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Research paper

Genome-wide identification of *turnip mosaic virus*-responsive microRNAs in non-heading Chinese cabbage by high-throughput sequencing

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ABSTRACT

Turnip mosaic virus (TuMV) is the most prevalent viral pathogen infecting most cruciferous plants. MicroRNAs (miRNAs) are around 22 nucleotides long non-protein-coding RNAs that play key regulatory roles in plants. Recent research findings show that miRNAs are involved in plant–virus interaction. However we know little about plant defense and viral offense system networks throughout microRNA regulation pathway. In this study, two small RNA libraries were constructed based on non-heading Chinese cabbage (*Brassica campestris* ssp. *chinensis L. Makino*, NHCC) leaves infected by TuMV and healthy leaves, and sequenced using the Illumina-Solexa high-throughput sequencing technology. A total of 86 conserved miRNAs belonging to 25 known miRNA families and 45 novel ones were identified. Among them, twelve conserved and ten new miRNAs were validated by real-time fluorescence quantitative PCR (qPCR). Differential expression analysis showed that 42 miRNAs were down-regulated and 27 miRNAs were up-regulated in response to TuMV stress. A total of 271 target genes were predicted using a bioinformatics approach, these genes are mainly involved in growth and resistance to various stresses. We further selected 13 miRNAs and their corresponding target genes to explore their expression pattern under TuMV and/or cold (4 °C) stresses, and the results indicated that some of the identified miRNAs could link TuMV response with cold response of NHCC. The characterization of these miRNAs could contribute to a better understanding of plant–virus interaction throughout microRNA regulation pathway. This can lead to finding new approach to defend virus infection using miRNA in Chinese cabbage.

1. Introduction

Endogenous small RNAs (sRNAs) are important regulators of gene expression at the transcriptional and post-transcriptional levels in eukaryotes and influence almost all aspects of plant biology processes both in animals and plants (Eamens et al., 2008). There are two major classes of endogenous sRNAs in plants: microRNAs (miRNAs) and short-interfering RNAs (siRNAs) (Baumberger and Baulcombe, 2005).

So far, miRNAs are the best understood sRNAs. In plants, miRNAs are endogenous non-coding RNAs of 21 to 24 nucleotides (nt) in length (Eddy, 2001; Reinhart et al., 2002). They differ from other sRNAs in their biogenesis. Plant miRNAs are generated from hairpin-structured non-coding transcripts by DICER-LIKE 1 (DCL1), which cleaves a short (21 bp) duplex from the stem region and guide post-transcriptional gene regulation mainly via mRNA cleavage (Kurihara et al., 2006). The duplex is incorporated into an AGO1 complex and then the miRNA* strand is degraded. The mature miRNA strand guides the AGO1 complex (RNA-induced silencing complex, RISC) to protein-coding RNAs, which are cleaved by AGO1 at a specific position (opposite the 10th and 11th nucleotides of the miRNA) (Fagard et al., 2000; Morel et al., 2002). Plant miRNAs suppress gene expression mainly by directing cleavage of their highly complementary target transcripts. Recent studies suggest that translational repression may be also common in plants (Schwab et al., 2006; Balmer and Mauch-Mani, 2013; Martínez de Alba et al.,

Abbreviations: ARF17, auxin response factor 17; CMV, cucumber mosaic virus; CP, coat protein; DCL1, DICER-LIKE 1; ERF, ethylene-responsive transcription factor; FPKM, fragments per million mapped reads; GO, Gene Ontology; JA, jasmonic acid; miRNA, MicroRNA; NHCC, non-heading Chinese cabbage; PR, Pathogenesis-Related; qPCR, real-time fluorescence quantitative PCR; RISC, RNA-induced silencing complex; siRNAs, short-interfering RNAs; sRNAs, small RNAs; TCP, teosinte branched 1/cycloidea/pcftcp; TuMV, Turnip mosaic virus.

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2013). Due to their evolutionary conservation, miRNAs have been found to exist in both plants and animals. Conserved miRNA molecules can also be found in ferns, mosses and fungi (Boopathi, 2013; Delaux et al., 2013).

In plants, miRNAs not only post-transcriptionally regulate their own targets but also interact with each other in regulatory networks to affect many aspects of development, such as developmental timing, senescence, leaf morphogenesis, reproductive development, plant stresses responses and so on (Brodersen et al., 2008; Dhir and Proudfoot, 2013). The number of newly discovered miRNAs is growing rapidly, to date, 18,226 mature miRNAs from a total of 153 species have been discovered and deposited in the public miRNA database miRBase (Release 18.0, http://microrna.sanger.ac.uk/sequences/index.shtml) (Kozomara and Griffiths-Jones, 2014). These miRNAs include 4014 miRNAs from 50 flowering plant species such as *Oryza sativa* (581), *Glycine max* (362), *Populus trichocarpa* (234), *Arabidopsis thaliana* (291), and *Physcomitrella patens* (229) (Kozomara and Griffiths-Jones, 2014). As non-conserved miRNAs are often expressed at a lower level than conserved miRNAs, many non-conserved miRNAs were not found in small-scale sequencing projects. However, high-throughput sequencing technologies have allowed the identification of many non-conserved miRNAs in several species (Fahlgren et al., 2007; Sunkar et al., 2008; Szittya et al., 2008; Pantaleo et al., 2010).

Turnip mosaic virus (TuMV) belongs to the potato Y virus family (Garcia-Ruiz et al., 2010). It mainly infects *cruciferous* and some ornamental plant species and its harm is only less than the *cucumber mosaic virus* (CMV) for vegetable production (García-Arenal et al., 2001; Ohshima et al., 2002). TuMV can produce diverse symptoms in different types of host plants, such as mosaic, necrosis or dwarf (Green and Deng, 1985). So far, several genes have been found that involved in *Brassica* TuMV resistance such as resistance genes *TuRB01*, *TuRB0lb*, *TuRB03*, *TuRB04*, *TuRB05*, *Retr01*, *ConTR01* and susceptible gene *TuRB02* (Jenner et al., 2000, 2003). Previous researches have revealed that *CP* gene accumulation in plant cells could inhibit the replication of the virus infection, thereby reducing the symptoms or delays the onset time of virus, causes the plant to obtain a high level of resistance (Abel et al., 1986). The obtained *CP* transgenic disease-resistant plants contain cabbage, tobacco, tomato, potato and so on (Abel et al., 1986; Hemenway et al., 1988; Brown et al., 2001; Tatineni et al., 2014). Zhu et al. firstly transferred *TuMV-CP* gene into Chinese cabbage, the obtained transgenic plants showed obvious resistance, and can delay the onset of 20–30d, after onset showed only mild mosaic, and virus content in plant were also significantly lower than normal plants (Zhu et al., 2012).

Non-heading Chinese cabbage (*Brassica campestris* ssp. *chinensis* L. Makino, NHCC) is a host of TuMV, and its production is seriously influenced by TuMV. To deeply explore the molecular mechanism of the NHCC TuMV resistance is essential. In this study, we identified miRNAs and their targets using high-throughput sequencing methods from both TuMV infected and healthy NHCC plants. The differential expression of miRNAs observed between them suggested that biogenesis of miRNAs could be influenced in the plant TuMV resistance.

2. Materials and methods

2.1. Plant materials

The non-heading Chinese cabbage cultivar 'Xiangqingcai' was used for both Illumina-Solexa sequencing and real-time quantitative PCR (qPCR) analysis. Seedlings of 'Xiangqingcai' were grown in a glasshouse at 22–25 °C with a 16 h light/8 h dark photoperiod and relative humidity of 75%. Then five-leaf stage seedlings were infected with TuMV, two weeks later, leaves from TuMV-infected and healthy plants were collected respectively for subsequent small RNA sequencing. For qPCR analysis, the five-leaf stage seedlings were subjected to three independent treatments as the following: healthy seedlings with TuMV infection, healthy seedlings with cold treatment (4 °C) for 2 h, TuMV infected seedlings with cold treatment (4 °C) for 2 h.

2.2. Total RNA isolation, small RNA library preparation and sequencing

RNA was extracted using RNeasy plant mini kit (Qiagen, Beijing, China) that refers to the manufacturer's instructions. Total RNA quantity and purity of both samples were assayed with the NanoDrop ND-2000 spectrophotometer (Thermo Fisher, Waltham, USA) at 260/280 nm (ratio >2.0). The total RNA balanced mix sample was fractionated by 15% denaturing polyacrylamide gel electrophoresis, and then the small RNA fragments between 18 and 28 nt were isolated from the gel. The small RNA molecules were ligated to a 5′ adaptor and a 3′ adaptor by T4 RNA ligase. Subsequently, the adapter-ligated small RNAs were converted to cDNA by RT-PCR following the Illumina protocol. The purified cDNA libraries were subjected to Solexa/Illumina sequencing in Biomarker Technologies (Beijing, China).

2.3. Bioinformatic analyses

The clean reads were filtered for miRNA prediction with the ACGT101-miR-v3.5 package (LC Sciences, Houston, USA). Firstly, reads matched rRNA, tRNA, snRNA, snoRNA, and other ncRNAs deposited in Rfam (http://www.sanger.ac.uk/software/Rfam) and the GenBank noncoding RNA database (http://www.ncbi.nlm.nih.gov/) were discarded (Griffiths-Jones et al., 2008). Then the retained reads were mapped onto the genome of Chinese cabbage (Wang et al., 2011) using MapMi software under default parameters. New candidate miRNAs were identified by folding the flanking genome sequence of unique small RNAs using miRBase Version 18.0, followed by the prediction of secondary structures by Mfold program (Zuker, 2003). Differentially expressed miRNAs in the two libraries were identified by the online service IDEG6 (http://telethon.bio.unipd.it/bioinfo/IDEG6_form/). Audic and Claverie, Fisher's exact test and chi-squared 2×2 were used for the selection of differential expression genes (DEGs), with the selection threshold of 0.01 (http://telethon.bio.unipd.it/bioinfo/IDEG6_form/detail.html#AC).

2.4. Real-time quantitative PCR

We performed qPCR to explore the validation and stress expression patterns of identified miRNAs and their putative target genes. The expression of selected miRNAs was assayed in Col and TuMV-in lines of Chinese cabbage by Platinum SYBR Green based qPCR (Invitrogen, 11733–038) with the High Specificity miRNA QuantiMir RT Kit (RA610A-1, System Biosciences) on Step One™ Real-Time PCR System (Applied Biosystems). The primers of these miRNAs and 2 internal control genes (U6 snRNA and actin) are available in Supplementary Table S8. The expression of selected target genes was assayed in Col and TuMV-in lines of Chinese cabbage by qPCR. Primers used are listed in Supplementary Table S8. The qPCR reactions were performed as follow: 94 °C for 30 s, and then 40 cycles at 94 °C for 10 s, 58 °C for 30 s. All the gene expression data were obtained from three individual biological/technical replicates and processed according to strict statistical methods.

3. Results

3.1. SRNA populations in the TuMV resistance of NHCC

To study the possible gene differences involved in the pathogen defense processes possibly caused by sRNA in NHCC, the sRNA libraries were respectively constructed with the total RNAs from TuMV virus infected (TuMV-in) and healthy (CK) plant leaves. Deep sequencing by Illumina-Solexa high-throughput sequencing technology generated a total number of 13,421,729 and 13,922,599 raw reads from TuMV-in