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

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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]. *miR398* 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) 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. Differentially 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 and 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-miRn91a-3p and bra-miRn91b-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 et 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, they 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 $\times$ 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 $|\log_2(\text{FC})| \geq 1.00$ and $\text{FDR} \leq 0.05$ 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 ddH₂O. 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^{-ΔΔCt} method [61]. Three technical replicates were ran for each sample. The primers used for qRT-PCR are listed in Table S1.

Abbreviations

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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Tables

Table 1 Summary of RNA sequencing data.

Terms Treatments	Raw-reads	Clean-reads	Q30(%)	Known miRNAs	Novel miRNAs	Total
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	96.83	184	112	296
6 hpi-3	29047164	28255193	96.84	182	112	294
12 hpi-1	16152755	15828390	96.75	188	112	300
12 hpi-2	12952599	12639773	97.33	177	111	288
12 hpi-3	17679735	17156727	97.56	181	112	293
24 hpi-1	15964220	15611975	97.33	184	111	295
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	13000697	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	Rebase	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	6907542	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	11238442	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	10573898	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	11871790	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	0.00152	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	$300 \leq \text{length} < 1000$	$\text{length} \geq 1000$
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.

Figure 4

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 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, 24 hpi, 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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