MicroRNAs in the response of non-heading Chinese cabbage to Hyaloperonospora parasitica (downy mildew) infection

Chengzhen Sun
Nanjing Agricultural University

Die Hu
Nanjing Agricultural University

Yuan Wang
Nanjing Agricultural University

Huawei Tan
Nanjing Agricultural University

Tongkun Liu
Nanjing Agricultural University

Changwei Zhang
Nanjing Agricultural University

Xilin Hou
Nanjing Agricultural University

Ying Li (mailto:yingli@njau.edu.cn)
Nanjing Agricultural University

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Abstract

Background MicroRNAs (miRNAs) are a subset of endogenous small non-coding ~21 nucleotide RNAs, which are playing important roles in development, abiotic stress, and pathogen responses. Downy mildew, caused by Hyaloperonospora parasitica, is a severe fungal disease in non-heading Chinese cabbage (Brassica rapa ssp. chinensis). However, miRNAs and their targets involved in the response of non-heading Chinese cabbage to downy mildew were still unclear. Results Here, fifteen small RNA libraries were generated from non-heading Chinese cabbage leaves at different time points after H. parasitica inoculation. By deep sequencing, we identified 197 known miRNAs and 112 novel miRNAs. According to data analysis, 8 known miRNAs and 8 novel miRNAs were found to be responsive to downy mildew infection in non-heading Chinese cabbage. Expression levels of 12 predicted miRNAs target genes were determined by reverse transcription-quantitative polymerase chain reaction (qRT-PCR). In this study, we proposed a regulatory network showing that non-heading Chinese cabbage defense response to downy mildew by repressing cell wall pectin, lipid and cellulose catabolic process (PME12, PME17, PLAIVA and GDPDL4) and callose deposition (CYP79B2), repressing indole glucosinolate metabolic process (CYP81F3), repressing respiratory burst (PUB24), repressing programmed cell death (CRK5), and inducing defense related gene expression (ROS1, LECRK-VII.2, BAK7 and WRKY48). Conclusions This research identified miRNAs associated with downy mildew infection in non-heading Chinese cabbage. Target genes and network analysis contributed to understanding the interaction between plant and pathogen.

Background

Brassica rapa is a species that contains multiple oil and vegetable crops [1]. Non-heading Chinese cabbage (B. rapa ssp. chinensis) is one of them and widely cultivated in Southern China [2]. Downy mildew, caused by Hyaloperonospora parasitica, is a common disease in brassicas [3-6]. Applying fungicide is an efficient way to control downy mildew [7], but it might increase the probability of pathogen population forming fungicide resistance that makes the disease management even worse [8]. Therefore, it is necessary to develop more disease-resistant varieties, which needs a better understanding of how plants response to downy mildew infection.

In plants, small RNA-guided post-transcriptional regulatory mechanisms play important roles in many aspects, including flowering regulation [9], hormone responses [10, 11] and epigenetic control of transposable elements [12]. MicroRNAs (miRNAs) are small regulatory RNAs and found in diverse eukaryotic lineages [13]. They are produced from pri-miRNAs, containing a fold-back structure, via DICER-LIKE proteins processing [14]. Mature miRNAs are incorporated into an RNA-induced silencing complex (RISC) to interact with the complementary sites of the target gene transcripts, which negatively regulate the target genes expression by degrading or repressing the RNA transcripts [13, 15]. Besides regulating a range of essential cellular and biological processes, miRNAs have been shown to play crucial roles in responses to abiotic and biotic stresses, such as nutritional deficiency [16], drought [17], salinity [18], cold [19], heat [20], oxidative stress [21], heavy metal stress [22], and resistance to disease [23, 24]. In
Arabidopsis, overexpression of miR393 enhances plant resistance to bacterium Pseudomonas syringae pv. tomato DC3000 by decreasing transcripts of TIR1, AFB2 and AFB3 that are involved in auxin signaling pathway [25]. miR393 is downregulated by Mildew resistance locus a (Mla) in barley, and thus the accumulation of SOD1 targeted by miR398 induces hypersensitive reaction after powdery mildew infection [26].

Some achievements have been made in the study of miRNAs in Brassica rapa. Yu et al. [20] showed that miR398 was downregulated and its target gene CSD1 was upregulated under heat stress. Huang et al. [11] found that miR159 participated in vernalization. miR156 and miR166 were involved in heading process in Brassica rapa [27, 28]. Till now, there have been no reports on miRNAs associated with downy mildew infection in non-heading Chinese cabbage. Here, using Solexa Analyzer, we identified a set of small RNAs from downy mildew infected leaves of a resistant non-heading Chinese cabbage cultivar. Based on the small RNA sequencing and target genes prediction, we proposed a miRNA-mediated regulation model in non-heading Chinese cabbage upon H. parasitica infections.

Results

Classification of small RNAs response to H. parasitica in non-heading Chinese cabbage

To identify miRNAs responsive to downy mildew in non-heading Chinese cabbage, 15 sRNA libraries were constructed by using leaf samples collected from five different time points (0, 6, 12, 24 or 48 hpi). High-throughput sequencing generated of mainly 18 to 30 nucleotides in size raw reads for each library of ranged between 12.5 million and 31.4 million (Table 1), which presented a deep resource for extensive discovery of small regulatory RNAs. In total, >280 million raw reads were obtained (Table 1) and processed for miRNA identification. After removing poly(A) tails, short and low-quality reads, and adaptor contamination, a total of clean reads for each library of ranged between 12.1 million and 31.1 million (Table 1). Thereafter, we mapped clean reads to known RNAs in the Silva database, GtRNAdb database, Rfam database and Repbase database (Table 2), filtering rRNA, tRNA, scRNA, snRNA, snoRNA and repetitive sequences. rRNAs were the most abundantly annotated RNAs, accounting for about half of total reads except 3 libraries of 48 hpi, and the unannotated RNAs were potentially novel regulatory small RNAs (Table 2). Then, miRBase database (v22) was utilized to identify known miRNAs. The number of unannotated clean reads that mapped to miRBase were ranged between 458535 and 4961663 (Table 2), and the number of known miRNAs were ranged between 157 and 191 (Table 1). The novel miRNAs (Table 1) were obtained after miRDeep-P [29] analysis by using the unannotated reads that unmapped to miRBase, and the number of novel miRNAs were ranged between 91 and 112 (Table 1). The formation of mature bodies is realized by the shear of Dicer/DCL enzyme and mature plant miRNAs are always 21 nt or 24 nt [30]. The 21- and 24-nt sequences were dominant in all libraries, and the 21-nt ones were the most abundant (Fig. 1a, b).

Characteristics of miRNAs response to H. parasitica in non-heading Chinese cabbage
In total, we identified 197 known miRNAs (Table S2) with 117 of them not described in *B. rapa* (Table S2), and 112 novel miRNAs (Table S3). The number of miRNAs in length of 21/24nt were 140 and 81 respectively for the known miRNAs and novel miRNAs (Fig. 2a, b; Table S2, S3), respectively. Based on the sensitivity and specificity of Dicer/DCL at recognizing and cleaving pre-miRNAs [30], miRNA bias analysis was conducted. The results indicated that the mature miRNA sequences had A-U contents greater than the G-C contents throughout all transcripts except at position 7, 15, 17, 19, 21, 22, 23 and 24 in known miRNAs, and 3, 4, 6, 8, 9, 14, 18, 19 and 21 in novel miRNAs (Fig. 3a,b). The frequencies of U appeared initially were ranged from 12.9% to 100% in known miRNAs (Fig. 4a), while they were 2.0% to 100% in novel miRNAs (Fig. 4b). Approximately 40.7% were U at the first nucleotide for 21-/24-nt miRNAs, and all first nucleotides were U for 19-nt miRNAs in known miRNAs (Fig. 4a). Approximately 21% were U at the first nucleotide for 21-/24-nt miRNAs, and all the first nucleotides were U for 18-nt miRNAs in novel miRNAs (Fig. 4b).

**Differential expression of miRNAs in response to *H. parasitica***

To identify the response pattern of miRNAs to *H. parasitica* in non-heading Chinese cabbage, we compared the abundance of miRNAs in the inoculated libraries (6 hpi, 12 hpi, 24 hpi and 48 hpi) compared with the libraries at 0 hpi. Differential expressed miRNAs were identified by using DESeq2 [31] with $|\log_{2}(FC)| \geq 1$ and FDR $\leq 0.05$ as the significance cutoffs. The nomenclature was following the previous reports [11, 32]. A known miRNA was named as bra-miR#-5p or -3p, and the opposite strand was named as bra-miR#*-3p or -5p. A novel miRNA was named as bra-miRn#-5p or -3p, and the opposite strand was named as bra-miRn#*-3p or -5p. Total of 8 known miRNAs and 8 novel miRNAs were identified to be differentially expressed in non-heading Chinese cabbage response to *H. parasitica* (Table 3; Fig. 5; Table S4). To illustrate the differences in miRNAs expression levels after *H. parasitica*-inoculation, a heat map was constructed using Heatmap Illustrator (HemI) on the platform BMKCloud (www.biocloud.net) based on the TPM method [33]. At 6 hpi, only bra-miRn47-3p showed up-regulated expression; at 12 hpi, 6 miRNAs were down-regulated while bra-miRn38-3p was up-regulated; at 24 hpi, 4 miRNAs showed up-regulated expression and 4 miRNAs were down-regulated; at 48 hpi, 6 miRNAs were up-regulated and 2 miRNAs showed down-regulated expression (Table 3; Table S4). The differentially expressed miRNAs at different time points suggesting that these miRNAs might be induced or repressed in the response to *H. parasitica*. Eight of the differentially expressed miRNAs were assigned to known miRNA families (Table 3).

**Prediction and function analysis of miRNA target genes**

The software TargetFinder (1.6) were employed to predict the miRNA targets based on gene sequence information of known miRNAs and novel miRNAs in *Brassica* genomic Scaffold sequence (http://brassicadb.org/). Total of 2,722 target gene were predicted as target genes of 183 known miRNAs, and 1,662 genes were predicted as that of 85 novel miRNAs (Table 4). The entire set of miRNA target genes was annotated against the NR (ftp://ftp.ncbi.nih.gov/blast/db/), Swiss-Prot (http://www.uniprot.org/), GO (http://www.geneontology.org/), COG (http://www.ncbi.nlm.nih.gov/COG/),
KEGG (http://www.genome.jp/kegg/), KOG (http://www.ncbi.nlm.nih.gov/KOG/), and Pfam databases (http://pfam.xfam.org/) using BLAST with a cutoff of E-value < 1E-05. Among the 4,384 target genes, 4,360 were annotated (Table 4, 5). 3201 target genes were annotated in GO database, of which the number of target genes at length between 300 and 1000 bp was 778, and that was 2389 at the length over 1000 bp. Only 1632 target genes annotated in KEGG database, of which 385 were at the length between 300 and 1000 bp, and 1235 at the length over 1000 bp (Table 5).

To have a better understanding of differentially expressed miRNAs, the target genes of these miRNAs were subjected to GO enrichment analysis by Blast2GO program with default parameters. The results showed that these predicted targets could be classified into 8 molecular functions, 9 biological processes and 3 cellular components (Fig. 6). Most of them, which were classified as the binding category (9 targets in oxygen binding and 11 targets in heme binding), encode transcription factors. KEGG pathway enrichment analysis was carried out as well. 30 pathways were enriched with target genes of significantly differentially expressed miRNAs, which were divided into five biological processes, including cellular processes (12.5%), environmental information processing (5.36%), genetic information processing (35.73%), metabolism (44.69%) and organismal system (1.79%) (Fig. 7). Among these pathways, miRNA surveillance pathway involved the most target genes (Bra001821, Bra028900, Bra029681, Bra040031 and Bra005265), while plant-pathogen interaction pathway only contained one target gene (Bra041056) (Fig. 7; Table S4).

qRT-PCR analysis of predicted target genes

To explore the relationship between differentially expressed miRNAs and their target genes, expression patterns of several predicted target genes were analyzed via qRT-PCR. Bra036138 were predicted as target genes of bra-miR400b-3p (Table S4). When the abundance of bra-miR400b-3p were decreased at 12 hpi and 48 hpi (Table 3), the expression level of Bra036138 were increased (Fig. 8). Bra005264 were predicted as target genes of both bra-miRn91a-3p and bra-miRn91b-3p (Table S4). When the abundance of bra-miRn91a-3p and bra-miRn91b-3p were increased at 48 hpi (Table 3), the expression level of Bra005264 were increased (Fig. 8). These suggested that miRNA-mediated silencing of their potential target genes occur during the process of defense response in non-heading Chinese cabbage after H. parasitica infection. Bra000540 and Bra024465 were predicted as target genes of bra-miRn28-5p, but they had different expression pattern (Fig. 8). When the abundance of bra-miRn28-5p was increased at 24 hpi (Table 3), the expression level of Bra024465 showed increased while Bra000540 didn't (Fig. 8). It indicated that these target genes may not be regulated only by miRNAs upon downy mildew infection.

Discussion

In this study, over twelve million clean reads were obtained from each library (Table 1) and most of them were about 20- to 24-nt long (Fig. 1a) that are common lengths of miRNAs in plant [32]. In total, 197 known miRNAs:117 of them not described in B. rapa, and 112 novel miRNAs were identified in downy mildew-infected non-heading Chinese cabbage by deep sequencing (Fig. 2; Table S2, S3). Compared to
Arabidopsis, rice or other model plants, information about miRNA in *Brassica rapa* is limited in miRBase (http://www.mirbase.org). This might be the reason for why so many known miRNAs not described in *B. rapa* were identified in our research. The number of known miRNAs were ranged between 157 and 191 (Table 1). The novel miRNAs were obtained after miRDeep-P analysis by using the unannotated reads that unmapped to miRBase, and the number of novel miRNAs were ranged between 91 and 112 (Table 1). Compared with 0 hpi, 6 hpi, 12 hpi and 24 hpi, the number of known and novel miRNAs detected at 48 hpi was less than several other time points (Table 1). In addition, the number of miRNAs detected at 6 hpi was similar with the number of miRNAs detected at 12 hpi, but the number of differentially expressed miRNAs in response to downy mildew infection was only 1 at 6 hpi compared to 0 hpi while 7 at 12 hpi (Table 3). Since the germination time of downy mildew spores vary [34, 35], we assume that the state of spores at 6 hpi and 12 hpi were different.

The differentially expressed miRNAs we found are bra-miR156c-3p, bra-miR156g-3p, bra-miR167f-3p, bra-miR398-5p, bra-miR398b-5p, bra-miR400b-3p, bra-miR5721b-5p and bra-miR9552b-3p. Research showed that miR156 was involved in heat stress response [36] and miR167 regulated auxin signaling pathway [37]. miR398 family was up-regulated in response to water deficiency in Medicago truncatula [38], but showed opposite expression patterns in soybean after nematode infection [39]. miR400 was involved in different stress responses as well. In Arabidopsis, the abundance of miR400 was decreased after turnip crinkle virus infection and heat stress [40, 41]. Overexpression of miR400 led to more susceptible to bacteria and fungi and sensitive to heat [41, 42]. In our study, miR400 was down-regulated at 12 hpi and 48 hpi compared with the one at 0 hpi (Table 3, S4).

Two targets of bra-miR400b-3p were BAK7 and WRKY48 (Table S4), which responded to fungi invasion [43]. One target gene of bra-miRn38-3p was PLAIVA (Table S4), which was involved in the plant defense through free fatty acid and lysophospholipid signaling molecules [44]. miRNA bra-miR9552b-3p were predicted to target GDPDL4 (Table S4), which coding a protein SHV3 play important role in cell wall organization [45]. Two target genes of miRNAs bra-miRn28-5p and bra-miRn90-5p were PME12 and PME17 (Table S4), and PME activity is important for the virulence of the necrotrophic fungal pathogen *Botrytis cinerea* by regulate the dicot cell wall pectin content [46]. miRNAs bra-miR91a-3p and bra-miR91b-3p shared the same target genes, including CYP79B2, CYP81F3, PUB24, CRK5, ROS1 and LECRK-VII.2 (Table S4). CYP79B2 and CYP81F3 encoding enzymes of indole glucosinolates biosynthesis are coordinately induced in response to *P. brassicae* [47]. PUB24 acted as negative regulators of PTI in response to several distinct PAMPs [48]. Chen et al. [49] showed that overexpression of CRK5 could trigger hypersensitive response-like cell death in transgenic *Arabidopsis* plants. *Arabidopsis ros1* mutant displayed enhanced resistance to the biotrophic pathogen *H. arabidopsidis* [50]. LECRK-VII.2 coding a L-type lectin receptor kinase plays a critical role in transfer defense signaling [51]. PINP1, a predicted gene of bra-miR167f-3p (Table S4), regulates RNA silencing pathway in plants [52].

Therefore, we hypothesized a regulatory network of non-heading Chinese cabbage defense response to downy mildew by cell wall modification, metabolite regulation and defense-related gene expression. This
study would fill the gaps in miRNAs-mediated downy mildew resistance in non-heading Chinese cabbage and provide some clues to plant miRNA research (Fig. 9).

**Conclusions**

This research identified miRNAs associated with downy mildew infection in non-heading Chinese cabbage. Target genes and network analysis contributed to understanding the interaction between plant and pathogen. We laid the foundation for further study of miRNA design and transformation to elucidate the function and regulatory mechanisms of plant miRNAs in disease resistance.

**Methods**

**Plant materials and treatments**

A downy mildew-resistant cultivar (NHCC001) of non-heading Chinese cabbage (*B. rapa* ssp. *chinensis*) was chosen as plant material. NHCC001 derived from self-pollinated plants of at least 6 generations in our lab have been re-sequencing by Song at al. [53] (http://nhccdata.njau.edu.cn/). Seedlings were grown in the growth chamber under 25°C with a 12 h-light/12 h-dark cycle. Preparation and inoculation of *H. parasitica* inoculum were performed as described by Sun et al.[54]. Leaves were collected at 0, 6, 12, 24 and 48 hours post inoculation (hpi), frozen in liquid nitrogen and stored at -80°C until RNA extraction. For each time point, three biological replicates were involved.

**RNA extraction**

Total RNA was extracted from leaf samples by using a modified CTAB method and treated with DNA erasol to avoid DNA contamination. RNA purity was checked using a NanoPhotometer spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using a Qubit RNA Assay Kit and a Qubit2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Only RNA samples with a 260/280 ratio between 1.8 and 2.0, a 260/230 ratio between 2.0 and 2.5, 28S/18S > 1.5, and a RIN value (RNA integrity number) > 8.0 were used for subsequent sequencing.

**Construction and sequencing of Small RNA libraries**

A total of 1.5 μg RNA per sample was used as input material for the RNA sample preparations. Fifteen small RNA libraries (3 biological replicates for five time points) were generated using a NEBNext® Ultra™ II small RNA Sample Library Prep Kit for Illumina (NEB, USA) according to the manufacturer’s protocol. Because the small RNAs contained 5’phosphate groups and 3’hydroxyl groups, T4 RNA ligase was used to add adaptors at both ends. Libraries were constructed through a series of experiments including reverse transcription, PCR amplification and gel extraction. The quality of each library was assessed on an Agilent Bioanalyzer 2100 system before sequencing, and the small RNA sequencing was performed on an Illumina HiSeq X-ten platform, which generated 50-bp single-end reads. The sequencing work were
performed by the Biomarker Technologies Corporation (Beijing, China). The full data sets have been submitted to the Gene Expression Omnibus (GEO) database of NCBI under SRA accession SUB5672046, Bioproject: PRJNA544898.

**Analysis of small RNA sequencing data**

The sequencing reads were processed to remove adaptors and cleaned by Q30 value. Reads with >20% bases <Q30, and N base >10% were filtered, and sequences shorter than 18 nt or longer than 30 nt were also removed. Silva database, GtRNAdb database, Rfam database and Repbase database were applied to find out ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), and other non-coding RNA as well as repeat sequences by using Bowtie software [55]. The remaining reads were mapped to *Brassica rapa* genome (v1.5) (http://brassicadb.org/brad/) and analyzed by miRBase (v22) (http://www.mirbase.org) and miRDeep-P [29]. According to the identification method described by Jiang et al. [56], reads that matched to miRBase with no more than 3 mismatches were called as known miRNAs; otherwise, thier were classified into novel miRNAs.

**Differential expression analysis of miRNAs**

To quantify the abundance of each miRNA, the Transcripts Per Kilobase Million (TPM) value was defined as ‘Read count × 1,000,000’/’Mapped Reads’. Differential expression analysis of miRNAs were performed using the DESeq2 R package (1.10.1) with default parameters [31]. DESeq2 provided statistical routines for determining differential expression in digital miRNA expression data using a model based on the negative binomial distribution. The resulting P values were adjusted by using the Benjamini and Hochberg's approach for controlling the false discovery rate. miRNA with |log2(FC)| ≥ 1.00 and FDR ≤ 0.05 found by DESeq2 were assigned as differentially expressed.

**Prediction of potential targets**

The software TargetFinder (1.6) [57] were employed to predicted the miRNA targets based on sequence information of known miRNAs and novel miRNAs in *Brassica rapa* genome (v1.5) (http://brassicadb.org/brad/). The number of mismatches at complementary sites, where there was no gap, between miRNA sequence and potential mRNA target were less than 4 [58]. The miRNA targets were confirmed using psRNATarget online server (http://plantgrn.noble.org/psRNATarget/). The entire set of miRNA target genes was annotated against the NR (ftp://ftp.ncbi.nih.gov/blast/db/), Swiss-Prot (http://www.uniprot.org/), GO (http://www.geneontology.org/), COG (http://www.ncbi.nlm.nih.gov/COG/), KEGG (http://www.genome.jp/kegg/), KOG (http://www.ncbi.nlm.nih.gov/KOG/), and Pfam (http://pfam.xfam.org/) using BLAST with a cutoff of E-value < 1E-05 [58].

**GO and KEGG pathway enrichment analysis**

Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) was implemented by the GOseq R packages based on Wallenius non-central hyper-geometric distribution. KEGG [59] is a database resource for understanding high-level functions and utilities of the biological system, such as
the cell, the organism and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies (http://www.genome.jp/kegg/). We used KOBAS [60] software to test the statistical enrichment of differential expression genes in KEGG pathways.

**qRT-PCR validation**

cDNA was synthesized from 1 µg total RNA with the HiScript® II Q RT SurperMix for qPCR (+gDNA wiper) reagent Kit (Vazyme Biotche Co., Ltd) and diluted 1/10 by ddH$_2$O. qRT-PCR assay was carried out with 2 µL of cDNA and 10 µL of 2× TransStart® Tip Green qPCR SuperMix (Vazyme Biotche Co., Ltd) using the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Actin-7 was chosen as the normalizer and data were analyzed by using 2$^{-\Delta\Delta Ct}$ method [61]. Three technical replicates were ran for each sample. The primers used for qRT-PCR are listed in Table S1.

**Abbreviations**

| Abbreviation | Description                     |
|--------------|---------------------------------|
| EST          | Expressed sequence tag          |
| GO           | Gene ontology                   |
| hpi          | Hours post inoculated           |
| miRNA        | microRNA                        |
| qRT-PCR      | Reverse transcription-quantitative polymerase chain reaction |
| rRNA         | Ribosomal RNA                   |
| sRNA         | Small RNA                       |
| scRNA        | Small cytoplasmic ribonucleic acids |
| snRNA        | Small nuclear ribonucleic acids |
| snoRNA       | Small nuclear RNA               |
| snRNA        | Small nuclear ribonucleic acids |
| tRNA         | Transfer RNA                    |

**Declarations**

**Ethics approval and consent to participate**

Not applicable.
Consent for publication

Not applicable.

Availability of data and materials

The sequencing data of non-heading Chinese cabbage in this study were deposited in NCBI Sequence Read Archive (SRA) Sequence Database with accession number SRR9129318, SRR9129319, SRR9129320, SRR9129321, SRR9129322, SRR9129323, SRR9129324, SRR9129325, SRR9129326, SRR9129327, SRR9129328, SRR9129329, SRR9129330, SRR9129331, SRR9129332.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

C-ZS, DH, and YL conceived and designed the experiments. C-ZS, DH, YW, H-WT, T-KL, C-WZ and X-LH performed the experiments. CZS, DH, YW and H-WT analyzed the data. C-ZS, DH, YW and YL wrote and revised the paper. All authors read and approved the final version of manuscript.

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Author details

1 State Key Laboratory of Crop Genetics & Germplasm Enhancement, College of Horticulture, Nanjing Agricultural University, Nanjing 210095, PR China

2 College of Horticulture Science and Technology, Hebei Normal University of Science & Technology, Qinhuangdao 066004, PR China

References

1. Sun R. Economic/academic importance of *Brassica rapa*. The *Brassica rapa* 2015. doi:10.1007/978-3-662-47901-8_1.
2. Liu TK, Li Y, Zhang CW, Qian Y, Wang Z, Hou XL. Overexpression of Flowering locus c, isolated from non-heading Chinese cabbage (Brassica campestris chinensis makino), influences fertility in Arabidopsis. Plant Molecular Biology Reporter. 2012;30:1444-9.

3. Xiao D, Liu ST, Wei YP, Zhou DY, Hou XL, Li Y, Hu CM. cDNA-AFLP analysis reveals differential gene expression in incompatible interaction between infected non-heading Chinese cabbage and Hyaloperonospora parasitica. Horticulture Research. 2016;3:16034.

4. Neik TX, Barbetti MJ, Batley J. Current status and challenges in identifying disease resistance genes in Brassica napus. Frontiers in plant science. 2017;8:1788.

5. Zhang B, Li P, Su T, Li P, Xin X, Wang W, Zhao X, Yu Y, Zhang D, Yu S, Zhang F. BrRLP48, encoding a receptor-like protein, involved in downy mildew resistance in Brassica rapa. Frontiers in plant science. 2018;9:1708.

6. Bayer PE, Golicz AA, Tirmaz S, Chan C-KK, Edwards D, Batley J. Variation in abundance of predicted resistance genes in the Brassica oleracea Plant Biotechnology Journal. 2019;17:789-800.

7. van der Wolf JM, Michta A, van der Zouwen PS, de Boer WJ, Davelaar E, Stevens LH. Seed and leaf treatments with natural compounds to induce resistance against Peronospora parasitica in Brassica oleracea. Crop Protection. 2012;35:78-84.

8. Hollomon DW. Fungicide Resistance: 40 Years on and Still a Major Problem. Fungicide Resistance in Plant Pathogens. 2015. doi:10.1007/978-4-431-55642-8_1.

9. Nag A, Jack T. Sculpting the flower; the role of microRNAs in flower development. Plant Development. 2010;91:349-78.

10. Liu Q, Chen YQ. Insights into the mechanism of plant development: interactions of miRNAs pathway with phytohormone response. Biochemical and Biophysical Research Communications. 2009;384:1-5.

11. Huang F, Wu X, Hou X, Shao S, Liu T. Vernalization can regulate flowering time through microRNA mechanism in Brassica rapa. Physiol Plant. 2018;164:204-15.

12. Lisch D. How important are transposons for plant evolution? Nature Reviews Genetics. 2013;14:49-61.

13. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116:281-97.

14. Song X, Li Y, Cao X, Qi Y. MicroRNAs and their regulatory roles in plant–environment interactions. Annual Review of Plant Biology. 2019;70:489-525.

15. Voinnet O. Origin, biogenesis, and activity of plant microRNAs. Cell. 2009;136:669-87.

16. Zhu YY, Zeng HQ, Dong CX, Yin XM, Shen QR, Yang ZM. MicroRNA expression profiles associated with phosphorus deficiency in white lupin (Lupinus albus). Plant Science. 2010;178:23-29.

17. Akdogan G, Tufekci ED, Uranbey S, Unver T. MiRNA-based drought regulation in wheat. Functional & Integrative Genomics. 2016;16:221-33.

18. Gao S, Yang L, Zeng HQ, Zhou ZS, Yang ZM, Li H, Sun D, Xie FL, Zhang BH. A cotton miRNA is involved in regulation of plant response to salt stress. Scientific Reports. 2016;6:19736.
19. Koc I, Filiz E, Tombuloglu H. Assessment of miRNA expression profile and differential expression pattern of target genes in cold-tolerant and cold-sensitive tomato cultivars. Biotechnology & Biotechnological Equipment. 2015;29:851-60.

20. Yu X, Wang H, Lu Y, de Ruiter M, Cariaso M, Prins M, van Tunen A, He Y. Identification of conserved and novel microRNAs that are responsive to heat stress in *Brassica rapa*. Journal of Experimental Botany. 2012;63:1025-38.

21. Sunkar R, Kapoor A, Zhu JK. Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in *Arabidopsis* is mediated by downregulation of miR398 and important for oxidative stress tolerance. Plant Cell. 2006;18:2051-65.

22. Huang SQ, Peng J, Qiu CX, Yang ZM. Heavy metal-regulated new microRNAs from rice. J Inorg Biochem. 2009;103:282-7.

23. Li X, Wang X, Zhang S, Liu D, Duan Y, Dong W. Identification of soybean microRNAs involved in soybean cyst nematode infection by deep sequencing. PLoS ONE. 2012;7:e39650.

24. Yin XC, Wang J, Cheng H, Wang XL, Yu DY. Detection and evolutionary analysis of soybean miRNAs responsive to soybean mosaic virus. Planta. 2013;237:1213-25.

25. Navarro L, Dunoyer P, Jay F, Arnold B, Dharmasiri N, Estelle M, Voinnet O, Jones JDG. A Plant miRNA Contributes to Antibacterial Resistance by Repressing Auxin Signaling. Science. 2006;312:436-39.

26. Xu W, Meng Y, Wise RP. *Mla*- and *Rom1*-mediated control of microRNA398 and chloroplast copper/zinc superoxide dismutase regulates cell death in response to the barley powdery mildew fungus. New Phytologist. 2014;201:1396-1412.

27. Wang Z, Jiang DH, Zhang CW, Tan HW, Li Y, Lv SW, Hou XL, Cui XY. Genome-wide identification of turnip mosaic virus-responsive microRNAs in non-heading Chinese cabbage by high-throughput sequencing. Gene. 2015;571:178-87.

28. Ren W, Wang H, Bai J, Wu F, He Y. Association of microRNAs with types of leaf curvature in *Brassica rapa*. Frontiers in Plant Science. 2018;9:73.

29. Y XZ, Li L. miRDeep-P: a computational tool for analyzing the microRNA transcriptome in plants. Bioinformatics. 2011, 27:2614-15.

30. Chen X. Small RNAs and Their Roles in Plant Development. Annu Rev Cell Dev Biol. 2009;2009:21-44.

31. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology. 2014;15:550.

32. Zhang B, Unver T: A critical and speculative review on microRNA technology in crop improvement: current challenges and future directions. Plant Science. 2018;274:193-200.

33. Li B, Victor R, Stewart RM, Thomson JA, Dewey CN. RNA-Seq gene expression estimation with read mapping uncertainty. Bioinformatics. 2010;26:493-500.

34. Cohen Y, Ben-Naim Y, Falach L, Rubin E. Epidemiology of Basil Downy Mildew. Phytopathology. 2017;107: 1149-60.
35. Yin X, Liu RQ, Su H, Su L, Guo YR, Wang ZJ, Du W, Li MJ, Zhang X, Wang YJ. Pathogen development and host responses to *Plasmopara viticola* in resistant and susceptible grapevines: an ultrastructural study. Horticulture Research. 2017;4:17033.

36. Liu J, Feng L, Li J, He Z. Genetic and epigenetic control of plant heat responses. Frontiers in Plant Science. 2015;6:267.

37. Baldrich P, San Segundo B. MicroRNAs in Rice Innate Immunity. Rice. 2016;9:6.

38. Trindade I, Capitão C, Dalmay T, Fevereiro MP, Santos DM. miR398 and miR408 are up-regulated in response to water deficit in *Medicago truncatula*. Planta. 2010;231:705-16.

39. Tian B, Wang S, Todd TC, Johnson CD, Tang G, Trick HN. Genome-wide identification of soybean microRNA responsive to soybean cyst nematodes infection by deep sequencing. BMC Genomics. 2017;18:572.

40. Wu C, Li X, Guo S, Wong SM. Analyses of RNA-Seq and sRNA-Seq data reveal a complex network of anti-viral defense in TCV-infected *Arabidopsis thaliana*. Sci Rep. 2016;6:36007.

41. Yan K, Liu P, Wu CA, Yang GD, Xu R, Guo QH, Huang JG, Zheng CC. Stress-Induced Alternative Splicing Provides a Mechanism for the Regulation of MicroRNA Processing in *Arabidopsis thaliana*. Molecular Cell. 2012;48:521-31.

42. Park YJ, Lee HJ, Kwak KJ, Lee K, Hong SW, Kang H. MicroRNA400-guided cleavage of Pentatricopeptide repeat protein mRNAs Renders *Arabidopsis thaliana* more susceptible to pathogenic bacteria and fungi. Plant & Cell Physiology. 2014; 55:1660-8.

43. Libault M, Wan J, Czechowski T, Udvardi M, Stacey G. Identification of 118 *Arabidopsis* transcription factor and 30 ubiquitin-ligase genes responding to chitin, a plant-defense elicitor. Mol Plant Microbe Interact. 2007;20:900-11.

44. Rietz S, Dermendjiev G, Oppermann E, Tafesse FG, Effendi Y, Holk A, Parker JE, Teige M, Scherer GF. Roles of *Arabidopsis* patatin-related phospholipases A in root development are related to auxin responses and phosphate deficiency. Molecular Plant. 2010;3:524-38.

45. Shimpei H, Tadashi I, Toshiro M, Rumi T, Takashi K, Takuji W, Kazuo S, Takashi H. The glycerophosphoryl diester phosphodiesterase-like proteins SHV3 and its homologs play important roles in cell wall organization. Plant & Cell Physiology. 2008;49:1522.

46. Gerit B, Grundman RE, Suma S, William T, Fumiaki K, Jane G. Arabidopsis pectin methylesterases contribute to immunity against *Pseudomonas syringae*. Plant Physiology. 2014;164:1093-1107.

47. Klaus S, Eliane AM, Antony B, Felix M. Disease resistance of *Arabidopsis* to *Phytophthora brassicae* is established by the sequential action of indole glucosinolates and camalexin. Plant Journal. 2010;62:840-51.

48. Trujillo M, Ichimura K, Casais C, Shirasu K. Negative regulation of PAMP-triggered immunity by an E3 ubiquitin ligase triplet in *Arabidopsis*. Current Biology. 2008;18:1396-1401.

49. Chen K, Fan B, Du L, Chen Z. Activation of hypersensitive cell death by pathogen-induced receptor-like protein kinases from Plant Molecular Biology. 2004;56:271-83.
50. López Sánchez A, Stassen JH, Furci L, Smith LM, Ton J. The role of DNA (de)methylation in immune responsiveness of *Arabidopsis*. Plant Journal for Cell & Molecular Biology. 2016;88:361-74.

51. Wang Y, Bouwmeester K. L-type lectin receptor kinases: new forces in plant immunity. Plos Pathogens. 2017;13:e1006433.

52. Qiao Y, Shi J, Zhai Y, Hou Y, Ma W. Phytophthora effector targets a novel component of small RNA pathway in plants to promote infection. Proc Natl Acad Sci U S A. 2015;112:5850-5.

53. Song XM, Ge TT, Li Y, Hou XL. Genome-wide identification of SSR and SNP markers from the non-heading Chinese cabbage for comparative genomic analyses. BMC genomics. 2015;16:328.

54. Sun CZ, Wang L, Hu D, Riquicho ARM, Liu TK, Hou XL, Li Y. Proteomic analysis of non-heading Chinese cabbage infected with *Hyaloperonospora parasitica*. Journal of Proteomics. 2014;98:15-30.

55. Varallyay E, Valoczi A, Agyi A, Burgyan J, Havelda Z. Plant virus-mediated induction of miR168 is associated with repression of ARGONAUTE1 accumulation. Embo Journal. 2010;29:3507-19.

56. Jiang J, Lv M, Liang Y, Ma Z, Cao J. Identification of novel and conserved miRNAs involved in pollen development in *Brassica campestris chinensis* by high-throughput sequencing and degradome analysis. BMC Genomics. 2014;15:146.

57. Allen E, Xie Z, Gustafson AM, Carrington JC. MicroRNA-directed phasing during trans-acting siRNA biogenesis in plants. Cell. 2005;121:207-21.

58. Jiang L, Tian XK, Fu YX, Liao XZ, Wang GD, Chen FD. Comparative profiling of microRNAs and their effects on abiotic stress in wild-type and dark green leaf color mutant plants of *Anthurium andraeanum* 'Sonate'. Plant Physiology and Biochemistry. 2018;132:258-70.

59. Minoru K, Michihiro A, Susumu G, Masahiro H, Mika H, Masumi I, Toshiaki K, Shuichii K, Shujiro O, Toshiaki T. KEGG for linking genomes to life and the environment. Nucleic Acids Research. 2008;36:D480-4.

60. Mao X, Cai T, Olyarchuk JG, Wei L. Automated genome annotation and pathway identification using the KEGG Orthology (KO) as a controlled vocabulary. Bioinformatics. 2005;21:3787-93.

61. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^−ΔΔCT Methods. 2001;25:402-8.

Tables

Table 1 Summary of RNA sequencing data.
| Terms  | Raw-reads | Clean-reads | Q30(%) | Known miRNAs | Novel miRNAs | Total miRNAs |
|--------|-----------|-------------|--------|--------------|--------------|--------------|
| 0 hpi-1 | 25357929  | 24534370    | 96.64  | 191          | 112          | 303          |
| 0 hpi-2 | 18706440  | 18032537    | 96.68  | 187          | 110          | 297          |
| 0 hpi-3 | 16680531  | 16015410    | 97.13  | 185          | 112          | 297          |
| 6 hpi-1 | 31352907  | 30581650    | 95.42  | 187          | 112          | 299          |
| 6 hpi-2 | 31368791  | 31093323    | 96.68  | 184          | 112          | 296          |
| 6 hpi-3 | 29047164  | 28255193    | 96.84  | 184          | 112          | 296          |
| 12 hpi-1 | 15964220  | 15611975    | 97.35  | 184          | 111          | 295          |
| 12 hpi-2 | 12952599  | 12639773    | 97.33  | 177          | 111          | 288          |
| 12 hpi-3 | 17679735  | 17156727    | 97.56  | 181          | 112          | 293          |
| 24 hpi-1 | 16152755  | 15828390    | 96.75  | 188          | 112          | 300          |
| 24 hpi-2 | 12512784  | 12089354    | 97.56  | 181          | 111          | 292          |
| 24 hpi-3 | 15658685  | 15379638    | 97.19  | 180          | 107          | 287          |
| 48 hpi-1 | 16804798  | 15589522    | 97.36  | 157          | 91           | 248          |
| 48 hpi-2 | 13725355  | 1300697     | 97.19  | 166          | 104          | 270          |
| 48 hpi-3 | 15117175  | 14724610    | 97.28  | 166          | 98           | 264          |

Table 2 Classification of small RNAs filtrated.

| rRNA | scRNA | snRNA | snoRNA | tRNA | Repbase | Unannotated | Mapped Reads |
|------|-------|-------|--------|------|---------|-------------|--------------|
| 0 hpi-1 | 12663580 | 0 | 0 | 11072 | 1086155 | 128090 | 10645473 | 4961663 |
| 0 hpi-2 | 11791973 | 0 | 1 | 9710 | 569800 | 69758 | 5591295 | 2310475 |
| 0 hpi-3 | 11104287 | 0 | 0 | 8235 | 664304 | 45855 | 4192729 | 1892412 |
| 6 hpi-1 | 14795074 | 0 | 0 | 9499 | 2101880 | 109887 | 13565310 | 4618107 |
| 6 hpi-2 | 19063502 | 0 | 1 | 17442 | 2404168 | 69915 | 9538295 | 3233356 |
| 6 hpi-3 | 20251033 | 0 | 0 | 17907 | 1023294 | 55417 | 1890754 | 2324538 |
| 12 hpi-1 | 7534532 | 0 | 0 | 6354 | 1339760 | 67803 | 6879941 | 2613764 |
| 12 hpi-2 | 7959450 | 0 | 0 | 6634 | 904740 | 40862 | 3728087 | 1364869 |
| 12 hpi-3 | 311238442 | 0 | 0 | 11455 | 622265 | 54738 | 5229827 | 2082980 |
| 24 hpi-1 | 8379976 | 0 | 0 | 7991 | 855954 | 50917 | 6317137 | 1907110 |
| 24 hpi-2 | 7959606 | 0 | 0 | 7181 | 642724 | 32801 | 3447042 | 1164054 |
| 24 hpi-3 | 105738978 | 0 | 1 | 8016 | 772383 | 25205 | 4000135 | 1192736 |
| 48 hpi-1 | 13580693 | 0 | 0 | 4324 | 86207 | 6519 | 1911779 | 458535 |
| 48 hpi-2 | 10376312 | 0 | 0 | 4259 | 334601 | 13258 | 2272267 | 694750 |
| 48 hpi-3 | 311871790 | 0 | 0 | 5008 | 291416 | 8543 | 2547853 | 748102 |

Table 3 Differential expression of miRNAs in response to downy mildew in non-heading Chinese cabbage.
| Group                  | miRNA name     | PValue   | FDR       | log2FC  | miRNA family |
|------------------------|----------------|----------|-----------|---------|--------------|
| 0 hpi vs 6 hpi         | bra-miRn47-3p  | 1.07E-10 | 3.28E-08  | 2.32137 | -            |
| 0 hpi vs 12 hpi        | bra-miR156c-3p | 1.62E-05 | 0.00076   | -1.01267 | MIR156       |
|                        | bra-miR156g-3p | 1.62E-05 | 0.00076   | -1.01267 | MIR156       |
|                        | bra-miR398-5p  | 3.00E-07 | 4.57E-05  | -1.42354 | MIR398       |
|                        | bra-miR398b-5p | 1.03E-06 | 0.00010   | -1.59384 | MIR398       |
|                        | bra-miRn77-3p  | 1.05E-05 | 0.00077   | -1.55930 | -            |
|                        | bra-miR400b-3p | 2.93E-07 | 4.57E-05  | -1.72636 | MIR400       |
|                        | bra-miRn38-3p  | 0.00059  | 0.01781   | 1.10897  | -            |
| 0 hpi vs 24 hpi        | bra-miR398-5p  | 2.76E-05 | 0.00211   | -1.38707 | MIR398       |
|                        | bra-miR9552b-3p| 1.07E-05 | 0.0152    | 1.74364  | MIR9552      |
|                        | bra-miR398b-5p | 7.04E-09 | 2.15E-06  | -1.87591 | MIR398       |
|                        | bra-miRn47-3p  | 1.49E-05 | 0.00152   | -1.71563 | -            |
|                        | bra-miR5721b-5p| 0.00040  | 0.02449   | 1.14187  | MIR5721      |
|                        | bra-miRn77-3p  | 0.00101  | 0.03870   | -1.23149 | -            |
|                        | bra-miRn28-5p  | 0.00055  | 0.02796   | 1.21597  | -            |
|                        | bra-miRn90-5p  | 0.00093  | 0.03870   | 1.08662  | -            |
| 0 hpi vs 48 hpi        | bra-miRn91a-3p | 1.46E-05 | 0.00111   | 1.68792  | -            |
|                        | bra-miRn91b-3p | 1.46E-05 | 0.00111   | 1.68792  | -            |
|                        | bra-miRn36-3p  | 0.00071  | 0.02689   | 1.03825  | -            |
|                        | bra-miRn47-3p  | 6.30E-15 | 1.92E-12  | 3.25535  | -            |
|                        | bra-miR5721b-5p| 3.41E-05 | 0.00174   | 1.44024  | MIR5721      |
|                        | bra-miR167f-3p | 0.00122  | 0.03722   | -1.30663 | MIR167       |
|                        | bra-miR400b-3p | 0.00014  | 0.00593   | -1.44768 | MIR400       |
|                        | bra-miRn28-5p  | 1.33E-06 | 0.00020   | 1.55367  | -            |

Footnote: FDR, false discovery rate; FC, fold change; log2FC >0 represents up regulated and log2FC >0 represents down regulated; - , miRNA family without match.

Table 4 Statistics of the number of miRNA target genes.

| Types           | All miRNA | miRNA with Target | Target gene |
|-----------------|-----------|-------------------|-------------|
| Known miRNA     | 197       | 183               | 2722        |
| Novel miRNA     | 112       | 85                | 1662        |
| Total           | 309       | 268               | 4384        |

Table 5 Annotation analysis of target genes.
| Anno Database    | Annotated Number | 300≤length<1000 | length≥1000 |
|------------------|------------------|------------------|-------------|
| COG Annotation   | 1756             | 269              | 1483        |
| GO Annotation    | 3201             | 778              | 2389        |
| KEGG Annotation  | 1632             | 385              | 1235        |
| KOG Annotation   | 2409             | 522              | 1869        |
| Pfam Annotation  | 3732             | 809              | 2902        |
| Swissprot Annotation | 3479      | 772              | 2682        |
| EggNOG Annotation| 3946             | 921              | 2990        |
| Nr Annotation    | 4356             | 1113             | 3186        |
| All Annotated    | 4360             | 1114             | 3186        |

**Figures**

**Figure 1**

Length analysis of all reads obtained. a, The average of three biological replicates of clean reads of the length of miRNAs from 18 to 30 nt. b, The average of three biological replicates of mapped reads of the length of miRNAs from 18 to 30 nt. All miRNAs included known miRNAs and novel miRNAs. Y axis represents the number of reads and X axis represents the length of miRNAs.
Figure 2

Length analysis of all miRNAs obtained. a, Length analysis of known miRNAs identified. b, Length analysis of novel miRNAs identified. Y axis represents the number of miRNAs and X axis represents the length of miRNAs.
Figure 3

miRNA nucleotide bias at each position. a, Known miRNA. b, Novel miRNA. Y axis represents the percent of nucleotides and X axis represents the position of miRNAs.
miRNA first nucleotide bias. a, Known miRNA. b, Novel miRNA. Y axis represents the percent of nucleotides and X axis represents the position of miRNAs.

Figure 4
Figure 5

Heat map and hierarchical clustering analysis of differentially expressed miRNAs response to downy mildew in leaves of Non-heading Chinese cabbage. The heat map was generated with Log10 (TPM+1) values from 0 hpi, 6 hpi, 12 hpi, 24hpi, and 48 hpi samples. Each row indicates a miRNA whose converted expression value is plotted on a color scale with green representing lower expression and red representing higher expression.
Figure 6

GO annotation of targets of downy mildew responsive miRNAs in non-heading Chinese cabbage leaves. GO terms belonging to molecular functions, cellular components and biological processes are shown in blue, red and green, respectively. GO terms were sorted based on P values.

Figure 7

KEGG pathway enrichment of targets of downy mildew responsive miRNAs in non-heading Chinese cabbage leaves. X axis represents the percentage of annotated genes.
Figure 8

Expression patterns of target genes in leaves of Non-heading Chinese cabbage after inoculated with H. parasitica by qRT-PCR. Bra010644, Bra020821, Bra005264, Bra010372 and Bra001424 were the target genes of bra-miRn91a-3p and bra-miRn91b-3p; Bra000540 and Bra024465 were the target genes of bra-miRn28-5p; Bra036138 and Bra041056 were the target genes of bra-miR400b-3p; Bra011730 was the target genes of bra-miRn38-3p; Bra035208 was the target genes of bra-miR9552b-3p; Bra023387 was the
target genes of bra-miR167f-3p. Error bars show SE of triplicate assays. Asterisks indicate statistical differences compared with 0 h, as determined by Student’s t-test (*P < 0.05; **P < 0.01).

Figure 9

Hypothetical model of miRNA-mediated regulation of target genes in non-heading Chinese cabbage–H. parasitica interactions. The up- and down-regulation miRNAs are marked red and green background, respectively. The black T represents repress and the blue arrows represents induce.

Supplementary Files

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