RESEARCH PAPER

Genome-wide identification of *Brassica napus* microRNAs and their targets in response to cadmium

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Received 6 March 2012; Revised 12 April 2012; Accepted 16 April 2012

Abstract

MicroRNAs (miRNAs) are a distinct class of small RNAs in plants that not only regulate biological processes but also regulate response to environmental stresses. The toxic heavy metal cadmium (Cd) induces expression of several miRNAs in rapeseed (*Brassica napus*), but it is not known on a genome-wide scale how the expression of miRNAs and their target genes, is regulated by Cd. In this study, four small RNA libraries and four degradome libraries were constructed from Cd-treated and non-Cd-treated roots and shoots of *B. napus* seedlings. Using high-throughput sequencing, the study identified 84 conserved and non-conserved miRNAs (belonging to 37 miRNA families) from Cd-treated and non-treated *B. napus*, including 19 miRNA members that were not identified before. Some of the miRNAs were validated by RNA gel blotting. Most of the identified miRNAs were found to be differentially expressed in roots/shoots or regulated by Cd exposure. The study simultaneously identified 802 targets for the 37 (24 conserved and 13 non-conserved) miRNA families, from which there are 200, 537, and 65 targets, belonging to categories I, II, and III, respectively. In category I alone, many novel targets for miRNAs were identified and shown to be involved in plant response to Cd.

Key words: *Brassica napus*, cadmium, degradome, deep sequencing, microRNAs.

Introduction

Toxic heavy metals such as cadmium (Cd) and mercury (Hg) constitute major contaminants due to their significant release into environments through anthropogenic activities (e.g. use of trace metal-containing fertilizers, sewage sludge, and fungicides) (Alloway and Steinnes, 1999; Chen et al., 2009). Cd ranks first among the top seven metals (Cd, Cr, Cu, Hg, Ni, Pb, and Zn) released into ecosystems (Han et al., 2002). Soils contaminated with Cd have increasingly become a concern, because Cd is mobile in soils and readily accumulated by crops. Thus, it affects not only crop productivity, but also brings risks to food safety (McLaughlin et al., 1999). Overload of Cd in plants leads to its binding to apoplastic and symplastic target sites, which disrupts basic mineral nutrition or blocks cell division and development (Prasad et al., 2001; Sun et al., 2007; Ahmad et al., 2009). A secondary toxic response such as oxidative stress may be evoked through the generation of reactive oxygen species by Cd (Rodriguez-Serrano et al., 2006). Thus, it is of great importance to minimize Cd concentrations in soils.

The use of plants to remove heavy metals from soils, namely phyto remediation, has been considered as cost-effective and environmentally friendly and has been widely used in agricultural practice (Ebbs et al., 1997; Pilon-Smits and Pilon, 2002; Chen et al., 2009). This technique emphasizes hyper-accumulation of heavy metals from soils and translocation of the hazards to above ground, thus reducing the metal concentrations in soils to a minimum level (McGrath et al., 2002). Recently, an alternative
way to limit heavy metals entering the food chain without treating soils has been proposed (Grant et al., 2008; Liu, 2009). This concept refers to breeding and genetic techniques to minimize the heavy metal accumulation in edible parts (e.g. grains and seeds) of crops. With this approach, selection of desirable cultivars (or genotypes) that accumulate very low amount of heavy metals is crucial, and the genetic modification of plant traits with the capability of decreasing accumulation of potentially heavy metals is of significance.

To dissect the mechanism for the metal accumulation, the first step is to understand Cd-responsive genes and their regulation networks. Previous studies have shown that transcription of many genes in plants could be induced by Cd exposure (Herbert et al., 2006). Some genes encoding for metal transporters are responsible for Cd uptake and sequestration (Bovet et al., 2003). Recent studies have demonstrated that heavy metal-regulated gene expression can be also achieved at post-transcriptional levels by a group of microRNAs (Zhou et al., 2008, 2012; Huang et al., 2009, 2010; Lima et al., 2011; Wang et al., 2011; Chen et al., 2012; Khraiwesh et al., 2012). Using microarray, 19 Cd-responsive microRNAs (miRNAs) were identified and their target genes were predicted in rice (Oryza sativa) (Ding et al., 2011). Recently, high-throughput sequencing technology has become a powerful tool to permit the concomitant sequencing of millions of signatures in genomes of single tissue (Fahlgren et al., 2007; Kwak et al., 2009; Xue et al., 2009). This approach highlights the advantage of providing a more thorough qualitative and quantitative description of gene expression than microarray technology. Using this approach, 52 new miRNAs with ~21 nucleotides have been profiled from Medicago truncatula seedlings exposed to mercury, most being differentially regulated by the heavy metal (Zhou et al., 2012). These results indicate that mRNA-regulated gene silencing may be involved in plant tolerance to heavy metals.

Brassica napus is one of the most importantly economical and biofuel crops. As a member of Brassicaceae family, B. napus possesses several traits such as fast growth, high biomass, moderate metal accumulation in aerial parts, ease of harvest, and tolerance to metals, and therefore, it is a desirable candidate plant for phytoremediation (Salt et al., 1995; Clemens et al., 2002). Using a computational approach, Xie et al. (2007) identified 21 miRNAs in B. napus and showed that several miRNAs responded to heavy metals. Shortly afterwards, 36 B. napus miRNAs representing 11 miRNA families were cloned using conventional sequencing (Wang et al., 2007). Furthermore, 13 miRNAs (nine families) were cloned from a small RNA library of B. napus seedlings with exposure to Cd and deficiency in sulphate (Huang et al., 2010). To date, a growing number of miRNAs from B. napus have been discovered using various advanced technologies (Bultz et al., 2008; He et al., 2008; Pant et al., 2009; Wei et al., 2010; Zhao et al., 2012). However, heavy metal-regulated miRNAs and their target genes have not been thoroughly identified in B. napus.

This study used the deep-sequencing technology developed by Solexa/Illumina to profile many more small RNAs and identify 84 conserved and non-conserved miRNAs from B. napus. It analysed miRNA abundance from four small RNA libraries created from Cd-treated and Cd-free roots and shoots. Deep sequencing of four degradome libraries allowed the identification of 802 targets for 37 miRNA families, of which 200, 537, and 65 in categories I, II, and III, respectively, were characterized. Some of the miRNA targets were identified as new transcripts involved in regulation of plant tolerance to Cd.

Materials and methods

Plant culture and treatment

Seeds of B. napus (line Texuan 4) were surface sterilized and germinated on a plastic net floating on 1/4-strength modified Hoagland nutrient solution (Huang et al., 2010). The plants were grown hydroponically for 14 d and then transferred to the same nutrient solution containing 0, 40, or 80 µM CdCl₂ for 0, 6, 24, or 48 h. Plants were grown with a 14/10 light/dark cycle at 24 ± 1 °C and 200 µmol m⁻² s⁻¹ light intensity. After treatment, roots and shoots were separately harvested and immediately frozen in liquid nitrogen.

Construction and sequencing of small RNA libraries

The creation of the small RNA libraries was based on the procedure of Kwak et al. (2009). Total RNA was isolated from frozen shoots and roots of B. napus with Trizol (Invitrogen). Four sets of total RNA were prepared from samples of Cd-free roots (R–Cd), Cd-treated roots (R+Cd), Cd-free shoots (S–Cd), and Cd-treated shoots (S+Cd). Each RNA sample was derived from the original RNA pool prepared from Cd-free or Cd-treated tissue (roots or leaves) at each time point (0, 6, 24, and 48 h). RNA samples were quantified and equalized so that equivalent amounts of RNA from each treatment were analysed. Total RNA was purified by electrophoretic separation on 15% TBE-urea denaturing polyacrylamide gel, and small RNA regions corresponding to the 18–30 nucleotide bands in the marker lane were excised and recovered. Each library underwent flow-cell cluster generation and bridge amplification (Solexa/Illumina). The sequencer, during automated cycles of extension, recorded fluorophore excitation and determined the sequence of bases for each cluster.

Analysis of small RNA sequencing data

Raw sequence reads were processed into clean full-length reads by the BGI small RNA pipeline. Unique small RNA sequences were mapped to the known B. napus miRNA sequences (Wang et al., 2007; Xie et al., 2007; Bultz et al., 2008; He et al., 2008; Pant et al., 2009; Huang et al., 2010). Small RNAs deposited at the Rfam and GenBank databases were identified using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The remaining unique small RNA sequences were mapped to the expressed sequence tags (EST) and tentative consensus (TC) sequences of the B. napus Gene Index (BrGI release 5.0, http://www.ncbi.nlm.nih.gov/nucest?term=Brassica_napus, http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=oilseed_rape) with no mismatch. miREAP (http://sourceforge.net/projects/mireap/) was used to extract the precursor sequences and check the base-pairing between the predicted miRNA and miRNA*. Mfold (http://mfold.rna.albany.edu/?q=mfold-RNA-Folding-Form, Zuker, 2003) was used to predict each precursor structure. The criteria were used for selecting the new miRNAs were according to Meyers et al. (2008).

Sequencing of degradome libraries and data analysis

The degradome libraries were constructed according to the method described by Addo-Quaye et al. (2008) and German et al. (2008). Poly(A) RNA was extracted from each sample of total RNA using the Oligotex kit (Qiagen). Polyadenylated transcripts possessing 5’-monophosphates were ligated to RNA adapters consisting of a MmeI recognition site at its 3’ end. After ligation, first-strand cDNA was generated using oligo d(T) and amplified using five PCR cycles. The PCR product was purified and digested with MmeI. The digested PCR product was then ligated to a 3’ double DNA adapter, amplified 18 PCR cycles, and gel-purified for Solexa/Illumina sequencing.

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Sequenced tags (18–21 nucleotides) were normalized after trimming sequence adapters and filtering the low-quality tags. The sliced miRNA targets were identified and classified into categories using the CleaveLand pipeline (Addo-Quaye et al., 2008, 2009a). Unique reads were normalized to give reads per million and subsequently mapped to annotated cDNA sequences from BnGI release 5.0 or *B. napus* precursors for miRNA analysis.

**Northern blotting**

For detection of miRNAs, 15 µg total RNA from samples was subjected to denaturing electrophoresis on 15% polyacrylamide gels. Carboxymethylated cross-linking of RNA to Hybond-NX was performed according to Pall et al. (2007). Membranes were hybridized with DNA oligonucleotides complementary to miRNA sequences, labelled with γ-32P-ATP using T4 polynucleotide kinase (Invitrogen) (Supplementary Table S1, available at JXB online). Blots were hybridized overnight at 37 °C in ULTRAhyb-Oligo hybridization buffer (Ambion) and washed twice with 0.2 × SSC and 0.1% SDS at 37 °C for 30 min. The membranes were exposed to phosphor imager plates.

**Statistical analysis**

Each result in this study is the mean of at least three replicated treatments and each treatment contained at least nine seedlings. Statistical analysis using a rigorous algorithm described previously (Audic and Claverie, 1997) was performed to identify small RNAs differentially expressed between libraries. For small RNAs, the Cd-stress library-derived sequences from BnGI release 5.0 or B. napus precursors for miRNA analysis. To identify small RNAs from *B. napus*, seedlings (2-week-old) were exposed to Cd at 0, 40, or 80 µM for 6–48 h. Shoots and roots were separately collected and small RNAs from the samples were isolated and pooled to generate four small RNA libraries for Cd-free roots (R–Cd), Cd-treated roots (R+Cd), Cd-free shoots (S–Cd), and Cd-treated shoots (S+Cd). Each library was individually sequenced using a Solexa/Illumina analyser. High-throughput sequencing generated 18,163,038 primary reads for R–Cd, 20,417,921 for R+Cd, 17,493,993 for S–Cd, and 18,482,210 for S+Cd, respectively (Table 1). After removal of low-quality reads, a total of 17,605,178, 19,592,894, 16,987,042, and 18,035,749 clean reads, corresponding to 5,978,720, 6,476,119, 3,131,102, and 5,753,497 unique signatures, remained for the R–Cd, R+Cd, S–Cd, and S+Cd libraries, respectively. The small RNA sequences were matched to the EST database at NCBI and TC sequence database at the Dana-Farber Cancer Institute gene index project of *B. napus*. When total reads were analysed, 24.45–43.52% reads could be matched to the EST and TC databases, respectively (Table 1). For unique reads, only 7.14–10.25% could be matched to the EST and TC databases. A large percentage of sequences failed to map because the *B. napus* genome has not yet been completely sequenced.

**Table 1.** Categorization and abundance of small RNA and degradome reads from Cd-free and Cd-treated roots and shoots of *B. napus*

| Library type                  | R–Cd         | R+Cd         | S–Cd         | S+Cd         |
|------------------------------|--------------|--------------|--------------|--------------|
| **Small RNA**                |              |              |              |              |
| Total raw reads              | 18,163,038   | 20,417,921   | 17,493,993   | 18,482,210   |
| Total clean reads            | 17,605,178   | 19,592,894   | 16,987,042   | 18,035,749   |
| Unique clean reads           | 5,978,720    | 6,476,119    | 3,131,102    | 5,753,497    |
| Total miRNA reads            | 3,268,352    | 3,491,958    | 9,045,499    | 5,295,055    |
| Total rRNA reads             | 964,750      | 1,498,791    | 966,51       | 816,473      |
| Total tRNA reads             |              |              |              |              |
| Total clean reads mapping to ESTs and TC sequences | 4,305,117 (24.45) | 5,180,501 (26.44) | 7,392,893 (43.52) | 5,532,517 (30.68) |
| Unique clean reads mapping to ESTs and TC sequences | 427,175 (7.14) | 466,292 (7.20) | 320,785 (10.25) | 461,185 (8.02) |
| **Degradome**                |              |              |              |              |
| Total raw reads              | 16,945,142   | 14,821,751   | 15,592,037   | 14,862,060   |
| Total clean reads            | 14,352,241   | 13,054,929   | 15,418,988   | 14,674,354   |
| Unique clean reads           | 817,705      | 804,892      | 5,602,100    | 6,277,974    |
| Total clean reads mapping to ESTs and TC sequences | 9,801,211 (68.29) | 9,234,518 (70.74) | 11,213,044 (72.72) | 10,805,736 (73.64) |
| Unique clean reads mapping to ESTs and TC sequences | 379,910 (46.46) | 411,845 (51.17) | 3,367,425 (60.11) | 3,813,251 (60.74) |
| Clean reads mapping to ESTs and TC sequences | 64,197 (64.19) | 65,710 (64.70) | 79,713 (78.48) | 79,050 (77.83) |
| Total clean reads mapping to miRNA precursors | 27,296 | 21,554 | 5286 | 7125 |
| Clean reads mapping to miRNA precursors | 38 | 49 | 69 | 79 |

Values are n or n (%) EST, expressed sequence tag; TC, tentative consensus; R–Cd, Cd-free roots; R+Cd, Cd-treated roots; S–Cd, Cd-free shoots; S+Cd, Cd-treated shoots.

**Results**

**Analysis of sequences from libraries**

To identify small RNAs from *B. napus*, seedlings (2-week-old) were exposed to Cd at 0, 40, or 80 µM for 6–48 h. Shoots and roots were separately collected and small RNAs from the samples were isolated and pooled to generate four small RNA libraries for Cd-free roots (R–Cd), Cd-treated roots (R+Cd), Cd-free shoots (S–Cd), and Cd-treated shoots (S+Cd). Each library was individually sequenced using a Solexa/Illumina analyser. High-throughput sequencing generated 18,163,038 primary reads for R–Cd, 20,417,921 for R+Cd, 17,493,993 for S–Cd, and 18,482,210 for S+Cd, respectively (Table 1). After removal of low-quality reads, a total of 17,605,178, 19,592,894, 16,987,042, and 18,035,749 clean reads, corresponding to 5,978,720, 6,476,119, 3,131,102, and 5,753,497 unique signatures, remained for the R–Cd, R+Cd, S–Cd, and S+Cd libraries, respectively. The small RNA sequences were matched to the EST database at NCBI and TC sequence database at the Dana-Farber Cancer Institute gene index project of *B. napus*. When total reads were analysed, 24.45–43.52% reads could be matched to the EST and TC databases, respectively (Table 1). For unique reads, only 7.14–10.25% could be matched to the EST and TC databases. A large percentage of sequences failed to map because the *B. napus* genome has not yet been completely sequenced.
The lengths of the small RNA sequences ranged from 18 to 28 nucleotides, but the 21- and 24-nt sequences were dominant in all libraries, and the 24-nt small RNAs were most abundant (Supplementary Fig. S1). This result was consistent with Dicer-derived products and most of the previous reports from other plant species (Sunkar and Zhu, 2004; Lelandais-Brière et al., 2009; Jeong et al., 2011). The patterns for 21- and 24-nt small RNA distribution were similar, but the abundances were not identical. For example, unique small RNAs were sequenced less often in S–Cd plants than in S+Cd plants, with decreases of about 11 and 45% for 21- and 24-nt small RNAs, respectively (Supplementary Fig. S1). This observation suggests that expression of small RNAs in shoots could be modulated by Cd exposure.

The proportions of common and specific small RNAs were further analysed between pairs of libraries (between roots and shoots, or between Cd-free and Cd-treated plants). For total small RNAs in all pairs of libraries, 69.99–75.61% were common to both libraries and 7.22–19.54% were specific to one library, respectively (Fig. 1 and Supplementary Fig. S2). However, for unique small RNAs, the opposite was found: there were larger proportions of specific sequences than those of common sequences. For example, analysis comparing Cd treatment in shoots showed that more than 60% of unique small RNAs were specific to the S+Cd library, whereas only 27.55% were specific to the S-Cd library (Fig. 1C). This tendency was also true for roots, in which 44.74% unique small RNAs were specific to the R+Cd library and 40.14% were specific to the R-Cd library (Fig. 1D). These results indicate that the expression of unique small RNAs was changed by Cd exposure.

Analysis of miRNA populations and abundances

To identify miRNAs from rapeseeds, the small RNA data sets (18–24 nt) were mapped to all publicly available miRNA sequences from *B. napus* and other species with two or fewer nucleotide mismatches (Wang et al., 2007; Xie et al., 2007; Buhtz et al., 2008; Pant et al., 2009; Huang et al., 2010; Griffiths-Jones et al., 2008). The alignment resulted in 3,268,352, 3,491,958, 9,045,499, and 5,295,055 matches for the R–Cd, R+Cd, S–Cd, and S+Cd libraries, respectively (Table 2). Among the miRNA populations, the 21-nt miRNAs were the most abundant and accounted for 69.91–75.63% of each library. The 20-nt miRNAs were the second-most abundant, comprising 22.46–28.49% of each library. The other miRNAs, with 18, 19, or 22–24 nt, comprised less than 2% of each library.
Choong et al., 2007; Moldovan et al., 2010) and Arabidopsis lyrata (Ma et al., 2010). Also, miR894 has been shown to exist only in Physcomitrella patens (Fattash et al., 2007). These miRNA families had a moderate or low abundance in the libraries. miR824, miR857, miR894, and miR2911 were preferentially expressed in roots, whereas miR1140 was preferentially expressed in shoots.

Identification of new miRNAs

To identify previously undiscovered miRNAs, a standard computation pipeline was applied based on the recently published criteria for plant microRNAs (Meyers et al., 2008). With this filter, 20–24-nt small RNA sequences were mapped to the B. napus EST database with no mismatch of nucleotides. All reads with low abundance (<10) were removed from the data set (Lister et al., 2009; Zhou et al., 2012). The data sets were also subjected to a query of the non-coding RNA sequences deposited in the GenBank and Rfam databases (Griffiths-Jones et al., 2008). Sequences matching rRNA, tRNA, snRNA, and snoRNA were removed. The consensus surrounding the regions of each sequence was retrieved and secondary structures were obtained (Zuker, 2003). All filtered small RNAs that could fold into a stem–loop structure were considered as miRNA candidates. Finally, 19 new loci belonging to eight conserved miRNA families and one non-conserved miRNA family were identified (Table 4 and Supplementary Table S3). These miRNAs were characterized by star strands (miRNA*) and have not been reported before. Additionally, 1731 miRNA homologues, exhibiting high similarity with miRNAs from other species, were identified using the criteria of no more than two nucleotide mismatches (Supplementary Table S4). However, these miRNAs had no B. napus ESTs or TC sequences to match and consequently their secondary structures could not be obtained.

Differential expression of miRNAs in response to cadmium

To identify the response of miRNAs to Cd, this study compared the abundance of miRNAs between any two libraries. To analyze differential expression of each miRNA family, reads were normalized on the basis of transcripts per million. Most miRNAs were differentially expressed in Cd-treated roots and shoots compared with the controls, but not all miRNA expression was significantly regulated by Cd (Table 3). In roots, there were eight miRNA families, whose expression were significantly regulated by Cd exposure ($P < 0.01$), including miR159, miR394, miR398, miR857, and miR2111 (Table 3) and miR172f, miR319d, and miR398b (Table 4). Of these, miR398, miR857, and miR172f were up-regulated by Cd exposure and the others were negatively regulated by Cd. In shoots, 13 miRNA families (miR158, miR159, miR161, miR162, miR164, miR171, miR319, miR394, miR395, miR400, miR858, miR1885, and miR2111) (Table 3) and five newly identified miRNAs (miR156m, miR158a, miR167f–h, miR167i, and miR319c) (Table 4) were found to be significantly regulated by Cd treatment ($P < 0.01$), of which four miRNA families (miR158, miR161, miR400, and miR1885) and two miRNA members (miR156m and miR158a) were up-regulated, and the others were down-regulated, by Cd exposure. In contrast, most miRNAs were found to be differentially expressed between roots and shoots under normal or Cd-stress conditions. Treatment with Cd could also result in altered expression between roots and shoots.

To confirm the expression of miRNAs identified by deep sequencing, 14 miRNAs with high and moderate sequencing counts were randomly selected for validation by RNA gel blotting. As shown in Fig. 2, all tested miRNAs were detected; only miR396 and miR400 showed very weak signals. Expression patterns were compared between RNA gel blotting and deep sequencing, and most of the results were comparable. miR156 and miR403 were more abundantly expressed in shoots than in roots. In shoots, expression of miR158 was up-regulated by Cd exposure, whereas expression of miR390 was down-regulated by Cd exposure. In roots, both miR397 and miR408 were induced by the presence of Cd. However, expression pattern of miR167 using Northern blotting was not in agreement with that from deep sequencing.

Identification of miRNA targets

Identification of miRNA targets is a prerequisite to understand the functions of miRNAs. At the time of writing, only some dozens of miRNAs from B. napus have been reported (Wang et al., 2007; Xie et al., 2007; Pant et al., 2009; Huang et al., 2010). Also, very few miRNA targets have been experimentally characterized (Huang et al., 2010). To identify more targets in B. napus, the present study performed a genome-wide analysis of miRNA-cleaved mRNAs using a recently developed high-throughput degradome sequencing technology (Addo-Quaye et al., 2008; German et al., 2008). This approach emphasizes detection of

| miRNA length (nt) | R–Cd | R+Cd | S–Cd | S+Cd |
|------------------|------|------|------|------|
| 18               | 3467 (0.11) | 5947 (0.17) | 8591 (0.09) | 4353 (0.08) |
| 19               | 12,573 (0.38) | 17,658 (0.51) | 31,048 (0.34) | 18,376 (0.35) |
| 20               | 733,937 (22.46) | 811,814 (23.25) | 2,576,903 (28.49) | 1,481,750 (27.98) |
| 21               | 2,471,928 (75.63) | 2,611,854 (74.8) | 6,323,660 (69.91) | 3,731,808 (70.48) |
| 22               | 42,182 (1.29) | 41,070 (1.18) | 96,782 (1.07) | 54,731 (1.03) |
| 23               | 3560 (0.11) | 2831 (0.08) | 8081 (0.09) | 3886 (0.07) |
| 24               | 705 (0.02) | 784 (0.02) | 434 (0) | 151 (0) |
| Total            | 3,268,352 (100) | 3,491,958 (100) | 9,045,499 (100) | 5,295,055 (100) |

Values are number of reads (%). R–Cd, Cd-free roots; R+Cd, Cd-treated roots; S–Cd, Cd-free shoots; S+Cd, Cd-treated shoots.
This study sequenced 14,821,751–16,945,142 signatures for each of the four libraries (Table 1). After removal of low-quality reads, adaptor contaminants, and shorter (<19 nt) reads, a total of 13,054,929–15,418,988 clean reads, corresponding to 804,892–6,277,974 unique reads, were obtained. The distribution of the total and unique reads between any two libraries and their lengths are presented in Supplementary Figs. S3 and S4. Mapping of the unique sequences to the *B. napus* cDNA database generated 64,197, 65,710, 79,713 and 79,050 ESTs and TC sequences for the R–Cd, R+Cd, S–Cd, and S+Cd libraries, respectively (Table 1).

The sliced targets for conserved and non-conserved miRNAs were identified according to the CleaveLand pipeline (Addo-Quaye *et al*., 2009a). Abundance of the sequences was plotted on each transcript (Supplementary Figs. S5 and S6). The degraded transcripts could be grouped into three categories based on cleavage products guided by miRNAs on a large scale and has been successfully used for characterizing hundreds of conserved and non-conserved miRNA targets from other plant species, e.g. rice (Li *et al*., 2010; Zhou *et al*., 2010), grapevine (Pantaleo *et al*., 2010), *M. truncatula* (Branscheid *et al*., 2011; Zhou *et al*., 2012), and soybean (Song *et al*., 2011).

### Table 3. Abundance of conserved and non-conserved miRNA families from Cd-free and Cd-treated roots and shoots of *B. napus*

| miRNA family | R–Cd | R+Cd | S–Cd | S+Cd | Log₂(R+Cd/R–Cd) | Log₂(S+Cd/S–Cd) | Log₂(S–Cd/R–Cd) | Log₂(S+Cd/R+Cd) |
|--------------|------|------|------|------|-----------------|-----------------|-----------------|-----------------|
| **Conserved miRNA** | | | | | | | | |
| 156/157 | 774,283 | 846,332 | 3,717,697 | 2,089,876 | -0.03 | -0.92 | 2.32* | 1.42* |
| 158 | 88,826 | 107,966 | 14,349 | 46,564 | 0.13 | 1.61* | -2.58* | -1.09* |
| 159 | 6676 | 2584 | 7648 | 2979 | -1.52* | -1.45* | 0.25 | 0.32 |
| 160 | 5023 | 5411 | 4497 | 3122 | -0.05 | -0.61 | -0.11 | -0.67 |
| 161 | 218 | 314 | 97 | 599 | 0.37 | 2.54* | -1.12* | 1.05* |
| 162 | 1978 | 2154 | 9406 | 3181 | -0.03 | -1.65* | 2.30* | 0.68 |
| 164 | 21,068 | 23,906 | 121,164 | 33,924 | 0.03 | -1.92* | 2.58* | 0.82 |
| 165/166 | 216,066 | 187,271 | 269,046 | 186,617 | -0.36 | -0.61 | 0.37 | 0.11 |
| 167 | 1,910,348 | 2,003,033 | 4,010,133 | 2,266,764 | -0.09 | -0.91 | 1.12* | 0.30 |
| 168 | 174,898 | 219,716 | 601,579 | 478,276 | 0.17 | -0.42 | 1.83* | 1.24* |
| 169 | 21,256 | 23,023 | 13,945 | 9901 | -0.04 | -0.58 | -0.56 | -1.10* |
| 171 | 509 | 588 | 6273 | 3205 | 0.05 | -1.06* | 3.67* | 2.57* |
| 172 | 5723 | 7653 | 11,749 | 17,034 | 0.26 | 0.45 | 1.09* | 1.27* |
| 319 | 1453 | 1468 | 39 | 5 | -0.14 | -3.05* | -5.17* | -8.08* |
| 390 | 2990 | 4744 | 2822 | 1972 | 0.51 | -0.60 | -0.03 | -1.15* |
| 391 | 495 | 513 | 13,579 | 8823 | -0.10 | -0.71 | 4.83* | 4.22* |
| 393 | 83 | 111 | 231 | 338 | 0.27 | 0.46 | 1.53* | 1.73* |
| 394 | 78 | 39 | 184 | 24 | -1.16* | -3.03* | 1.29* | -0.58 |
| 395 | 68 | 62 | 78 | 41 | -0.29 | -1.01* | 0.25 | -0.48 |
| 396 | 2066 | 1879 | 6906 | 4343 | -0.29 | -0.76 | 1.79* | 1.33* |
| 397 | 3087 | 5702 | 1533 | 1390 | 0.73 | -0.23 | -0.96 | -1.92* |
| 398 | 23 | 108 | 188 | 302 | 2.08* | 0.60 | 3.08* | 1.60* |
| 399 | 48 | 71 | 100 | 72 | 0.41 | -0.56 | 1.11* | 0.14 |
| 408 | 9,554 | 19,831 | 220,195 | 122,181 | 0.90 | -0.94 | 4.58* | 2.74* |
| **Non-conserved miRNA** | | | | | | | | |
| 400 | 408 | 422 | 123 | 522 | -0.11 | 2.00* | -1.68* | 0.43 |
| 403 | 3580 | 3783 | 6430 | 7382 | -0.07 | 0.11 | 0.90 | 1.08* |
| 824 | 3363 | 4259 | 741 | 1021 | 0.19 | 0.38 | -2.13* | -1.94* |
| 827 | 246 | 306 | 360 | 342 | 0.16 | -0.16 | 0.60 | 0.28 |
| 857 | 164 | 365 | 52 | 64 | 1.00* | 0.21 | -1.61* | -2.39* |
| 858 | 9 | 5 | 28 | 3 | -1.00 | -3.31* | 1.69 | -0.62 |
| 860 | 78 | 59 | 30 | 39 | -0.56 | 0.29 | -1.33* | -0.48 |
| 894 | 10,926 | 15,229 | 1389 | 1404 | 0.32 | -0.07 | -2.92* | -3.32* |
| 1140 | 721 | 950 | 2358 | 1929 | 0.24 | -0.38 | 1.76* | 1.14* |
| 1863 | 41 | 69 | 41 | 43 | 0.60 | -0.02 | 0.05 | -0.56 |
| 1885 | 569 | 531 | 161 | 563 | -0.25 | 1.72* | -1.77* | 0.20 |
| 2111 | 112 | 62 | 41 | 11 | -1.01* | -1.98* | -1.40* | -2.38* |
| 2911 | 1318 | 1439 | 308 | 200 | -0.03 | -0.71 | -2.05* | -2.73* |

Values are number of reads. * indicates significant differences in expression between two treatments (P < 0.01 and \( \log_2 N \) ≥ 1). R–Cd, Cd-free roots; R+Cd, Cd-treated roots; S–Cd, Cd-free shoots; S+Cd, Cd-treated shoots.
| miRNA     | Mature sequence (5'–3') | R–Cd   | R+Cd   | S–Cd   | S+Cd   | \( \log_2(\frac{R+Cd}{R-Cd}) \) | \( \log_2(\frac{S+Cd}{S-Cd}) \) | \( \log_2(\frac{S-Cd}{R-Cd}) \) | \( \log_2(\frac{S+Cd}{R+Cd}) \) | Total miRNA* |
|-----------|------------------------|--------|--------|--------|--------|--------------------------------|-------------------------------|-------------------------------|--------------------------------|----------------|
| miR156g–l | UGACAGAAGAGAGUGAGCAC   | 650,821| 710,264| 2,443,327| 1,284,542| -0.03                          | -0.91                         | 1.96*                         | 1.08*                         | 2               |
| miR156m   | UUGACAGAAGAAGAGAGAC    | 4517   | 5125   | 2845   | 98,985 | 0.03                            | 5.03*                         | -0.62                         | 4.39*                         | 1               |
| miR158a   | UUUCAAUAUGUAAGCAAAGCA  | 47,104 | 56,696 | 11,996 | 42,611 | 0.16                            | 1.74*                         | -1.92*                        | -0.34                         | 6               |
| miR160b   | GCGUACAGAGUAAGCAAGAU   | 326    | 239    | 883    | 540    | -0.60                          | -0.80                         | 1.49*                         | 1.30*                         | 813             |
| miR160c   | UCUGUGGUCCUGAGAGUCCA   | 11     | 14     | 26     | 8      | 0.19                            | -1.79                         | 1.29                          | -0.69                         | 3               |
| miR167f–h | UGAAGCUGCCAGAAGAUUC    | 588    | 717    | 14,284 | 6090   | 0.13                            | -1.32*                        | 4.65*                         | 3.21*                         | 2               |
| miR167i   | UGAAGCUGCCAGAAGAUCUU   | 14,135 | 17,376 | 31,548 | 13,010 | 0.14                            | -1.36*                        | 1.21*                         | -0.30                         | 15              |
| miR168c   | UCUGUGGUCCAGGUGGAGAAA  | 17     | 14     | 33     | 22     | -0.43                          | -0.67                         | 1.01                          | 0.77                          | 4               |
| miR172f   | GAAGUUGUGAAGGUGCUA     | 11     | 45     | 96     | 141    | 1.88*                          | 0.47                          | 3.18*                         | 1.77*                         | 9               |
| miR319c   | GAGCUUUCUUGUGUGCUCAC   | 1111   | 1417   | 38     | 3      | 0.20                            | -3.75*                        | -4.82*                        | -8.76*                        | 570             |
| miR319d   | UUUGGAUGAGGGGAGGAGCU   | 72     | 21     | 0      | 1      | -1.93*                         | -0.90                         | -6.12*                        | -4.27*                        | 1               |
| miR398b   | GGUGAGAUAUGUGAAGACAUG  | 21     | 96     | 145    | 253    | 2.04*                          | 0.72                          | 2.84*                         | 1.52*                         | 28              |
| miR860a–b | UCAGUUGAGUGAGAUGAUA    | 78     | 59     | 30     | 39     | -0.56                          | 0.29                          | -1.33*                        | -0.48                         | 2               |

Values are number of reads. * indicates significant differences in expression between pairs of libraries \( P < 0.01 \) and \( |\log_2(N)| \geq 1 \). R–Cd, Cd-free roots; R+Cd, Cd-treated roots; S–Cd, Cd-free shoots; S+Cd, Cd-treated shoots.
the relative abundance of the tags sequenced at the target sites (Addo-Quaye et al., 2008). Based on the criteria, category I species were the most abundant degradome tags, in which the expected site is cleaved by corresponding miRNAs; category II comprised degradome sequences with more than one raw read at the position, abundance at position less than the maximum but higher than the median for the transcript; and category III contained all of the other transcripts sliced by miRNAs. Apparently, category I targets always had much higher degradome tags and lower false rates of miRNA-guided cleavage. In total, 802 non-redundant targets of 37 (24 conserved and 13 non-conserved) miRNAs were obtained. There were 200, 537, and 65 targets in categories I, II, and III, respectively (Table 5 and Supplementary Tables S5 and S6). The distribution pattern is similar to recent reports in other plants (Li et al., 2010; Pantaleo et al., 2010; Zhou et al., 2010, 2012). For category I transcripts, they could also be present in category II or III. Taking the R–Cd library as an example, there were 12 and 16 miRNA targets in categories II and III that could be detected in category I in the other three libraries (Table 5).

Table 5. Summary of the miRNA target categories from Cd-free and Cd-treated roots and shoots of B. napus

| Category | R–Cd | R+Cd | S–Cd | S+Cd | Total non-redundant targets |
|----------|------|------|------|------|-----------------------------|
| I        | 36   | 12   | 16   | 85   | 108                         |
| II       | 41   | 30   | 63   | 16   | 305                         |
| III      | 12   | 17   | 25   | 12   | 55                          |
| Total    | 36   | 53   | 58   | 81   | 116                         |

Categories are defined according to Addo-Quaye et al. (2008). R–Cd, Cd-free roots; R+Cd, Cd-treated roots; S–Cd, Cd-free shoots; S+Cd, Cd-treated shoots.

Table 6. Analysis of the miRNA targets between pairs of libraries of B. napus

| R–Cd vs. R+Cd | S–Cd vs. S+Cd | R–Cd vs. S–Cd | R+Cd vs. S+Cd |
|---------------|---------------|---------------|---------------|
| Common (I, II, III) | 82 (43, 37, 2) | 353 (126, 221, 6) | 93 (44, 49, 0) | 111 (67, 42, 2) |
| Only in Cd-free library | 66             | 166            | 55             | 75             |
| Only in Cd-treated library | 104            | 209            | 426            | 451            |

R–Cd, Cd-free roots; R+Cd, Cd-treated roots; S–Cd, Cd-free shoots; S+Cd, Cd-treated shoots.
between any two libraries (Table 6). Apart from the common targets, there were more specific targets detected in Cd-exposed than in Cd-free libraries. This suggests that treatment with Cd intensified the cleavage of miRNA targets, resulting in the accumulation of sliced transcripts.

Because targets belong to category I usually have sites that are more accurately cleaved by miRNAs, this group of targets was analysed in more detail. As shown in Table 7 and Supplementary Fig. S5, of the 24 highly conserved miRNA families, 22 (except for miR391 and miR398) were identified to target 177 transcripts. Most of the miRNAs had multiple targets, except for miR161 and miR399 which had only one. miR156 had the highest number of targets with 28 transcripts, from which 23 transcripts encode different proteins. Also, there were 19 targets identified for miR167, of which 12 come from different gene families. By contrast, miR165, miR319, miR390, miR394, and miR395 had only two targets. Most of the targets for conserved miRNAs were conserved. miR395 targeted a plasma membrane sulphate transporter and an ATP sulphurylase, both of which have been well described previously (Kawashima et al., 2009; Liang et al., 2010). However, some of the conserved miRNAs may also target non-conserved or novel transcripts. For instance, a transcript encoding a malate synthase was identified as a new target for miR396. Phosphatase, a putative new target for miR394, was also identified in this study. Moreover, some transcripts targeted by conserved miRNAs are involved in plant response to environmental stresses, including those encoding for laccase (TC164751, miR397), NRAMP-type metal transporters (CD826328 and GT073274, miR167), and monothiol glutaredoxin (TC185396, miR164).

Identification of target miRNAs for non-conserved miRNAs

There were 23 category I targets identified for seven non-conserved miRNA families (Table 7). The target distribution and abundance varied from one library to another (Supplementary Table S5), suggesting that cleavage by miRNAs could be mediated by metal stress. miRNAs in category I targeted genes that are involved in diverse biological functions. In addition, some miRNA targets were identified as environmentally responsive genes. Apart from the conserved targets, some new targets were identified. For instance, miR400 targeted a transcript encoding for a putative salt-inducible protein; miR408 targeted an ascorbate oxidase; and miR860 targeted an enolase. These targets are closely associated with plant tolerance to environmental stresses (Cho et al., 2006; Ameline-Torregrosa et al., 2008; Zörb et al., 2010). Additionally, one target for miR414 was identified to encode for an ubiquitin carrier protein. However, most of the detected transcripts have not been functionally annotated. The non-conserved miRNAs usually had relatively less targeted transcripts than the conserved miRNAs.

Analysis of pre-miRNA degradome patterns

The plant homologue of Dicer or Dicer-like 1 (DCL1) cleaves both primary miRNA transcripts (pri-miRNAs) and miRNA precursors (pre-miRNAs) in the nucleus (Kim et al., 2010). Similarly to AGO-catalysed slicing, the remnants with 5’-monophosphate of pri-miRNAs and pre-miRNAs by DCL1 dicing may be identified by parallel analysis of RNA ends (PARE) or degradome sequencing (German et al., 2008; Addo-Quaye et al., 2009b; Li et al., 2010). A total of 27,296, 21,554, 5286, and 7125 degradome signatures were perfectly mapped to 38, 49, 69, and 79 (conserved and non-conserved) pre-miRNAs in the R–Cd, R+Cd, S–Cd, and S+Cd libraries, respectively (Table 1). In all, 82 of 94 (87.23%) unique pre-miRNAs of B. napus identified from this study had one or more mapping degradome reads. The abundance of degradome signatures corresponding to pre-miRNAs at the four typical sites, the starts and ends of miRNA and miRNA*, was frequently higher than that at other sites, suggesting that DCL1 processes the primary miRNA transcripts precisely (Supplementary Fig. S7). There were 64 unique pre-miRNAs, including 15 of 19 newly identified pre-miRNAs with degradome signatures at the starts/ends of miRNA/miRNA*, corresponding to 26, 17, 52, and 55 in the R–Cd, R+Cd, S–Cd, and S+Cd libraries, respectively (Table 8, Supplementary Tables S3 and S7 and Supplementary Fig. S7). Pre-miRNA degradome patterns can distinguish pri-miRNA transcripts from siRNA-generating transcripts (Li et al., 2010). The present analysis demonstrates that miRNAs that are generated from the 64 precursors are bona fide miRNAs. Of the 64 unique pre-miRNAs, 59 (92.2%) had degradome signatures at the miRNA start, which was higher than those at the miRNA end (19), the miRNA* start (21), and the miRNA* end (11) (Table 8). These results indicate that the 5′ remnants cleaved by DCL1 at the miRNA start are usually stable and beneficial for the generation of miRNA mature sequences.

Discussion

As post-transcriptional regulators, miRNAs have been found in all eukaryotic plants and are involved in response to various environmental stresses (Zhang et al., 2006; Khraiwesh et al., 2012). To identify more miRNAs and those in response to heavy metals from B. napus, high-throughput sequencing was performed. This study identified 84 miRNAs (including new members of miRNAs) and 1731 miRNA homologues from B. napus. Of these, 75 were identified as conserved. This group of miRNAs shares several common features with those from other plant species. First, the conserved miRNAs usually showed higher expression abundance. Taking the Cd-free root and shoot libraries as an example, the average read counts for the conserved miRNA families were 135,284 and 376,393, respectively, whereas those for non-conserved miRNA families were 1657 and 928, respectively (Table 3). Second, the conserved miRNAs had more family members than the non-conserved miRNAs. The average number of family members for the conserved miRNAs was 3.13, whereas for non-conserved miRNA families was 1.8 (Supplementary Tables S2 and S3). Third, more targets (e.g. category I) were identified for the conserved miRNAs than for the non-conserved miRNAs (Table 7). Also, most targets for conserved miRNAs were associated with developmental processes and transcription regulation, and less were associated with response to environmental stress and signal transduction. These results are consistent with previous reports in A. thaliana.
Table 7. Category I targets identified from any of the four degradomes from Cd-free and Cd-treated roots and shoots of B. napus

| miRNA     | Target genes | Score | Target category | Target gene annotation                                      |
|-----------|--------------|-------|-----------------|-------------------------------------------------------------|
|           |              |       | R–Cd | R+Cd | S–Cd | S+Cd |
| Conserved miRNA | | | | | | |
| miR156    | TC210178     | 1     | I    | I    | I    | I    | Squamosa promoter-binding-like protein 2 |
| miR156    | TC182990     | 1     | I    | I    | I    | I    | Squamosa promoter-binding-like protein 2 |
| miR156    | TC169034     | 1.5   | no   | no   | I    | I    | Squamosa promoter-binding-like protein 3 |
| miR156    | TC177533     | 1     | no   | no   | I    | I    | Squamosa promoter-binding-like protein 3 |
| miR156    | TC195915     | 1     | no   | I    | I    | I    | Squamosa promoter-binding-like protein 10 |
| miR156    | TC200337     | 1     | no   | I    | I    | I    | Squamosa promoter-binding-like protein 10 |
| miR156    | ES997975     | 1     | no   | I    | I    | I    | Squamosa promoter-binding-like protein 15 |
| miR156    | TC213662     | 3.5   | no   | no   | I    | no   | Glutathione-γ-glutamylcysteinyl transferase 2 |
| miR156    | TC204681     | 3.5   | no   | no   | no   | I    | 40S ribosomal protein Sa-1 |
| miR156    | EV002651     | 4     | no   | II   | no   | I    | Probable pleiotropic drug resistance protein 5 |
| miR156    | TC175179     | 4     | no   | no   | I    | no   | RING-U-box superfamily protein |
| miR156    | TC168211     | 3.5   | no   | I    | II   | II   | OST3/OST6 family protein |
| miR156    | TC174107     | 3     | I    | no   | no   | no   | Chromosome chr5 scaffold_2 |
| miR156    | FG560749     | 3.5   | no   | I    | no   | no   | ATGSL03 (GLUCAN SYNTHASE-LIKE 3); 1,3-beta-glucan synthase/transferase |
| miR156    | TC171252     | 3.5   | no   | I    | II   | II   | Transcriptional regulator |
| miR156    | TC205146     | 3     | II   | II   | I    | I    | unknown protein |
| miR156    | TC168656     | 3     | no   | no   | I    | II   | SAE1-S9-protein |
| miR156    | TC194880     | 3     | no   | no   | II   | I    | GATA transcription factor 27 |
| miR156    | FG576933     | 3     | no   | no   | no   | I    | Genomic DNA |
| miR156    | TC195666     | 3.5   | no   | I    | II   | no   | Serine/threonine-protein kinase Nek3 |
| miR156    | ES904551     | 4     | no   | I    | II   | I    | Eukaryotic aspartyl protease family protein |
| miR156    | TC183712     | 3.5   | I    | I    | no   | II   | Luminal-binding protein 2 precursor |
| miR156    | TC211628     | 3.5   | no   | no   | no   | I    | Unknown binding protein |
| miR156    | ES952034     | 3.5   | I    | no   | no   | no   | Probable histone H2A.1 |
| miR156    | TC185930     | 3.5   | no   | I    | no   | no   | Ferredoxin thioredoxin reductase |
| miR156*   | CD81244      | 3     | I    | no   | no   | II   | DEAD-box ATP-dependent RNA helicase 3 |
| miR156*   | ES265305     | 4     | no   | I    | no   | no   | A subfamily of OB folds |
| miR157    | TC171779     | 3     | I    | no   | no   | no   | RING-H2 finger protein |
| miR157    | DY030585     | 3.5   | no   | no   | I    | no   | Chromosome undetermined scaffold_30 |
| miR157    | TC165728     | 3.5   | no   | no   | I    | no   | Chromosome undetermined scaffold_30 |
| miR157    | EE506890     | 4     | no   | I    | II   | no   | RSZp21 protein |
| miR157    | TC171167     | 3.5   | I    | no   | no   | no   | Expressed protein |
| miR157    | TC175876     | 3.5   | I    | no   | no   | no   | Expressed protein |
| miR157    | CD813575     | 3.5   | no   | I    | no   | no   | Chromosome undetermined scaffold_227 |
| miR157    | ES907812     | 3.5   | I    | no   | no   | II   | Thioredoxin M-type 3 |
| miR157    | EL625648     | 3.5   | no   | no   | I    | II   | Uncharacterized protein |
| miR158    | TC181466     | 3.5   | no   | no   | I    | II   | DEAD-box ATP-dependent RNA helicase 6 |
| miR158    | TC183657     | 3.5   | no   | no   | I    | II   | DEAD-box ATP-dependent RNA helicase 6 |
| miR159    | TC190748     | 2.5   | no   | I    | I    | I    | Similarity to NAM |
| miR159    | EV087133     | 2.5   | I    | I    | I    | I    | MYB65 |
| miR159    | CX195998     | 3.5   | no   | no   | I    | I    | Genomic DNA |
| miR159    | DW999433     | 3.5   | no   | no   | I    | I    | YDL167c ARP1 singleton partial |
| miR159    | FG567250     | 3.5   | no   | no   | I    | I    | ABC transporter |
| miR159    | GR446300     | 3.5   | no   | no   | I    | I    | Chromosome chr17 scaffold_12 |
| miR159    | TC186567     | 4     | no   | I    | II   | no   | Chromosome chr18 scaffold_1 |
| miR159    | DY030757     | 3.5   | no   | no   | III  | I    | Chromosome chr12 scaffold_47 |
| miR160    | TC193317     | 1     | I    | I    | I    | I    | Auxin response factor 16 |
| miR160    | GT084423     | 3.5   | no   | no   | no   | I    | Unknown |
| miR160    | TC201448     | 0.5   | I    | no   | I    | I    | Auxin response factor 17 |
| miR160*   | TC165518     | 0.5   | I    | I    | I    | I    | Auxin response factor 17 |
| miR160*   | TC183439     | 4     | no   | no   | no   | I    | Expressed protein |
| miR160*   | TC188717     | 4     | no   | no   | no   | I    | Chromosome chr8 scaffold_23 |
| miR161    | FG573058     | 2.5   | no   | no   | no   | no   | Pentatricopeptide repeat-containing protein |
| mRNA     | Target genes | Score | Target category | Target gene annotation                                      |
|----------|--------------|-------|-----------------|-------------------------------------------------------------|
| miR162  | EE408149     | 4     | no              | no              | no              | I               | RING/U-boxdomain-containing protein               |
| miR162' | TC203508     | 4     | no              | III             | II             | I               | Cytochrome P450-like protein                      |
| miR164  | TC168009     | 1     | I               | no              | I              | I               | Protein CUP-SHAPED COTYLEDON 1                    |
| miR164  | TC186885     | 1.5   | no              | no              | I              | I               | NAM (No apical meristem)-like protein            |
| miR164  | TC211305     | 1     | no              | no              | I              | I               | NAC domain-containing protein 21/22              |
| miR164  | TC185396     | 4     | no              | I               | I              | I               | Monothiol glutaredoxin-S12                        |
| miR164  | TC203633     | 4     | I               | no              | II             | no              | Sorting nexin 1                                 |
| miR164  | TC163443     | 4     | III             | III             | I              | no              | Carbohydrate-binding X8 domain-containing protein |
| miR164  | TC210593     | 4     | no              | no              | no             | I               | Chalcone synthase                               |
| miR164  | EV183736     | 4     | no              | no              | I              | no              | Phosphate starvation response regulator 1        |
| miR164  | EE438989     | 3     | no              | no              | II             | I               | Unknown                                        |
| miR164  | TC186668     | 3     | no              | no              | I              | Unknown                                        |
| miR164  | ES914070     | 3.5   | no              | no              | I              | II              | Unknown                                        |
| miR165  | EV102172     | 2.5   | no              | no              | I              | no              | Transcriptional regulator                       |
| miR165  | EE562244     | 3.5   | I               | I               | I              | I               | Class III HD-Zip protein 1                       |
| miR166  | TC167613     | 2     | II              | I               | I              | I               | Homeodomain-leucine zipper protein               |
| miR166  | TC192563     | 2     | no              | II              | I              | I               | HD-zip protein                                  |
| miR166  | TC162295     | 3     | no              | no              | I              | no              | Development and lipid accumulation within the tapetum |
| miR166  | TC166514     | 3     | no              | no              | I              | no              | Development and lipid accumulation within the tapetum |
| miR166  | TC189133     | 3     | no              | no              | I              | no              | Development and lipid accumulation within the tapetum |
| miR166  | TC196490     | 3     | no              | no              | I              | II             | Development and lipid accumulation within the tapetum |
| miR166  | ES911720     | 3     | no              | no              | no             | I               | At1g10410/F14N23_31 Protein of unknown           |
| miR166  | ES963909     | 4     | no              | no              | I              | no              | Unknown                                        |
| miR166  | EE424026     | 4     | I               | II              | II             | I               | Peptide chain release factor subunit 1–3         |
| miR166  | EV172600     | 3     | no              | II              | I              | I               | Unknown                                        |
| miR166  | TC181758     | 3     | no              | II              | I              | II             | Uncharacterized protein                         |
| miR166* | EV089744     | 3.5   | no              | no              | I              | II             | Uncharacterized protein                         |
| miR167  | TC163509     | 3.5   | no              | I               | II             | I               | Auxin response factor 8                          |
| miR167  | TC179576     | 3.5   | II              | II              | I               | I               | Auxin response factor 8                          |
| miR167  | TC212888     | 3.5   | II              | II              | I               | I               | Auxin response factor 8                          |
| miR167  | TC183925     | 3.5   | no              | I               | I               | I               | ARF6                                           |
| miR167  | TC200079     | 3.5   | no              | I               | I               | I               | ARF6                                           |
| miR167  | TC208397     | 3.5   | no              | I               | I               | I               | ARF6                                           |
| miR167  | TC188972     | 3.5   | no              | no              | I               | I               | Putative U2 snRNP auxiliary factor small subunit |
| miR167  | TC205461     | 4     | no              | no              | no             | I               | Auxin efflux carrier component 1                |
| miR167  | FG560824     | 4     | I               | I               | no              | no              | Probable WRKY transcription factor 21            |
| miR167  | TC164111     | 4     | I               | I               | no              | no              | Probable WRKY transcription factor 21            |
| miR167  | EE562388     | 4     | II              | I               | no              | no              | Uncharacterized protein                         |
| miR167  | TC196372     | 4     | no              | no              | I               | no              | Unknown                                        |
| miR167  | TC204819     | 4     | no              | no              | I               | no              | Invertase-like protein                          |
| miR167  | CD826328     | 3     | no              | I               | no              | no              | Metal transporter Nramp1                        |
| miR167  | GT073274     | 3     | no              | I               | no              | no              | Metal transporter Nramp1                        |
| miR167  | EL623555     | 3     | no              | I               | no              | no              | F-box only protein 6                            |
| miR167  | GT076997     | 3.5   | I               | no              | no              | no              | Uncharacterized protein                         |
| miR167  | TC163902     | 3.5   | II              | no              | I               | I               | Peptidase M1 family protein                     |
| miR167  | TC178278     | 3.5   | II              | no              | II             | I               | Peptidase M1 family protein                     |
| miR168  | TC193360     | 3.5   | no              | no              | I               | no              | S-adenosyl-L-methionine-dependent methyltransferases superfamily protein |
| miR168  | TC204355     | 3     | II              | no              | II             | I               | Hypothetical protein                            |
| miR168  | TC196158     | 3.5   | I               | no              | I               | I               | Involved in cation homeostasis and transport     |
| miR168  | ES952129     | 4     | no              | no              | no             | I               | Unknown                                        |
| miR168  | TC161728     | 3     | I               | III             | III             | III             | NAC-domain protein 5–7                           |
| miR168  | TC207530     | 3.5   | no              | no              | II             | I               | Chromosome undetermined SCAF10321                |
| miR169  | TC161690     | 2.5   | no              | I               | I               | I               | CCAAT-binding factor B subunit homologue         |
| miR169  | TC200850     | 3     | I               | no              | I               | I               | CCAAT-binding factor B subunit homologue         |
| miRNA | miRNA* | Target genes | Score | Target category | Target gene annotation |
|-------|--------|--------------|-------|-----------------|------------------------|
| miR169 | miR169* | TC183411 | 2.5 | no | I | I | CCAAT-binding factor B subunit homologue |
| miR169 | miR169* | TC204571 | 2.5 | no | I | I | CCAAT-binding factor B subunit homologue |
| miR169 | miR169* | TC2184180 | 4 | no | I | no | no | Chromosome chr11 scaffold_13 |
| miR169 | miR169* | TC202311 | 4 | no | I | no | no | Chromosome chr11 scaffold_13 |
| miR169 | miR169 | EE543166 | 1.5 | no | no | I | I | CCAAT-binding factor B subunit homologue |
| miR169 | miR169 | EV064177 | 2.5 | I | no | I | I | CCAAT-binding factor B subunit homologue |
| miR169 | miR169 | TC212312 | 1.5 | no | no | II | I | Isoform 2 of Q8SQD7 |
| miR169 | miR169 | TC169941 | 2.5 | no | no | I | I | Nuclear transcription factor Y subunit A-1 |
| miR169 | miR169* | ES991856 | 4 | no | no | I | no | Serine/threonine protein phosphatase 7 inactive homologue |
| miR169 | miR169* | TC167595 | 3 | no | I | II | II | Ubiquinol-cytochrome C chaperone family protein |
| miR169 | miR169* | TC188279 | 3 | no | I | II | II | Ubiquinol-cytochrome C chaperone family protein |
| miR171 | miR171 | FG563769 | 4 | no | no | no | I | Nucleoside diphosphate kinase family protein |
| miR171 | miR171 | TC191279 | 1 | no | no | I | I | Ap2 SCARECROW-like protein |
| miR172 | miR172 | ES922267 | 3 | no | I | no | no | Chromosome chr18 scaffold_1 |
| miR172 | miR172 | TC184340 | 0.5 | no | no | I | I | AP2-like ethylene-responsive transcription factor |
| miR172 | miR172 | DY020927 | 0.5 | no | I | I | I | AP2-like ethylene-responsive transcription factor |
| miR172 | miR172 | TC200318 | 0.5 | no | II | I | I | AP2-like ethylene-responsive transcription factor |
| miR172 | miR172 | TC184400 | 1.5 | no | III | I | I | Ethylene-responsive transcription factor |
| miR172 | miR172 | TC161595 | 3.5 | no | no | I | II | Shaggy-related protein kinase theta |
| miR172 | miR172 | TC192206 | 1.5 | no | II | I | I | Ethylene-responsive transcription factor |
| miR172 | miR172 | TC195815 | 0.5 | no | no | I | I | AP2-like transcriptional factor |
| miR172 | miR172 | TC196185 | 0.5 | no | no | I | I | Floral homeotic protein APETALA 2 |
| miR172 | miR172 | TC205794 | 0.5 | no | no | I | I | AP2-like transcriptional factor |
| miR172 | miR172 | TC209791 | 0.5 | no | no | I | I | Floral homeotic protein APETALA 2 |
| miR172 | miR172 | DY012557 | 2 | no | no | I | II | Eukaryotic translation initiation factor 3 subunit E-interacting protein |
| miR172* | miR172* | TC177968 | 2.5 | I | no | no | II | Unknown protein; CONTAINS InterPro DOMAIN |
| miR172* | miR172* | TC183087 | 3 | no | no | I | no | Serine/arginine-rich protein |
| miR319 | miR319 | TC166304 | 2.5 | II | I | I | I | TCP family transcription factor |
| miR319 | miR319 | TC178420 | 2.5 | II | I | I | I | TCP family transcription factor |
| miR390 | miR390 | TC164858 | 4 | II | I | II | I | Encodes a trans-acting siRNA (tasi-RNA) |
| miR390 | miR390 | TC175812 | 3.5 | I | no | no | no | Rhomboid family |
| miR393 | miR393 | EV007466 | 1 | I | II | I | I | Protein AUXIN SIGNALING F-BOX 3 |
| miR393 | miR393 | EV038237 | 1 | I | I | I | I | Protein AUXIN SIGNALING F-BOX 3 |
| miR393 | miR393 | TC175423 | 1 | no | III | I | I | Protein AUXIN SIGNALING F-BOX 3 |
| miR393 | miR393 | TC184499 | 1 | I | II | I | I | Protein AUXIN SIGNALING F-BOX 3 |
| miR393 | miR393 | TC188384 | 1 | I | II | I | I | Protein AUXIN SIGNALING F-BOX 3 |
| miR393 | miR393 | TC175098 | 2.5 | no | no | I | I | Protein TRANSPORT INHIBITOR RESPONSE 1 |
| miR393 | miR393 | TC180163 | 2.5 | no | no | I | I | Protein TRANSPORT INHIBITOR RESPONSE 1 |
| miR393 | miR393 | TC176250 | 3 | no | no | I | I | Similarity to DNA-binding protein |
| miR393 | miR393 | TC181533 | 2.5 | no | II | I | I | GRR1-like protein 1 |
| miR394 | miR394 | TC197402 | 1 | no | no | I | II | F-box only protein 6 |
| miR394 | miR394 | GR443433 | 4 | I | no | no | no | Protein phosphatase 2C-like protein |
| miR395 | miR395 | TC167317 | 3 | no | no | I | I | ATP sulphurylase precursor |
| miR395 | miR395 | TC196344 | 1.5 | no | I | no | no | Plasma membrane sulphate transporter |
| miR396 | miR396 | EE557600 | 2 | no | no | I | I | Transcription activator |
| miR396 | miR396 | ES980066 | 3.5 | no | no | no | no | Uncharacterized protein |
| miR396 | miR396 | FG570467 | 3 | no | no | I | I | BHLH transcription factor like protein |
| miR396 | miR396 | ES923674 | 2.5 | no | no | I | I | BHLH transcription factor like protein |
| miR396 | miR396 | GT083908 | 1.5 | no | no | I | I | ORF1a polyprotein Gill-associated virus |
| miR396 | miR396 | TC171496 | 2.5 | no | no | I | I | Emb|CAB41081.1 |
| miR396 | miR396 | TC177516 | 2.5 | no | no | III | I | Hypothetical protein |
| miR396 | miR396 | TC193012 | 4 | no | no | II | I | Chromosome chr19 scaffold_66 |
| miR396 | miR396 | TC197898 | 3.5 | no | no | no | I | Malate synthase |
| miR396 | miR396 | TC174358 | 4 | no | no | I | II | Ulp1 protease family protein |
M. truncaula, and other plant species (Rajagopalan et al., 2006; Fahlgren et al., 2007; Lenz et al., 2011; Chen et al., 2012; Zhou et al., 2012).

In addition to identifying small RNAs, the high-throughput sequencing also provides a basis to estimate expression levels of B. napus miRNAs. Identification of millions of sequences allowed the number of reads to be estimated and the miRNA abundances compared between any two libraries. The abundances of the identified miRNAs varied from one library to another. Transcript levels of conserved and non-conserved miRNA families were differentially regulated by Cd exposure (Table 3). Compared with miRNAs in roots, more miRNAs in shoots were significantly regulated by Cd exposure. This suggests that more miRNAs in shoots would be involved in plant response to Cd. This study also found that, under normal conditions, most of the miRNA families (70.27%, 26/37) were differentially expressed in roots and shoots (Table 3). Under Cd stress, the patterns of miRNA expression in shoots and roots were altered. For instance, miR162, miR164, and miR860 showed significant differences in levels of expression in roots and shoots under normal conditions whereas their expression levels were not significantly different under Cd stress. The contrasting situation (i.e. differences in expression between root and shoots with Cd exposure) was observed for miR169, miR390, miR397, and...
miR403, suggesting that regulation of miRNA biogenesis is most likely to be altered by heavy metals.

In *B. napus*, most miRNA targets have been predicted, but only a few of them have been identified using the 5′-RACE method (Huang et al., 2010). To accelerate the identification of miRNA targets in *B. napus*, this study carried out a genomewide analysis of the degradome and identified numerous target transcripts for conserved and non-conserved miRNAs. For all miRNAs, 802 non-redundant targets were identified. There were 200, 537, and 65 targets that could be grouped to categories I, II, and III, respectively. Importantly, the 200 targets belonging to category I are the most close to the authentic transcripts sliced by miRNAs (Addo-Quaye et al., 2009a). Most are conserved, including transcripts encoding for transcription factors, proteins for development processes, and intermediates in hormone-dependent pathways, all of which are found in other plant species (Addo-Quaye et al., 2009b; Li et al., 2010; Pantaleo et al., 2010; Zhou et al., 2010, 2012; Song et al., 2011; Zheng et al., 2012; Zhang et al., 2012). Unexpectedly, some new transcripts involved in plant response to heavy metals were identified for the conserved miRNAs. These miRNA target genes encoding critical enzymes or proteins for Cd tolerance (Table 7). miR156 targets a transcript encoding a glutathione-γ-glutamylcysteinyltransferase (GGT). GGT, along with phytochelatin synthase, constitutes a major mechanism to detoxify heavy metals (e.g. Cd and Hg) in plant cells by chelating them with phytochelatin–metal complexes into vacuoles (Cobbett, 2000). The miRNA-mediated GGT gene expression is probably involved in plant tolerance to toxic heavy metals. Glutaredoxins (Grxs) are thiol-disulphide oxidoreductases present in most prokaryotic and eukaryotic organisms (Fernandes and Holmgren, 2004). Recent studies show that monothiol glutaredoxin is able to regulate oxidative stress in higher plants (Cheng et al., 2011). The present study also found a target for miR164 encoding a monothiol glutaredoxin, suggesting that miR164-guided cleavage of monothiol glutaredoxin could be involved in mediation of plant response to Cd-induced oxidative stress. In addition, an ABC transporter and two natural resistance-associated macrophage proteins (NRAMP)-type metal transporters were identified for miR159 and miR167, respectively, which play an important role in metal uptake and translocation in plants; modification of these transporter activities may confer plant tolerance to metal stress. (Bovet et al., 2003; Talke et al., 2006; Krämer et al., 2007).

Although a number of target transcripts were detected for most of the conserved and non-conserved miRNAs in this study, there were several miRNAs for which targets were not identified. This was particularly observed for those non-conserved miRNAs. It is possible that expression of the targets sliced by the non-conserved miRNAs was too low to be detected. Another possibility is that not all plant miRNAs regulate their targets using cleavage. Instead, they may silence their target’s activity via translational repression (Brodersen and Voinnet, 2006). Also, the spatial/temporal differences in expression, or very low expression of a miRNA, may result in insufficient degradation of targets.

In conclusion, this study identified a large number of conserved and non-conserved miRNAs from *B. napus* seedlings with or without heavy metal exposure. Comparative analysis of four libraries, from treated and control roots and shoots, showed that expression of some miRNAs was differentially regulated by Cd exposure. These miRNAs may be directly or indirectly involved in processes leading to plant tolerance to Cd. This study detected 13 non-conserved miRNAs, some of which being regulated by Cd exposure. No species-specific miRNAs were identified, possibly because only a small proportion (24.45–43.52%) of small RNAs could be mapped to ESTs and TC sequences of *B. napus* or because of a limitation of the tissues collected for sequencing. With the completion of the sequencing of the *B. napus* genome in the near future, more non-conserved or species-specific miRNAs may be discovered. Notably, many high-quality target transcripts were identified for the conserved and non-conserved miRNAs, particularly important are those possibly involved in regulation of plant response to Cd stress. Identification of these targets will help uncover the regulatory mechanism for plant tolerance to Cd.

### Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Table S1. Probe sequences used for Northern blotting to validate miRNAs

Supplementary Table S2. All known conserved and non-conserved miRNAs and their transcript abundance

Supplementary Table S3. New conserved and non-conserved miRNAs identified from *B. napus*

Supplementary Table S4. miRNA homologues with known miRNAs in other plant species in miRBase

Supplementary Table S5. Category I targets for miRNAs in details identified from degradome

Supplementary Table S6. Category II and III targets for miRNAs in details identified from degradome
Supplementary Table S7. Observed frequencies and patterns of degradome reads on the new and known miRNA precursors
Supplementary Fig. S1. Distribution of total and unique small RNAs according to length
Supplementary Fig. S2. Venn diagrams for analysis of total and unique small RNAs between Cd-free roots and shoots or between Cd-treated roots and shoots of B. napus
Supplementary Fig. S3. Venn diagrams for analysis of total and unique reads of degradomes between any two libraries
Supplementary Fig. S4. Distribution of total (A) and unique (B) reads of degradomes
Supplementary Fig. S5. t-plots for category I targets of miRNAs identified from degradome
Supplementary Fig. S6. t-plots for category II and III targets of miRNAs identified from degradome
Supplementary Fig. S7. Degradome signature abundance corresponding to miRNA precursors

Acknowledgements
This research was supported by the National Natural Science Foundation of China (31071343), the China Postdoctoral Science Foundation (special grant: 201003593), and the Priority Academic Program Development of Jiangsu Higher Education Institutions (grant no. 200910).

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