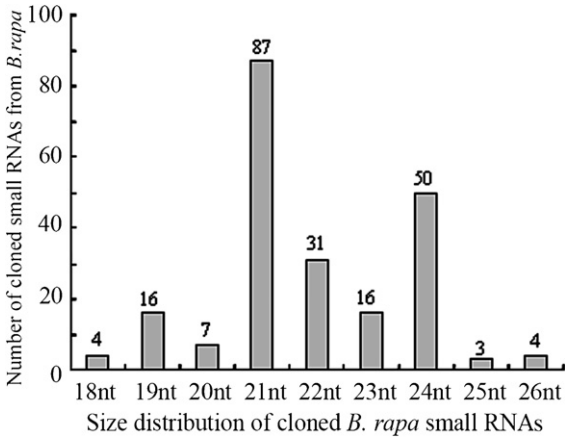

Fig. 1. Size distribution of sequenced small RNAs from *Brassica rapa*.

Thirty-five percent (83/237) of *B. rapa* miRNA sequences did not match the *Arabidopsis* genome sequence. Given the high levels of sequence homology between *Brassica* and *Arabidopsis* in the transcribed regions, the unmatched small RNAs are likely to be specific to *Brassica*. To search new *Brassica* miRNAs and to further confirm the existence of miRNA pairs among our cloned bra-miRNAs, we performed a prediction of the miRNA precursor hairpin structure as described below.

3.2. Hairpin structure prediction of conserved miRNAs in B. rapa

miRNAs originate from longer, non-protein-coding RNAs called precursors that have the ability to form hairpins, and the miRNA sequence is within one of its arms. First, the precursor structures for all cloned bra-miRNAs and bra-miRNA*s were tested based on the available genomic sequence of *Brassica*. With the exception of bra-miR390, whose sequence was not available in the *Brassica* database, hairpin structures could be predicted for the surrounding sequences of all other cloned bra-miRNAs and bra-miRNA*s, using MFOLD (http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi). Thirteen hairpin structures were predicted from 18 loci of *B. rapa* and *B. oleracea* (Fig. 2 and Supplementary data 1).

Both bra-miR172 and bra-miR172* were found in one precursor sequence (Fig. 2), a finding that further supports the existence of miRNA pairs. Both precursor sequences of bra-miR160* and bra-miR398* contain the plant conserved sequences of miR160 and miR398 [1–3,7], implying that bra-miR160 and bra-miR398 might be produced in *Brassica*. The Bra-miR160* sequence is homologous to ath-miR160a*, while there is no homology between bra-miR398* and ath-miR398a/b/c*.

3.3. Expression and target analysis of B. rapa conserved miRNAs

To examine the relative abundance and tissue specificity of cloned *Brassica* miRNAs, we analyzed the expression patterns of bra-miRNAs using northern hybridization in leaves, flowers and siliques of *B. napus*. Different tissues of *A. thaliana Columbia* were included as a control. As shown in Fig. 3A, miR157, miR159 and miR160 expressed ubiquitously with no dramatic differences in leaves, flowers or siliques in *Brassica*, which was the same result as that from the miR159 found in *Arabidopsis*. There was a higher accumulation of miR167 in *Brassica* leaves and a lower accumulation of this miRNA in both flowers and siliques, while a high amount of accumulation was observed in both leaf and sillique of *Arabidopsis*. The different accumulation patterns suggested that the patterns in the spatial and temporal expression of the conserved miRNAs in *Brassica* and *Arabidopsis* were different. The different tissue specificity was further supported by analyses of the accumulation of other miRNAs. Both miR164 and miR171 were highly accumulated in flowers of *Brassica*, whereas, in *Arabidopsis*, the two miRNAs were more highly accumulated in the siliques than in other tissues. miR172 was found to be expressed exclusively in the *Brassica* flower, and it was expressed ubiquitously at a higher level in the flowers and stems of *Arabidopsis*. Bra-miR390b, which has a 2 nt mismatch with ath-miR390, was highly accumulated in *Brassica* flowers. The stress-related ath-miR398 [17], which was not present in our cloned population, was detected at a low level in *Arabidopsis* leaves and siliques, but not in any *Brassica* tissues. The difference in tissue specificity for conserved miRNAs between *Brassica* and *Arabidopsis* suggests that even the older miRNAs might possess

Table 1
Brassica rapa miRNAs identified by cloning and verified by RNA blot

miRNA	Sequences (5′–3′)	Size nt	Homologue in Arabidopsis	Consensus with the homologue in Arabidopsis	RNA blot
bra-miR157a	TTGACAGAAGATAGAGAGCAC	21	ath-miR157a/b	Match	Yes
bra-miR159a	TTTGGATTGAAGGGAGCTCTA	21	ath-miR159a	Match	Yes
bra-miR160a[i]	TGCCTGGCTCCCTGTATGCCA	21	ath-miR160a/b/c	Match	Yes
bra-miR160a*	GCGTATGAGGAGCCATGCATA	21	ath-miR160a*	Match	Yes
bra-miR164a	TGGAGAAGCAGGGCACGTGCA	21	ath-miR164a/b	Match	Yes
bra-miR167b	TGAAGCTGCCAGCATGATCTA	21	ath-miR167a/b	Match	Yes
bra-miR171a	TTGAGCCGTGCCAATATCACG	21	ath-miR171b/c	Match	–
bra-miR171b	TGATTGAGCCGCGTCAATATC	21	ath-miR171a ath-miR170	1 nt mismatch 1 nt mismatch	–
bra-miR171c	TGATTGAGCCGCGCCAATATC	21	ath-miR171a	Match	Yes
bra-miR172a	AGAATCTTGATGATGCTGCAT	21	ath-miR172a	Match	Yes
bra-miR172b*	GCAGCACCATTAAGATTCACA	21	ath-miR172b*	Match	Yes
bra-miR390b	AGCTACAGGAGGGATAGCGCC	21	ath-miR390a	2 nt mismatch	Yes
bra-miR398a[i]	TGTGTTCTCAGGTCTCCCCTTG	21	ath-miR398a	1 nt mismatch	Yes
bra-miR398a*	GAGTGTCATGAGAACACGGA	21	No	–	Yes
bra-miR1885 (bra-2010)	CATCAATGAAAGGTATGATTCC	22	No	–	Yes

i: both miRNA sequences obtained from related precursor sequences by compared to ath-miRNAs.

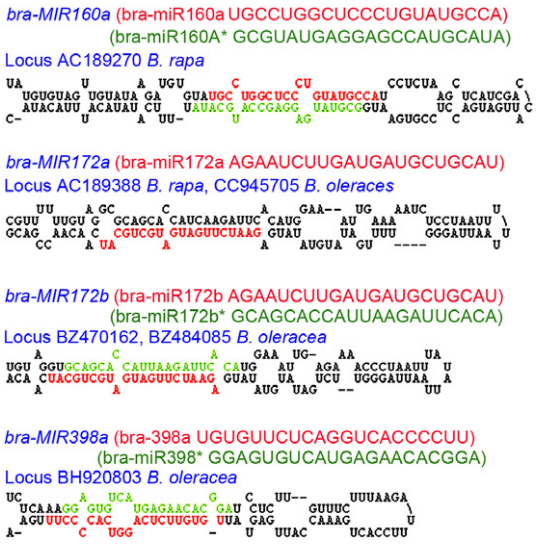

Fig. 2. Predicted stem-loop structures of three cloned miRNAs* and their opposite miRNAs in *B. rapa*. The mature miRNAs are in red, and the miRNA*s are in green. Sequences derived from the *Brassica* genome loci are indicated (see supplementary data for precursor stem-loop structures of all cloned miRNAs, except for miR390).

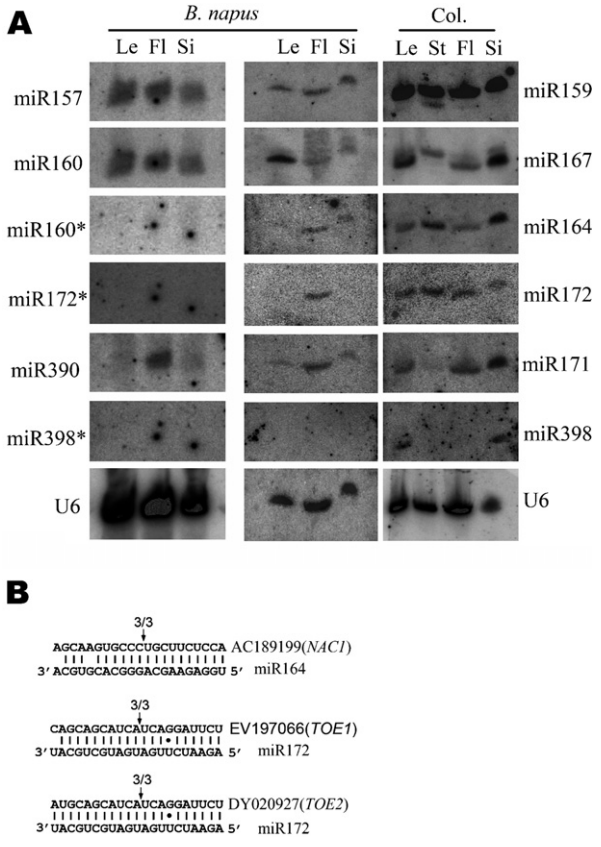

Fig. 3. Northern analysis of cloned conserved miRNAs and target identification in *Brassica*. (A) Detection of conserved miRNAs in different tissues from *B. napus* and *A. thaliana* Columbia (Col.). Leaves (Le), flowers (FL), stem (St) and siliques (Si) are indicated on the top, corresponding miRNAs and miRNAs* as indicated, and U6 RNA hybridization is used as a loading control. (B) 5′RACE detection of miRNAs targets in *Brassica*. The arrows indicate the cleavage sites of the *Brassica NAC1*, *TOE1* and *TOE2* mRNA.

their own precise regulation processes through the tissue-dependent miRNA biogenesis and target selection in different plant species. The three * strands: miR160*, miR172* and miR398* were not detected in hybridization assays, suggesting that miRNA*s might be isolated by cloning but might not necessarily be detected by normal northern assays.

Putative targets in *Brassica* for some conserved miRNAs have been predicted through a computational approach [2,18] without experimental verification. Thus, we searched through sequences in the *Brassica* database for the NAC domain transcription factor family of genes and for the *APETALA2*-like transcription factor family of genes. These families of genes are targets of miR164 and miR172 in *Arabidopsis*, respectively, and verified the miRNA cleavage sites by 5′-RACE. Specific 5′-RACE PCR products were obtained for loci AC189199 (homologous to NAC1 At1g56010, miR164 target), EV197066 (homologous to TOE1 At2g28550, miR172 target) and DY020927 (homologous to TOE2 At5g60120), but not for locus CD838953 (homologous to CUC1 At3g15170, miR164 target). miRNA-directed cleavage sites were confirmed by sequencing of PCR products (Fig. 3C).

3.4. Expression analysis of novel miRNAs in Brassica

To search for potential new *B. rapa* miRNAs from the cloned population, approximately 400 nt of surrounding sequences of small RNAs that match the *Brassica* database perfectly were analyzed using the MFOLD program to predict hairpin structures. Three 21 nt and one 22 nt small RNA sequences (bra-158, -174, -178 and -2010) reside within the arms of a predicted hairpin structure (Locus AC189642); bra-158 and bra-174 have only one nucleotide difference at the small RNA terminus and were counted as one, named bra-158a/b. Bra-178 and bra-2010 formed a pair of duplexes, resembling a miRNA/miRNA* duplex. Among them, the bra-2010 also positions in another hairpin structure (AM391091/EV179238) (Fig. 4A), and both hairpin-forming transcripts share 80.1% similarity in their nucleotide sequences. The lengths of 21 and 22 nt make them unlikely to be repeat-associated siRNA, which are normally 24 nt in length and require RDR2 (RNA-dependent RNA polymerase 2) and DCL3 for their biosynthesis [2]. A recent study of non-conserved miRNAs by high-throughput sequencing of *Arabidopsis* miRNAs supports the inverted duplication model of miRNA evolution, which proposes that miRNA genes arose from inverted duplications of their target genes [2,14]. Over time, these inverted duplications accumulate mutations that cause them to become highly divergent from their source except in small regions corresponding to the miRNA and miRNA* sequences and result in multigene conserved families across many species. In contrast, some non-conserved miRNAs contain a high homology with target genes throughout the miRNA precursor [2,11,14].

Since the full genome sequence is not available for *Brassica*, we used the two hairpin-forming sequences as precursor sequences in BLASTN searches against the TAIR *Arabidopsis* gene database. Both sequences match a large family of TIR–NBS–LRR class disease resistance (*R*) genes imperfectly. We then searched potential targets for the three small RNAs against the TAIR *B. napus* and the *Arabidopsis* mRNA using the miRU program (http://bioinfo3.noble.org/miRNA/miRU.htm). Surprisingly, two of the small RNAs (bra-158a/b and -2010) may target the same family of the disease-resistant *R* proteins encoded by *Arabidopsis* mRNAs (Fig. 5A). We had two specula-

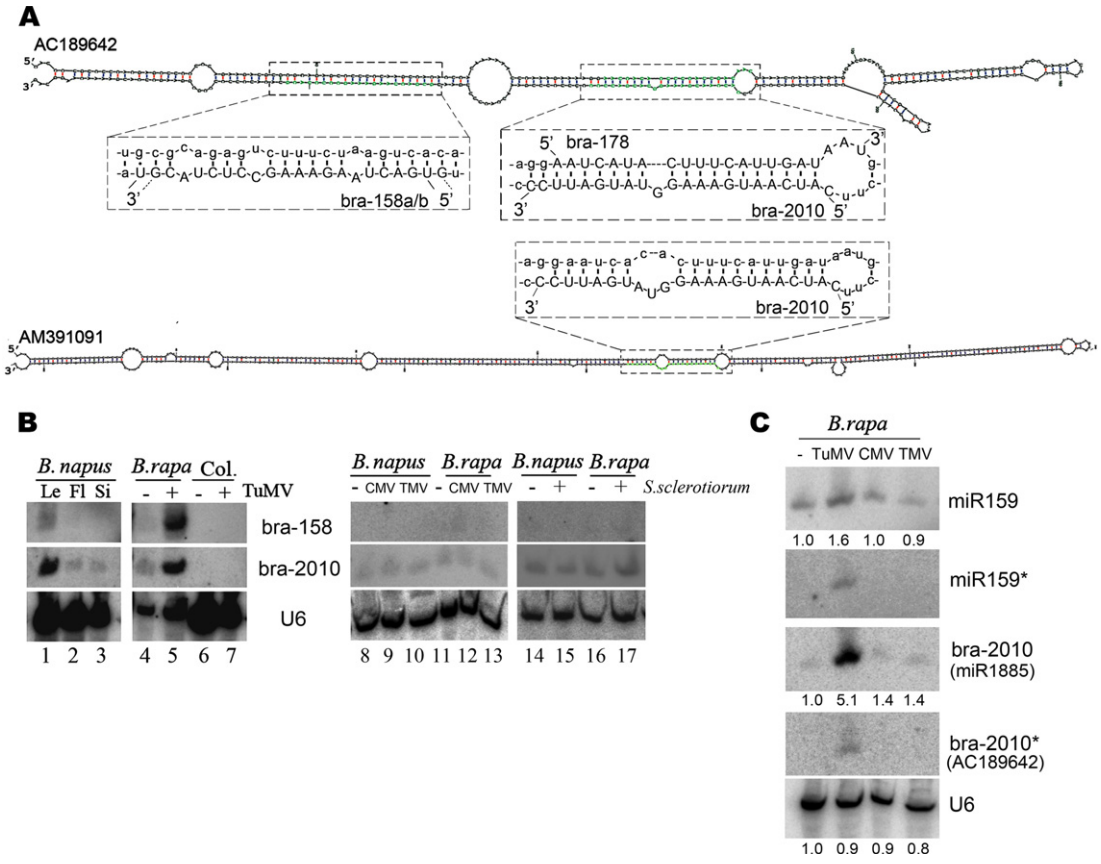

Fig. 4. Predicted stem-loop structures of three potential miRNAs and their expression analysis in *Brassica*. (A) Predicted stem-loop structures from two *Brassica* loci containing the potential miRNAs, in which sequences (in upper case) and imperfect paring duplexes are presented in the inserts. (B) Detection of bra-158 and -2010 accumulation in *B. napus*, *B. rapa* and Columbia with or without pathogenic infection, with specific LNA nucleotide probes. Three viruses and a necrotrophic fungal pathogen are indicated. (C) Detection of accumulation of miR159/miR159* and bra-2010/bra-2010* in *B. rapa* with or without viral infection. Numbers below the gels indicate relative miR159 and bra-2010 accumulation levels.

tions based on the inverted duplication hypothesis for the de novo generation of new miRNA genes discovered in *Arabidopsis* [2,11,14] and on finding tandem gene duplications and duplication of individual or small groups of genes to unlinked loci (ectopic duplication) of *R* genes in *Arabidopsis* [19–21]. Firstly, we speculated that the two hairpin-forming sequences were probably transitional loci derived from *Brassica* protein-coding genes homologous to disease-resistant genes in *Arabidopsis*. Secondly, we speculated that one or more of these small RNAs might evolve into true and new miRNA(s) if their silencing activities were advantageous. A northern blot analysis was performed to determine the three small non-conserved RNA cloned. In agreement with the low abundance of non-conserved miRNAs [11,14,22], none of the three were detected under the normal northern hybridization conditions using corresponding antisense oligonucleotides as probes (data not shown). Therefore, we used more sensitive lock-modified DNA (LNA) probes to detect bra-158 and -2010. As shown in Fig. 4B, both bra-158 and -2010, and particularly bra-2010, were highly accumulated in *Brassica* leaves (lanes 1) and had low accumulations in *Brassica* flowers and siliques (lanes 2 and 3).

3.5. Increased accumulation of novel miRNAs in TuMV-infected *Brassica*

To determine whether both bra-158 and -2010 were related to disease resistance, we infected *B. rapa* and *Columbia* with *Turnip mosaic virus* (TuMV). Both bra-158 and -2010 were greatly induced by TuMV infection (Fig. 4B, cf. lanes 4 and 5). No hybridization signal was detected in *Columbia* with or without TuMV infection (lanes 6 and 7). Given that the haploid genome equivalent of *B. rapa* was closely related to *Arabidopsis*, the hybridization results suggested that bra-158 and -2010 were specific in *Brassica*, probably related to the response to pathogen stress.

We further inoculated *B. napus* and *B. rapa* with another two viruses that can infect *Brassica*, the *Cucumber mosaic virus* (CMV) and the*Tobacco mosaic virus* (TMV). Systemic diseases in developed leaves were collected, and virus accumulations were confirmed by northern blot analysis with related Coat Protein-encoding sequences (data not shown). Neither bra-158 nor bra-2010 was induced by either virus (Fig. 4B, lanes 9–10 and 12–13) compared with that in the mock-inoculated plants (lanes 8 and 11). A necrotrophic fungal pathogen *Sclerotinia sclerotiorum* was also used to challenge *B napus* and *B. rapa*. Induction of bra-158 and bra-2010 were not observed in systemic necrosis-infected leaves (Fig. 4B, lanes 14–17). These results suggested that the induction of these small RNAs might be TuMV-specific.

TuMV is known to encode P1/HC-Pro, a viral silencing suppressor that has been shown to inhibit miRNA function, and, in many cases, P1/HC-Pro-expressing transgenic plants show increased accumulation of both the mature miRNAs and miRNA*s [1,2,23]. TuMV-induced accumulation of the small RNAs can be caused by increased expression either through

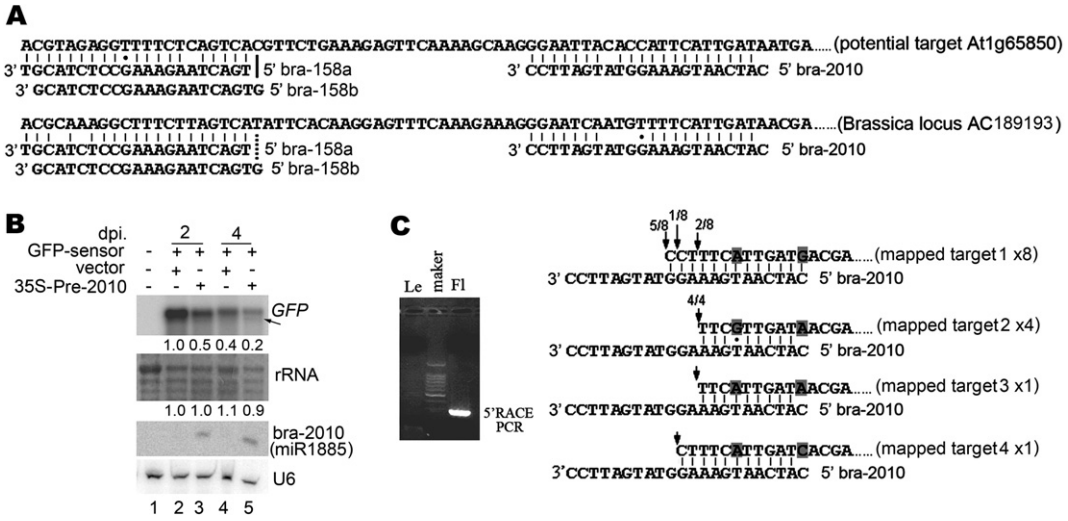

Fig. 5. Target predictions and identification via 5′RACE assay and transient expression system. (A) One of the predicted potential targets in *Arabidopsis* and *Brassica*, respectively, for bra-158a/b and bra-2010 is presented. (B) Detection of bra-2010 expression and bra-2010-mediated GFP-sensor transcript reduction in the transient expression system. Co-infiltration of GFP-sensor with 35S-Pre-2010 or vector control is indicated. Dpi indicates day-post infiltration. Numbers below the gels indicate relative *GFP* mRNA levels. The 3′cleavage fragment of the GFP-sensor is indicated with an arrow. (C) Potential targets in *Brassica* for bra-2010 are identified by 5′RACE detection (left). Regions from four mapped targets obtained from 5′RACE PCR sequences that are complementary to bra-2010 are presented (right). Sequenced target clones and cleavage sites/frequency are indicated. Nucleotide differences in sequenced targets are highlighted with gray boxes.

an unknown mechanism or as a result of silencing suppressor activity of P1/HC-Pro. To examine this TuMV-induced accumulation, we first examined accumulation of miR159 and miR159* in TuMV-infected *Brassica*. An increase of both miR159 and miR159* was detected (Fig. 4C, cf. lanes 1 and 2), suggesting that the silencing suppressor activity of P1/HC-Pro was functional in TuMV-infected *Brassica*. We then selected star strains of bra-2010 in both AC189642 and AM391091 loci (Fig. 4A) to design probes in northern blot analysis. Bra-2010* (AC189642) (equal to bra-178), but not bra-2010* (AM391091), was readily detected and increased in TuMV-infected *Brassica* (Fig. 4C, cf. lanes 1 and 2, and data not shown). This result suggested that the increase of bra-2010 could, at least in part, result from P1/HC-Pro's silencing suppressor activity. However, taking into account that bra-2010 was induced much more strongly than miR159 in infected plants, we suggest that an unknown mechanism could be also involved in the increase of bra-2010 during TuMV-infection in *Brassica*. The detection of bra-2010* (AC189642) in TuMV-infected *Brassica* also suggested that the bra-2010 could be a true miRNA derived from the AC189642 precursor locus but not the AM391091 locus. Our results might also suggest that another cloned novel small RNA, bra-178, was merely a star strand of bra-2010 (bra-2010*). In CMV- and TMV-infected *Brassica*, no increase was observed in either miR159 or miR159* (Fig. 4C, lanes 3–4). This is consistent with bra-2010, which did not show an increase in accumulation in these CMV- and TMV-infected plants. Both CMV and TMV also encode silencing suppressors, the Cmv-2b and the 126 kDa proteins, respectively [24–26]. *Arabidopsis* infected with CMV has been shown to increase accumulation of both mature miRNAs and miRNAs* due to the Cmv-2b silencing suppressor activity [27,28]. Our results also suggest that the mode of viral silencing suppressor activities could show host specificity.

Plant miR393 have been shown to be induced by a flagellin-derived peptide (flg22) to restrict *Pseudomonas syringae* growth by repressing auxin signaling [29]. However, plant miRNAs that were specifically elicited by plant virus infections through mechanisms other than the effects of viral silencing suppressors have not been reported. The bra-2010 precursor locus AC189642 promoter-GUS construct is currently being conducted for further study to address this question.

3.6. Analysis of putative targets of novel miRNAs in Brassica

The non-conserved bra-158 and -2010 were further investigated as potential novel miRNAs. A sequence of the best putative target of these small RNAs, At1g65850, which had scores of 2 and 2.5, respectively, was used to search the homologous sequence against the *Brassica* database. Specifically, locus AC189193 from *B. rapa* was screened (Fig. 5A). The downstream regions of the putative target sites in AC189193 were selected to design outer and inner primers for 5′RACE assays. A specific 5′RACE PCR band was obtained in *Brassica* flowers but not in leaf tissues (Fig. 5C). Sequencing results from fourteen 5′RACE PCR clones showed that four different sequences belonged to the same family of genes that were homologous to *Arabidopsis* TIR–NBS–LRR class disease-resistant proteins. All of these four mapped targets were highly homologous with, but did not derive from, the AC189193 locus sequence (Fig. 5C). Specific cleavage sites were obtained for bra-2010, but not for bra-158, for all the sequenced clones, although the cleavage sites were varied (Fig. 5C). This result was not expected. We speculated that the cleavage sites were probably for bra-158a/b because they were both 21 nt in length, and the first nt U for bra-158a made it a more valid miRNA. However, our result could not rule out that bra-158 was a true functional miRNA in targeting the disease-resistant transcripts in *Brassica*, as the bra-2010 target site was downstream (by about 24 bp) of that for bra-158. Thus, the target site would obstruct the detection of bra-158 cleavage sites via the 5′RACE approach.

To test the authenticity of bra-2010-mediated cleavage of the mapped targets, homologous regions downstream of bra-2010 cleavage sites in the mapped targets were selected to design outer and inner primers for 5′RACE to obtain upstream se-

quences covering the bra-2010 target region. In this way, a specific 5′RACE PCR band was obtained. Sequencing of 5′RACE PCR clones confirmed that the sequence was derived from the mapped target 2, which had a unique cleavage site for bra-2010 (Fig. 5C). The sequence was then used to create a GFP-sensor construct for a transient assay. The bra-2010 precursor AC189642 sequence was also amplified from *Brassica* and cloned under the 35S promoter, which produced construct 35S-Pre-2010. Co-expression of the GFP-sensor with 35S-Pre-2010 in *N. benthamiana* leaves resulted in reduction of GFP transcript accumulation and GFP fluorescence compared to co-expression of the GFP-sensor with vector control (Fig. 5B and data not shown). A 3′cleavage fragment of the GFP-sensor was detected by a northern blot analysis to co-express the GFP-sensor with 35S-Pre-2010 but did not co-express with vector control (Fig. 5B). Specific expression of bra-2010 was also detected (Fig. 5B). There were no other small RNA detected using a mixture of six specific oligo probe sequences complementary to regions beyond the bra-2010 sequence. Moreover, neither bra-158 nor bra-2010* (AC189642) were detected when specific oligo probes were used (data not shown). Our results further strongly indicated that the AC189642 locus was a veritable miRNA precursor encoding bra-2010, which resulted in degradation of the GFP-sensor transcript. We could not exclude other *R* gene transcripts being also targets of bra-2010 in Brassica. Nevertheless, together with the analysis results from the specific LNA nucleotide probes (Fig. 4B and C), our data indicated that bra-2010 was a true, functional, and novel miRNA specific to *Brassica*, which could be induced by TuMV infection and targeted TIR–NBS–LRR class disease-resistant transcripts for cleavage. The bra-2010 was, therefore, nominated as bra-miR1885. The similarity in sequences between the bra-miR1885 precursor locus (AC189642) and target transcript genes suggests that bra-*MIR1885* was a new evolutionary miRNA gene that resulted from gene duplication events from TIR–NBS–LRR class disease-resistant protein-coding gene sequences. These sequences likely became bra-miR1885 targets. Gene families rapidly evolving under stress constraints, such as these complex disease-resistant *R* gene clusters, being prime candidates for young small RNA-generating loci in plants has been proposed [30]. The young small RNA-generating loci might evolve into new and genuine miRNA-encoding genes if the new miRNA silencing activities were advantageous. Further pathogenesis should be undertaken to study in detail the expression patterns of bra-miR1885 and bra-miR1885-mediated regulation of specific TIR–NBS–LRR transcripts in response to pathogen stress and, particularly, to TuMV infection.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.06.011.

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