Cloning and characterization of microRNAs from *Brassica napus*

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Abstract A library containing approximately 40,000 small RNA sequences was constructed for *Brassica napus*. Analysis of 3025 sequences obtained from this library resulted in the identification of 11 conserved miRNA families, which were validated by secondary structure prediction using surrounding sequences in the *Brassica* genome. Two 21 nt small RNA sequences reside within the arm of a pre-miRNA like stem–loop structure, making them likely candidates for novel non-conserved miRNAs in *B. napus*. Most of the conserved miRNAs were expressed at similar levels in a F1 hybrid *B. napus* line and its four double haploid progeny that showed marked variations in phenotypes, but many were differentially expressed between *B. napus* and Arabidopsis. The miR169 family was expressed at high levels in young leaves and stems, but was undetectable in roots and mature leaves, suggesting that miR169 expression is developmentally regulated in *B. napus*.

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1. Introduction

Non-coding RNAs are abundant in eukaryotic cells, of which small RNAs constitute a family of regulatory RNAs of 18–28 nt in length [1]. These small RNAs can be divided into four classes, namely microRNA (miRNA), small interfering RNA (siRNA), tiny non-coding RNA (tncRNA), and small modulatory RNA (smRNA) [2]. siRNA and miRNA are the two major types, both of which are produced by RNase III-like enzymes called DICER-LIKE in plants [3]. They are incorporated into silencing complexes that contain Argonaute proteins to guide repression of target genes [4]. siRNAs are processed from long, double-stranded RNA (dsRNA) precursors and direct gene silencing through both mRNA degradation and chromatin modification [5]. In plants, siRNAs play a major role in defense against viruses and other invasive nucleic acids as well as in controlling developmental pathways [6,7].

miRNAs are chemically and functionally similar to siRNAs but are derived from local stem–loop structures in the genome. The majority of miRNA genes exist as independent transcriptional units, which are transcribed by RNA polymerase II into long primary transcripts (pri-miRNAs) [8–10]._pri-miRNAs fold into secondary stem–loop structures which are processed in a two-step manner [8]. In animals, pri-miRNAs are trimmed in the nucleus to generate approximately 70 nt mRNA precursors (pre-miRNAs) with fold-back structures by a multiprotein complex called microprocessor, in which Drosha (an RNase III-like enzyme) and Pasha (a double-stranded RNA binding protein) are critical components [11,12]. The pre-miRNAs are exported to the cytoplasm and subsequently cleaved by Dicer to generate mature miRNAs [13]. Plants do not appear to encode a Drosha ortholog, and the nuclear-localized DICER-LIKE 1 (DCL1) appears to be responsible for processing both pri- and pre-miRNAs [10]. Three other factors, HYL1, SERRATE and HEN1, are also required for the processing and maturation of miRNAs in plants [14–17]. The mature single-stranded miRNA is loaded into the RNA induced silencing complex (RISC) [18], where it regulates the transcript level or expression of target genes by directing mRNA cleavage or translational inhibition [8].

miRNAs have recently been shown to play critical roles at each major stage of plant development [19], regulating a number of key pathways. They typically act at the core of a gene regulatory network, targeting genes that are themselves regulators, such as those encoding transcription factors and F-box proteins that are involved in organ morphogenesis and plant development [20–23]. For example, miR165/miR166 is involved in the determination of the adaxial/abaxial pattern in developing leaves [24], miR172 governs floral organ development [25], and miR-JAW regulates the level of TCP-family transcripts controlling leaf development [26]. In addition, it has recently been shown in Arabidopsis, rice, and *Populus trichocarpa* that some miRNAs are stress regulated and may be involved in cell responses to abiotic stresses such as salinity, cold, and dehydration [27–29].

The structural and functional characterization of miRNA genes is important in addressing genetic and molecular mechanisms controlling phenotype determination and adaptation to growth conditions on which yield potential and stability depend [30,31]. Apart from Arabidopsis, rice, and *P. trichocarpa*, cloning of miRNAs has recently been reported for *Glycine max*, *Medicago truncatula*, *Saccharum officinarum*, *Sorghum bicolor*, and *Zea mays*. In the past 4 years, the total number of
miRNAs has dramatically increased as the approaches for identifying species-specific miRNAs are enhanced [32], and miRNA families have been deposited in the RNA Registry at www.sanger.ac.uk/Software/Rfam/.

In *B. napus*, where the phenomenon of hybrid vigor is used in cropping systems, phenotypic characteristics are strongly influenced by heterozygosity. With the long-term goal of exploring the possible involvement of miRNAs in the hybrid-vigor phenomenon, we have constructed a small RNA cDNA library of *B. napus* and identified 11 miRNA families and two candidates for novel miRNAs. We have also explored the potential functions of these miRNAs by comparing their expression levels in four phenotypically different haploid lines and their parental F1 line.

2. Materials and methods

2.1. Plant materials

The *B. napus* lines used in this study included a F1 hybrid that originated from hybridization between the hybrid-vigor DH lines Quantum and No2127, and its DH progeny 098, 151, 181, 225 generated by anther culture using pollen from the F1 line. For each line, the tissues examined were derived from at least four independent plants, which were grown in the glasshouse at 22–25 °C with a 14 h light/10 h dark photoperiod. All plant materials were immediately frozen in liquid nitrogen and stored at −80 °C before use.

2.2. Construction of *B. napus* small RNA libraries

Total RNA was isolated from whole plants of pooled 20-day-old seedlings of the five different lines using the Trizol reagent. To enrich for small RNAs, larger RNA was removed by precipitation with 5% polyethylene-glycol and 0.5 M NaCl in ice for 10 min followed by centrifugation at 12,000 rpm for 10 min. Small RNAs were then recovered from the supernatant by precipitation with 3 vol of ethanol and 0.1 vol of 3 M NaOAc. The small RNAs were fractionated in 12% urea–polyethyleneglycol and 0.5 M NaCl in ice for 10 min followed by centrifugation at 12,000 rpm for 10 min. Small RNAs were then recovered from the gel with 0.4 M NaCl, and used for cloning.

For cloning of the purified small RNAs, essentially the same protocol developed in the Bartel laboratory was followed [33]. Basically, 5’ phosphorylated DNA adaptors and DNA/RNA mixed adaptors were sequentially ligated to the 3’ and 5’ ends, and the products reverse-transcribed to generate the first strand of cDNA, which was then amplified by PCR. The PCR products were digested with BanI and concatamerized with T4 DNA ligase. DNA fragments of 200–400 bp in size were gel-purified and ligated into pGEM-T Easy and transformed into *Escherichia coli* using electroporation. DNA sequencing was performed using BigDye Terminator v3.1 (Applied Biosystems) following the manufacturer’s instruction.

2.3. Northern blot analysis of microRNA expression in *B. napus*

Total RNA and small RNAs were isolated from the five *B. napus* lines and Arabidopsis (Col-0) following the same procedure as described above. The small RNAs were separated in 15% urea–polyacrylamide gel, transferred to Hybond-N+ filter, and hybridized with 32P-labeled DNA oligonucleotides complementary to the detected miRNAs (sequences of the oligonucleotides are available upon request). The hybridization was carried out overnight at 30 °C in the phosphate buffer containing 125 mM Na2PO4 (pH7.2), 250 mM NaCl2, 7% SDS, and 50% formamide, and washed three times for 30 min with 2× SSC and 0.2% SDS at 37 °C. The hybridization signal was detected using a phospho-imager.

2.4. Cloning of microRNA precursor

Total RNA from 4-week-old *B. napus* seedlings was extracted using Trizol reagent. Approximately, 5 μg of total RNA and 0.5 μg of oligo dT20 were used for reverse transcription with SuperScriptII reverse transcriptase (Invitrogen). PCR was performed using 1 μl of the initial RT reaction with the following pairs of gene-specific primers for bna-miR161 designed against the sequences of predicted microRNA precursors from Brassica: F 5’AAAAACTCGGGTTTTGACCTG and R 5’CCTTACAAAAGTATCACCTC (bna-miR161). The PCR product was ligated into pGEM-T Easy and sequenced.

3. Results and discussion

3.1. Cloning and identification of *B. napus* miRNAs

A small RNA library generated using RNA from 20-day-old seedlings of *B. napus* contained approximately 10000 clones. Sequencing 800 of these clones resulted in 3025 small RNA sequences, with each clone containing 3–7 small RNA inserts. The size of the inserts varied from 10 to 30 nt; among the 3025 small RNAs, 24.3% are 21 nt in size, 9.3% are 22 nt, 19.1% are 23 nt and 29.3% are 24 nt, with the remaining 18% either smaller than 21 nt or larger than 24 nt (Fig. 1).

Thus, 21–24 nt RNAs represent the predominant species (more than 80%), with 24 nt RNAs being the most abundant class, consistent with the distribution patterns of small RNAs from other species [34–36].
<table>
<thead>
<tr>
<th>miRNA</th>
<th>Sequences (5′–3′)</th>
<th>Reads</th>
<th>Size</th>
<th>Homologue in Arabidopsis or rice</th>
<th>Consensus with the homologue</th>
<th>RNA blot</th>
<th>Target family</th>
<th>Predicted targets in B. napus database</th>
</tr>
</thead>
<tbody>
<tr>
<td>bna-miR157a</td>
<td>UUGACAGAAGAGAAGAGACAC</td>
<td>2</td>
<td>21</td>
<td>ath-miR157a,b</td>
<td>Match</td>
<td>Yes</td>
<td>SBP-like [20,30,45]</td>
<td>TC4614, TC4049</td>
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<td>bna-miR159a</td>
<td>UUGGAAGGAAAAAGAAGACUU</td>
<td>1</td>
<td>21</td>
<td>ath-miR159a;</td>
<td>Match</td>
<td>Yes</td>
<td>MYB33, MYB65 [26,46]</td>
<td>TC1619</td>
</tr>
<tr>
<td>bna-miR161a</td>
<td>UCAAAUGCAUGAAGAAGACUA</td>
<td>1</td>
<td>21</td>
<td>ath-miR161a2</td>
<td>1 nt mismatch</td>
<td>NO</td>
<td>PPR [6]</td>
<td>NO</td>
</tr>
<tr>
<td>bna-miR164a</td>
<td>UUGGAAGAAGAGAAGACUGC</td>
<td>4</td>
<td>21</td>
<td>ath-miR164a,b</td>
<td>Match</td>
<td>Yes</td>
<td>CUC1, CUC2 [23,47]</td>
<td>TC2548, TC1396, CD827550</td>
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<tr>
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<td>UCGCUUGAGGCGACCAUCU</td>
<td>1</td>
<td>21</td>
<td>ath-miR166a</td>
<td>Match</td>
<td>Yes</td>
<td>PHB, PHV, REV, ATHB-8, ATHB-15 [21, 48–50]</td>
<td>CD827440</td>
</tr>
<tr>
<td>bna-miR167a</td>
<td>UUGACAGAAGAGAAGAGACAC</td>
<td>1</td>
<td>21</td>
<td>ath-miR167a;</td>
<td>Match</td>
<td>Yes</td>
<td>ARF6, ARF8 [6,51]</td>
<td>TC3663, CD840352, TC1317, TC1549</td>
</tr>
<tr>
<td>bna-miR167b</td>
<td>UUGACAGAAGAGAAGAGACAC</td>
<td>46</td>
<td>21</td>
<td>ath-miR167a,b</td>
<td>Match</td>
<td>Yes</td>
<td>HAP2-like [27]</td>
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<tr>
<td>bna-miR169a</td>
<td>UUGACAGAAGAGAAGAGACAC</td>
<td>24</td>
<td>21</td>
<td>ath-miR169a</td>
<td>Match</td>
<td>Yes</td>
<td>AGO1 [22]</td>
<td>NO</td>
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<tr>
<td>bna-miR171a</td>
<td>UUGACAGAAGAGAAGAGACAC</td>
<td>68</td>
<td>21</td>
<td>ath-miR169h, i, j, k, l, m, n</td>
<td>Match</td>
<td>Yes</td>
<td>SCL6-III, SCL6-IV</td>
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<td>UUGACAGAAGAGAAGAGACAC</td>
<td>1</td>
<td>21</td>
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<td>Match</td>
<td>Yes</td>
<td>TAS3 [6]</td>
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<td>bna-miR397a</td>
<td>UUGACAGAAGAGAAGAGACAC</td>
<td>1</td>
<td>21</td>
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<td>Match</td>
<td>Yes</td>
<td>Laccases [27,53]</td>
<td>NO</td>
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<td>UUGACAGAAGAGAAGAGACAC</td>
<td>2</td>
<td>21</td>
<td>ath-miR171b,c</td>
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<td>CB686439; CD844359</td>
<td>NO</td>
</tr>
<tr>
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<td>UUGACAGAAGAGAAGAGACAC</td>
<td>1</td>
<td>21</td>
<td>ath-miR171b,c</td>
<td>Match</td>
<td>Yes</td>
<td>CB686439; CD844359</td>
<td>NO</td>
</tr>
</tbody>
</table>
As determined by BLASTN searches against the www.arabidopsis.org transcripts database, a large fraction (~65%) of the cloned small RNAs had sequences that did not match the Arabidopsis transcriptional products perfectly. Given the high levels of sequence homology between B. napus and Arabidopsis in the transcribed regions, many of these unmatched small RNAs are likely to be siRNAs from transposons and other repetitive sequences specific to Brassica. However, this group of cloned small RNAs could also contain novel miRNAs and regulatory siRNAs not conserved between Brassica and Arabidopsis. A smaller fraction of the sequenced small RNAs had sequences that match with known non-coding RNAs, including rRNAs (6.1%), tRNAs (7.2%) and retrotransposon RNAs (2.7%). mRNA breakdown products represented about 2.5% of the sequenced population.

Among the 3025 small RNA sequences, 411 (more than 13%) matched the conserved plant miRNAs perfectly, with another 19 showing near-perfect match. There were only eight reads of miRNA’s (the passenger strand; two for miR164 and six for miR166), representing less than 2% of the total microRNA population, which was much lower than the ~9% ratio for the published Arabidopsis miRNA’s [37]. The miRNAs could be classified into 11 conserved families, namely bna-miR157, bna-miR159, bna-miR161, bna-miR164, bna-miR166, bna-miR167, bna-miR168, bna-miR169, bna-miR171, bna-miR390, bna-miR397 (Table 1). Most (408) of the 430 B. napus miRNA sequences were 21 nt in length with the remainder being 22 nt or 20 nt long (Fig. 2A). This is similar to observations for miRNAs from other plant species [34], indicating that B. napus miRNAs are mostly processed by DCL1. Furthermore, a majority of the bna-miRNAs begin with a 5’ uridine, which is characteristic of miRNAs [38]. The absence of shorter (<20 nt) miRNA sequences in the cloned population indicates that no significant degradation had occurred during the isolation and cloning procedure.

The B. napus miRNA sequences varied greatly in their representation in the cloned population (Fig. 2B). bna-miR169 was the most abundant miRNA, represented by 18 members with 363 reads, of which miR169 accounted for 199 times. There were 50 reads for the bna-miR167 family consisting of five members that differ slightly in nucleotide sequence. Only one member of this family, bna-miR167b, appeared to be highly expressed as it accounted for 46 of the 50 reads. The other miRNAs were either cloned only a few (2–5) times (bna-miR157, bna-miR164, bna-miR171) or only once (bna-miR159, bna-miR161 bna-miR166, bna-miR168, bna-miR390, bna-miR397).

3.2. Secondary structure prediction of B. napus miRNA precursor sequences

In general, miRNAs can be distinguished from endogenous siRNAs on the basis of the ability of the miRNA-surrounding sequences to adopt a hairpin structure [19]; most of the 20–25
nt miRNAs are processed from a ~70 nt precursor in animals, forming a hairpin structure that contains mature miRNA in either of its arms [12]. Plant miRNA precursors range from

47 to 698 nt in size, with the majority (>80%) being larger than 100 nt. Based on the available genomic sequence of Brassica, hairpin structures could be predicted for the surrounding sequences of all the 11 conserved B. napus miRNA families; using MFOLD (http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi), 38 hairpin structures were predicted from 57 loci of Brassica (Supplementary data 1). The predicted precursor for bna-miR161a was validated by RT-PCR isolation of the B. napus RNA; the sequence of the RT-PCR product matched perfectly to that of the B. oleracea genome (NH596944, BZ512955) with the potential to form a hairpin structure (Supplementary data 4). The two hairpins containing miR169j and miR169k were predicted to arise from the same location in the genome (GenBank accession numbers AC189512 and AC189215) with only a 67 nt spacer separating them (Fig. 3). This suggests that bna-miR169j and bna-miR169k are derived from the same primary transcript. The predicted stem-loop structures of the 11 conserved B. napus miRNA families resemble those of the same miRNA families from other plant species such as Arabidopsis [39]. This result confirmed that these families of miRNAs are bona fide miRNAs and indicated that all the 11 miRNA families are conserved among the plant species studied so far.

3.3. Secondary structure prediction reveals a new candidate bna-microRNAs family

To identify new B. napus miRNAs from the sequenced population, the 20–24 nt small RNA sequences were used to search the Brassica database, and over 70% of these sequences match the Brassica genomic sequences perfectly. Approximately, 300 nt sequence surrounding each of these matched sites was analyzed using the MFOLD program to predict secondary structure. Two 21 nt small RNA sequences reside within the arm of a predicted stem–loop structure (Fig. 4), making them miRNA candidates (named as bna-miR1140a and bna-miR1140b). Searching the TAIR Brassica napus and the Arabidopsis mRNA using the miRU program at http://bioinfo3.noble.org/cgi-bin/miRNA/miRU.htm found no perfectly complementary mRNA sequences for bna-miR1140. Nevertheless, two B. napus miRNAs [encoding for a protein related to a large-conductance mechanosensitive channel protein (CB686439) and an unannotated protein (CD844359)], and two Arabidopsis mRNAs [encoding for a protein kinase (At5g41680) and aspartyl protease family protein (At2g36670)], contained sequences that could potentially be targeted by bna-miR1140 (Supplementary data 2). RNA blot analysis was performed with corresponding antisense oligonucleotides as probe but no signals of bna-miR1140 could be detected, suggesting that bna-miR1140 exists in low abundance in B. napus. In keeping with this lack of detection, recent studies show that non-conserved miRNAs are generally present at low abundance in plants [37,40].

3.4. bna-miR164b*

bna-miR164a appeared four times in the sequenced population, but no other miR164 members were identified. bna-miR164a is located on the 5′ arm of the predicted stem–loop structure (Fig. 5). A 21 nt sequence (CACGUGCUACUACCUAAC) appeared twice in the sequenced population, and five loci in the Brassica genome database matched it perfectly. These five sequences were all predicted to form stem–loop structures in which the 21 nt sequence is located on the 3′ arm of the stem–loop (Fig. 5). Surprisingly, although this 21 nt sequence is significantly divergent from the predicted bna-miR164a* sequence (CACGUGCUACUACCUAAC), its opposite strand has a sequence almost identical to that of bna-miR164a with only one nucleotide difference (Fig. 5). We therefore designated the 21 nt sequence CACGUGCUACUACCUAAC as bna-miR164b* and its opposite strand, although not present in the cloned population, as bna-miR164b. The bna-miR164b* sequence is divergent from the predicted bna-miR164a* sequence but identical to the Arabidopsis miR164a*/miR164b*/miR164c* sequence (Fig. 5). This cross-species conservation could imply that miR164b* is a functional miRNA in its own right; both strands of miRNA duplexes acting as functional miRNAs have been reported in animals [41–43].

3.5. The miR169 family

In Arabidopsis, miR169 represents the largest microRNA family, consisting of 4 types (sequence variants), which are encoded by 14 different genomic loci (http://asrp.cgrb.oregonstate.edu). Known target genes of miR169 in Arabidopsis include the CCAAT binding transcription factors HAP2A, HAP2B and HAP2C [27]. miRNA169 is represented by 6
types (at least 11 loci) in maize (zma), 9 types (16 loci) in rice (osa), 16 types (31 loci) in P. trichocarpa (ptc), and 5 types (9 loci) in S. bicolor (sbi) (http://microrna.sanger.ac.uk/sequences/) (Supplementary data 3). Our result showed that there are at least 18 types for the miR169 family in B. napus, more than in Arabidopsis. The sequences of the 4 most abundant bna-miR169 members, bna-miR169a, bna-miR169j, bna-miR169k and bna-miR169o, were all 21 nt in length (Table 1), suggesting that they are processed solely by DCL1. The other members of bna-miR169 had a relatively low frequency in the sequenced population, suggesting that they were expressed either at very low levels or in specific tissues or cells. Interestingly, a significant number of the low-abundance bna-miR169 sequences are 22 nt in length (Table 1; 1 for bna-miR169d, 7 for bna-miR169l, 4 for bna-miR169p, and 2 for miR169r). This could suggest that either the pri-miRNA structures cause DCL1 to cleave a 22 nt fragment, or they are processed by DCL2, which generates 22 nt siRNAs from viral or transgene-derived dsRNAs and may function only in specific tissues or cells. A recent report showed that DCL1 is not the only Dicer for miRNA processing in plants; at least two miRNAs in Arabidopsis were found to be dependent on DCL4 for their accumulation [44]. Several 24 nt miRNA species have been reported for Arabidopsis and rice [29], suggesting that DCL3 may also be involved in the processing of a small subset of miRNAs in plants.

A phylogenetic analysis of miR169 from six different plant species (Fig. 6) showed that the individual members tend to
be more closely related to those from other plant species than from the same plant species, suggesting that evolution of the miR169 family occurred before the separation of these species.

3.6. Expression analysis of the *B. napus* miRNAs

Understanding the spatial and temporal expression patterns of miRNAs could provide clues about their physiological functions. In a wide range of organisms, many miRNAs are differentially expressed at different developmental stages and in different tissues and cell types [27,29]. For instance, only a small number of miRNAs in Arabidopsis are expressed ubiquitously, whereas many others show preferential accumulation in specific tissues [34,39]. In an attempt to associate *B. napus* miRNAs with morphological differences, we examined the expression of 11 miRNA families plus miR164* in the five *B. napus* lines from which the small RNA library was constructed. These five lines consisted of a F1 DH line and its DH progeny. The original F1 line was derived from crosses between Quantum and No2127, and the progeny lines (designated as F2), 098, 151, 181 and 225, were derived from the F1 DH line using microspore culture and chromosome doubling. In contrast to the F1 DH population that showed uniform phenotypes among individual plants, the F2 population showed significant phenotypic variations, ranging from different flower color and flowering time to different plant stature, leaf morphology and yield (e.g. Fig. 7B). Small RNAs from mature leaves were used for the expression analysis, and small RNAs from Arabidopsis (Col-0) were included as a control.

As shown in Fig. 7A, there were no dramatic differences among the five different *B. napus* lines in the expression levels of the 11 miRNAs, although small variations could be seen for some, such as miR157, miR166, miR390 and miR397. For miR168, a prominent larger hybridizing band could be clearly seen in the F1 and F2-098 lines, but not in the other three lines, which could suggest differential expression of miR168 between the two groups of *B. napus* plants. Surprisingly, miR169 was undetectable by northern blot hybridization from mature leaves (Fig. 7A), in contrast to its high abundance in the cloned population. Presumably, this miRNA is preferentially expressed at a younger stage of *B. napus* development or in tissues other than leaves. To investigate this possibility, small RNAs were extracted from root, leaf, stem and shoot apex tissues of young (20-day-old) *B. napus* plants and analyzed by northern blot hybridization. As shown in Fig. 7C, bna-miR169 was readily detectable in young leaves and stems. Thus, miR169 appeared to be expressed predominantly at early stages of *B. napus* development. No hybridization signals were detected for miR169 in the roots of the 20-day-old plants, and very low levels of signals could be detected in the shoot apex (Fig. 7C), suggesting that miR169 expression is spatially regulated in *B. napus*. Also, in the young roots and shoot apex where mature miR169 could not be readily detected, a larger prominent band appeared, which presumably represents unprocessed miR169 precursor transcript (Fig. 7C). This result appears to suggest that the low expression levels of miR169 in roots and shoot apex result from impaired processing of miR169 precursors in these tissues. The high intensity of the miR159, miR164 and miR168 hybridizing bands in contrast to their low frequency in the sequenced population suggests that the expression of these miRNAs might also be developmentally controlled. The strong hybridizing signals for miR167, and the relatively weak signals for miR157, miR161, miR171, miR390 and miR397 appeared consistent.

![Fig. 7. Expression analysis of *Brassica napus* miRNAs among the F1 and F2 DH lines used for small RNA library construction. RNA from mature leaves of Arabidopsis (A. Ler) was included as control. Gel blots of total RNA isolated from mature leaves of 70-day-old plants (A) or from F1 20-day-old seedlings (C) were hybridized with labeled complementary DNA oligonucleotides. The bottom panels of A and C are ethidium bromide-stained tRNA and 5S rRNA bands used as loading control (bottom-left). (B) The typical phenotypes of the five lines.](image-url)
with their relatively high and low frequencies in the sequenced population, respectively.

Apart from miR161, miR166, miR167, miR171 and miR390, which showed similar expression levels in *B. napus* and Arabidopsis, significant differences existed in the expression of the other miRNAs between the two different species. miR159, miR164, miR168 and miR397 were expressed at much lower levels in Arabidopsis than in *B. napus*, while the opposite is true for miR157. This could either reflect a difference between the *B. napus* and Arabidopsis tissue types used for small RNA extraction, or an inherent difference between the two species in miRNA expression patterns.

Northern blot hybridization was also carried out on several of the endogenous siRNAs identified in the cloned population. However, no expression was detected for these siRNAs, presumably due to their low abundance in mature leaves of *B. napus* (data not shown).

In conclusion, we have cloned and identified 11 conserved micro RNA families and examined their expression patterns in five double haploid *B. napus* lines. Recently, Xie et al. used computational methods to predict *B. napus* microRNAs and identified 21 potential *B. napus* miRNAs [54]. Interestingly, only two of our cloned miRNAs (bna-miR169k and bna-miR171c) matched the predicted sequences perfectly, indicating that cloning and sequencing are indispensable for identifying miRNAs. In addition to the conserved miRNAs, we have identified a new candidate miRNA family (bna-miR1140) which appears to be unique to Brassica. The discovery of such non-conserved miRNAs in Brassica is important for identifying Brassica-specific miRNA-dependent regulatory processes. One future effort will be to look for more novel miRNAs in Brassica. Expression analysis and target validation will be critical for determining the biological functions of both the conserved and novel miRNAs in Brassica.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.07.010.

References


