Arch Virol (2017) 162:2591–2602
DOI 10.1007/s00705-017-3401-6

ORIGINAL ARTICLE

Genome-wide identification of cucumber green mottle mosaic virus-responsive microRNAs in watermelon

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Received: 6 February 2017 / Accepted: 2 May 2017 / Published online: 9 May 2017

Abstract Cucumber green mottle mosaic virus (CGMMV) is a damaging pathogen that attacks crop plants belonging to the family *Cucurbitaceae*. Little is known about the regulatory role of microRNAs (miRNAs) in response to CGMMV infection. To identify CGMMV-responsive miRNAs, two sRNA libraries from mock-inoculated and CGMMV-infected watermelon leaves were constructed and sequenced using Solexa sequencing technology. In total, 471 previously known and 1,809 novel miRNAs were obtained, of which 377 known and 246 novel miRNAs were found to be differentially expressed during CGMMV infection. The target genes for the CGMMV-responsive known miRNAs are active in diverse biological processes, including cell wall modulation, plant hormone signaling, defense-related protein induction, primary and secondary metabolism, regulation of virus replication, and intracellular transport. The expression patterns of some CGMMV-responsive miRNAs and their corresponding targets were confirmed by RT-qPCR. One target gene for miR156a-5p was verified by 5'-RNA-ligase-mediated rapid amplification of cDNA ends (5'-RLM-RACE) analysis. The results of this study provide further insights into the miRNA-mediated regulatory network involved in the response to viral infection in watermelon and other cucurbit crops.

Electronic supplementary material The online version of this article (doi:10.1007/s00705-017-3401-6) contains supplementary material, which is available to authorized users.

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Introduction

Watermelon [*Citrullus lanatus* (Thunb.) Matsum. & Nakai] (2n = 2x = 22) is an annual trailing plant in the family *Cucurbitaceae*. Watermelon fruit is a healthy food that contains proteins, carbohydrates, minerals, vitamins, and dietary fiber, and it is popular with consumers [1]. The peel and seeds of watermelon fruits are also edible. The annual production of watermelon is approximately 97 million tons worldwide, and about 67% of watermelon production is in China (http://faostat3.fao.org). However, watermelon production has been severely affected by cucumber green mottle mosaic virus (CGMMV).

CGMMV belongs to the genus *Tobamovirus* in the family *Virgaviridae* [2]. It is a damaging pathogen that infects cucurbit crops such as watermelon, cucumber, melon, and bottle gourd [2–4]. Plants infected with CGMMV exhibit characteristic mosaic symptoms on the leaves, show stunted growth, and produce distorted fruit [2, 5]. CGMMV has a far-ranging geographic distribution. It was first reported by Ainsworth in 1935 [6]. Since then, it has spread to more than 30 countries and regions in Europe [7, 8], Asia [9–11] the Middle East [12, 13], North America [4, 14] and Oceania [3]. CGMMV is easily transmitted by seeds, pollen, soil, water, and insect vectors, as well as mechanical contact [5, 15, 16]. Currently, there are no effective methods to control its spread. However, there is increasing evidence that miRNA-mediated RNA silencing has the potential to control viral diseases. miRNA-mediated RNA silencing is the process in which miRNAs target

viral suppressors of RNA silencing (VSRs), thereby contributing to viral resistance in plants [17, 18].

MicroRNAs (miRNAs), approximately 19–25 nucleotides in length, are a class of small, endogenous, non-coding RNAs that play roles in regulating gene expression [19]. Previous reports have revealed a general role for miRNAs in response to viral infection. In *Arabidopsis*, miR156 and miR164 were shown to be induced by expression of the VSR P1/HC-Pro, and miR171 was shown to accumulate in response to turnip mosaic virus (TuMV) and direct the cleavage of Scarecrow-like transcription factors [17]. In tobacco, miR6019 and miR6020 were reported to confer resistance to tobacco mosaic virus (TMV) by guiding the cleavage of the immune receptor N [20]. In rice, miR160, miR166, miR167, miR171, and miR396 were found to accumulate in response to rice stripe virus (RSV) infection [21]. These results imply that miRNAs are involved in host resistance against viral infection by targeting VSRs [18].

High-throughput sequencing technology has led to the identification of more miRNAs and clarification of their regulatory roles in response to viral infection. For example, in a previous study, two sRNA libraries from mock-inoculated and soybean mosaic virus (SMV)-infected soybean leaves were constructed and sequenced. Subsequent 5′-RNA-ligase-mediated rapid amplification of cDNA ends (5′-RLM-RACE) analyses showed that 12 targets of nine miRNAs (miR395, miR530, miR1510, miR1514, miR1515, miR1535, miR2109, miR3522 and miR2118-3p) responded to SMV infection [22]. A comparative analysis revealed that 79 previously known and 40 novel miRNAs were differentially expressed between mock-inoculated and cucumber mosaic virus (CMV)-infected tomatoes. Functional analysis demonstrated that the target genes of these differentially expressed miRNAs in CMV-infected tomatoes were related to the defense response and photosynthesis [23]. Although a few miRNAs were shown to be produced by cucumber plants infected with CGMMV [2], the lack of a mock-inoculated sample made it difficult to clarify the regulatory role of miRNA in response to CGMMV infection.

To identify the miRNAs involved in the response to CGMMV in watermelon, two sRNA libraries from mock-inoculated and CGMMV-infected watermelon leaves were constructed and sequenced using the Illumina Hiseq 4000 system. Known and novel miRNAs were found to be induced by virus infection, and their expression patterns were analyzed. Based on the results of a functional analysis, the possible roles of miRNAs in response to CGMMV are discussed. The results of this study increase our understanding of how miRNA-mediated regulatory networks function in response to viral infection in watermelon and other cucurbit crops.

Materials and methods

Plant materials

The watermelon (*Citrullus lanatus* L.) advanced inbred line 'JJZ-M' was used in this study. Seeds were planted in plastic pots and were kept at room temperature under insect-proof netting. Seedlings at the two-true-leaf stage were mechanically inoculated with the virus or mock-inoculated on the first two true leaves. The virus inoculum was prepared by macerating virus-infected bottle gourd leaves (1:5 w/v) in 5 mM sodium phosphate buffer (pH 7.2) with a mortar and pestle. Mock-inoculated plants were inoculated with sodium phosphate buffer only. Leaves were harvested separately at 25 days post-inoculation (dpi). The presence of CGMMV was confirmed by RT-PCR using CGMMV coat-protein-specific primers (forward, 5′-ATGGCTTACAATCCGATCACAC-3′; reverse, 5′-CTAAGCTTTCGAGGTGGTAGCC-3′).

Small-RNA library construction and high-throughput sequencing

Equal amounts of leaf samples from three plants were pooled for a single experiment. Total RNA was extracted using RNAiso reagent (Takara, Otsu, Japan). The quantity and quality of RNA samples were measured using a NanoDrop 2000 spectrophotometer (Thermo, USA) and by electrophoresis. Total RNA was separated and purified by 15% denaturing polyacrylamide gel electrophoresis. Then, sRNAs with lengths ranging from 18 to 30 nt were ligated to 5′ and 3′ adapters using T4 RNA ligase. The adapter-ligated fragments were reverse transcribed and amplified by PCR using a pair of complementary adapter primers. The purified PCR products were sequenced using an Illumina Hiseq 4000 instrument. Construction of the sRNA libraries and high-throughout sequencing were carried out by 1Gene (Hangzhou, China).

Bioinformatics analysis of the sequencing data of the small RNAs

Clean reads were obtained by filtering low-quality reads, polyAs, ex-length, 5′ adaptor contaminants, 3′ adaptor nulls, and insert nulls from the raw data. Using SOAP2 software [24], the clean reads were aligned against sequences at the Rfam database (http://www.sanger.ac.uk/Software/Rfam) [25] and NCBI GenBank (http://www.ncbi.nih.gov/GenBank/) [26], as well as the watermelon genome (http://www.icugi.org). Reads that were annotated as non-coding RNA or were classified as mRNA degradation fragments were removed. The remaining sRNA

sequences were subjected to BLASTn searches using the miRBase database version 21.0 (http://www.mirbase.org/index.shtml) [27]. Sequences with nearly perfect matches (fewer than two mismatches) were considered to be known miRNAs [28]. Small RNAs not mapped to any miRNAs in miRBase were classified as putative novel miRNAs. The stem-loop structures of pre-miRNAs were modeled using Mfold software [29].

Identification of differentially expressed miRNAs and prediction of their targets

To identify CGMMV-responsive miRNAs, a differential expression analysis of miRNAs was carried out. The expression of miRNAs was normalized to transcripts per million (TPM) [30]. The normalized value of miRNAs with an abundance of zero was set to 0.01. The *P*-value for each miRNA was calculated as described elsewhere [30, 31]. miRNAs with a *P*-value less than 0.01 and an absolute $\log_2$ value (CGMMV/mock) greater than 1 were considered to be differentially expressed. Target genes of differentially expressed miRNAs were predicted according to criteria established previously [32, 33]. The target genes were queried against the KEGG database to map them to KEGG reference metabolic pathways [34].

RT-qPCR validation of CGMMV-responsive miRNAs and their targets

To verify the expression patterns of CGMMV-responsive miRNAs and their targets, several miRNAs and their corresponding targets were selected for validation and quantification according to previously established methods [35]. For the miRNAs, about 2 μg of total RNA was reverse transcribed into single-strand cDNA using TransScript miRNA First-Strand cDNA Synthesis SuperMix (TransGen, Beijing, China). The reverse primer (GTGCAGGGTCCGAGGT) was universal, and the forward primers were designed based on miRNA-specific sequences. The internal reference was U6 snRNA (forward, GGGGACATCCGATAAAATT; reverse, TGTGCGTGTCATCCTTGC). For analysis of the corresponding targets, total RNA was reverse transcribed into cDNA using TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen) according to the manufacturer's instructions. The housekeeping gene encoding β-actin was used as an internal control (primers: forward, 5′-CCATGTATGTTGCCATCCAG-3′; reverse, 5′-GGATAGCATGGGGTAGAGCA-3′). Each reaction was performed in a total volume of 20 μl containing 10 μl of 2× TransStart Top Green qPCR SuperMix, 2.0 μl of diluted cDNA, 0.4 μl of 50× Passive Reference Dye I, and 0.2 μM each primer. The RT-qPCR conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 55 °C for 15 s, and 72 °C for 10 s using the StepOnePlus Real-Time PCR System (ABI, USA). All PCR products were denatured at 95 °C and cooled to 65 °C, and the fluorescence signals were monitored consistently from 65 °C to 95 °C as the temperature increased at 0.3 °C per second. All reactions were performed in triplicate. The fold change in gene expression was estimated in terms of threshold cycles using the $2^{-\Delta\Delta CT}$ method [36]. The primers used to amplify miRNAs and their targets by RT-qPCR are listed in Table S1.

5'-RLM-RACE validation of targets

To experimentally validate the targets for cleavage, total RNA was extracted from mock-inoculated and CGMMV-infected watermelon leaves using RNAiso reagent (Takara). Total RNA was subjected to 5′-RLM-RACE using a 5′-Full RACE kit (Takara). First, total RNA was treated with calf intestinal phosphatase to remove the 5' phosphate from all molecules containing free 5' phosphates (degraded mRNA, tRNA, ribosomal RNA, and genomic DNA). Then, the RNA was treated with tobacco acid pyrophosphatase to remove the cap structure from the full-length mRNA, leaving a 5'-monophosphate. The 5′ RACE adapter was ligated to the uncapped full-length mRNA using T4 RNA ligase (Takara). cDNA was synthesized using random 9-mers and M-MLV reverse transcriptase (RNase H^-). Nested PCR was performed with the 5′ RACE outer primer (5′-CATGGCTACATGCTGACAGCCTA-3′) and a gene-specific primer (GSP1), followed by second-round PCR with the 5′ RACE inner primer (5′-CGCGGATCCA-CAGCCTACTGATGATCAGTCGATG-3′) and another gene-specific primer (GSP2). The RACE products were cloned and sequenced for analysis.

Results

Confirmation of CGMMV infection

At 25 dpi, characteristic symptoms of CGMMV, including mosaic patterning on the leaves, shriveling of veins, and slight growth inhibition, were observed in the CGMMV-infected plants (Fig. 1b), while the mock-inoculated plants exhibited no disease symptoms (Fig. 1a). The presence/absence of CGMMV in both mock-inoculated and CGMMV-infected samples was confirmed by RT-PCR using coat protein-specific primers. Only the infected samples produced the specific 486-bp fragment (Fig. 1c).

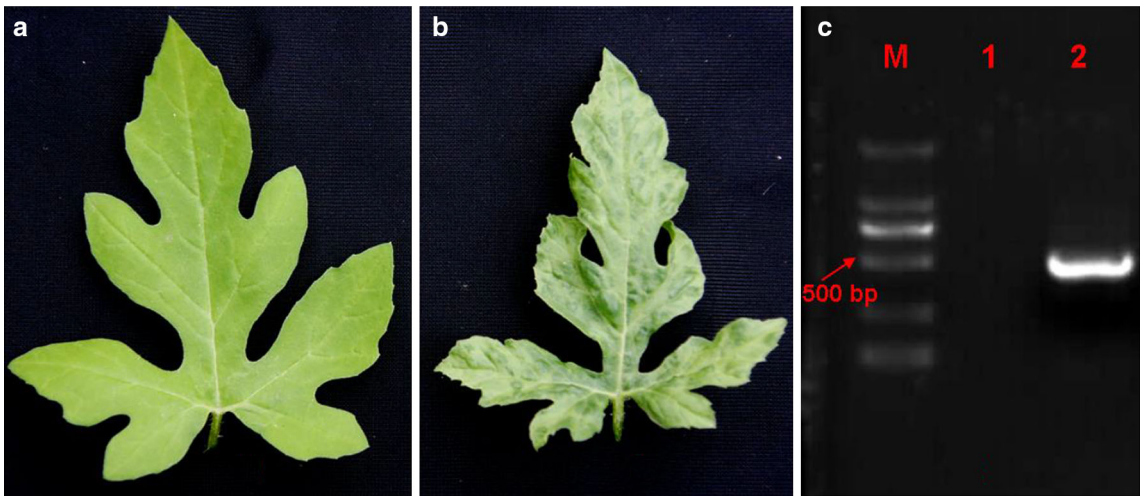


Fig. 1 Confirmation of CGMMV infection of watermelon seedlings. **a**. Healthy leaf from a mock-inoculated plant **b.** Leaf from a CGMMV-infected plant exhibiting typical symptoms of mottling **c.** confirmation of CGMMV status by RT-PCR. Lane M, DNA ladder (DL2000); lane 1, mock-infected sample; lane 2, inoculated sample

Overview of small-RNA sequencing results

About 34.32 M and 28.83 M raw reads were generated from the mock and CGMMV library, respectively. After eliminating poor-quality reads, 28.13 M (7.58 M unique sequences) and 26.13 M (5.63 M unique sequences) clean reads were obtained from the mock and CGMMV library, respectively. About 88.60% of total sRNAs in the mock library mapped to the watermelon genome, while only 39.91% of those in the CGMMV library mapped to the watermelon genome. More than 80% of unique sRNAs in both the mock and CGMMV libraries mapped to the watermelon genome (Table 1). Among them, 6,278,721 (53.29%) unique sequences were mock-library-specific, 4,366,163 (37.06%) unique sequences were CGMMV-library-specific, and 1,137,476 (9.65%) unique sequences were common to both libraries (Table 2). These sRNAs were annotated into ten categories (Table 3). Of these, 51,957 (0.69%) and 64,341 (1.14%) unique sRNAs were annotated as miRNAs in the mock and CGMMV library, respectively. More than 86% of unique sequences in both libraries were classified as unannotated sRNAs.

The length of the majority of sRNA reads was between 20 and 24 nt (Fig. 2). The abundance of these sRNA differed significantly between the two libraries. The proportions of 21- and 22-nt sRNAs were markedly higher in the CGMMV library than in the mock library, whereas the proportion of 24-nt sRNAs was much lower in the CGMMV library than in the mock library. This result suggested that the 21- and 22-nt sRNAs were induced by CGMMV infection and might play a role in the response to the virus.

Identification of known miRNAs in watermelon

The small RNA sequences were aligned with currently known and experimentally validated mature miRNAs deposited in miRBase21.0. After a homology search, a total

Table 1 Statistical analysis of sequencing data from the mock and CGMMV sRNA libraries from watermelon

Category	Mock	CGMMV
Total reads	34,325,694	28,831,544
Low-quality reads	32,936	27,682
3' adapter null	228,523	413,230
Insert null	81,354	35,478
5' contaminants	146,321	109,320
Ex-length	5,701,734	2,110,342
PolyA	2,053	3,503
Clean reads	28,132,773	26,131,989
Total sRNAs mapping to genome	24,925,121 (88.60%)	10,428,318 (39.91%)
Unique sRNAs	7,577,628	5,628,239
Unique sRNAs mapping to genome	6,374,983 (84.13%)	4,502,785 (80.00%)

Table 2 Summary of common and specific sequences between mock and CGMMV sRNA libraries

Category	Unique sRNAs	Percentage (%)	Total sRNAs	Percentage (%)
Total_sRNAs	11,782,360	100.00%	24,118,792	100.00%
CGMMV_&_mock	1,137,476	9.65%	8,777,576	36.39%
CGMMV_specific	4,366,163	37.06%	6,724,686	27.88%
mock_specific	6,278,721	53.29%	8,616,530	35.73%

Table 3 Distribution of small RNAs among different categories in watermelon

Category	Mock		CGMMV	
	Unique sRNAs	Total sRNAs	Unique sRNAs	Total sRNAs
Total	7,577,628 (100%)	28,132,773 (100%)	5,628,239 (100%)	26,131,989 (100%)
exon_antisense	61,193 (0.81%)	1,780,237 (6.33%)	87,780 (1.56%)	334,634 (1.28%)
exon_sense	211,092 (2.79%)	560,880 (1.99%)	123,060 (2.19%)	206,201 (0.79%)
intron_antisense	184,825 (2.44%)	395,566 (1.41%)	149,663 (2.66%)	223,433 (0.86%)
intron_sense	249,023 (3.29%)	609,393 (2.17%)	215,163 (3.82%)	394,064 (1.51%)
miRNA	51,957 (0.69%)	2,102,989 (7.48%)	64,341 (1.14%)	2,607,198 (9.98%)
rRNA	162,027 (2.14%)	4,865,252 (17.29%)	91,154 (1.62%)	1,030,187 (3.94%)
snRNA	7,922 (0.10%)	35,258 (0.13%)	8,644 (0.15%)	55,723 (0.21%)
snoRNA	5,195 (0.07%)	31,310 (0.11%)	3,434 (0.06%)	18,920 (0.07%)
tRNA	35,183 (0.46%)	1,428,054 (5.08%)	24,350 (0.43%)	496,896 (1.90%)
Unannotated	6,609,211 (87.22%)	16,323,834 (58.02%)	4,860,650 (86.36%)	20,764,733 (79.46%)

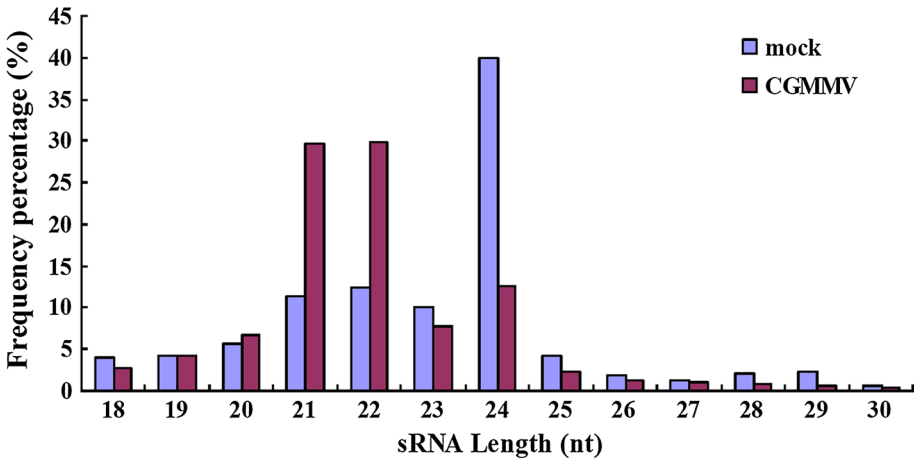


Fig. 2 Length distribution of sRNAs in the watermelon mock and CGMMV libraries. The *y*-axis indicates the percentage of sRNA reads in each library, whereas the *x*-axis corresponds to the nucleotide (nt) lengths of sRNAs

of 471 known miRNAs (353 known miRNAs in the mock library, 256 known miRNAs in the CGMMV library) belonging to 411 families were identified (Table S2). The majority of miRNA families (89.05%) contained only one member, while 12 miRNA families (miR1028, miR1520, miR156, miR164, miR165, miR166, miR171, miR172, miR1886, miR2111, miR319, and miR7696) had three members. The majority of known miRNAs, including 219 (62.03%) in the mock library and 185 (72.26%) in the CGMMV library, had more than 100 reads (Table S2). The known miRNAs showed a wide range of read counts. For example, miR159a, miR166h-3p, and miR3932b-5p showed extraordinarily high expression levels in both libraries, whereas miR9472-5p and miR5558-5p had read counts of less than ten.

Identification of novel candidate miRNAs in watermelon

Based on the criteria set describes previously [28], a total of 1,613 and 463 novel miRNA precursor candidates were identified for the mock and CGMMV library, respectively

(Table S3), representing a total of 1,809 unique miRNA sequences (Table S4). The length of the novel miRNA precursors ranged from 66 to 374 nt (average, 175 nt). The minimum folding free energies of the putative miRNA precursors ranged from −214.6 to −18.0 kcal/mol (average, −39.61 kcal/mol) (Table S3). The novel mature miRNAs had a length distribution ranging from 20 to 25 nt, with 23 nt accounting for the highest proportion (Table S4). Whereas the known miRNAs showed relatively high expression levels, the predicted novel miRNAs were often expressed at low levels. Only 45 and 66 novel miRNAs in the mock and CGMMV library, respectively, had more than 100 reads (Table S4). Using mfold software, the pre-miRNA sequences with more than 100 reads from the mock and CGMMV libraries were subjected to the typical stem-loop secondary structure prediction, and the hairpin-like secondary structures were generated (Fig. S1).

Identification of CGMMV-responsive miRNAs in watermelon

Next, the differentially expressed miRNAs after CGMMV infection were obtained by comparing the expression of miRNAs between the two libraries. A total of 377 known miRNAs and 246 novel miRNAs were identified as differentially expressed in response to CGMMV infection (Table S5). The miRNAs with the greatest change in expression levels were miR7696a-3p and miR1047-3p (−16.34-fold and 19.60-fold change, respectively). Among these differentially expressed miRNAs, 178 known and 158 novel miRNAs were detected only in the mock library, whereas 114 known and 54 novel miRNAs were detected only in the CGMMV library (Table S5), suggesting that these miRNAs might be induced or repressed during CGMMV infection in watermelon. These differentially regulated miRNAs may play crucial roles in response to CGMMV infection in watermelon. Further analyses indicated that different members of a certain miRNA family might have similar or different expression patterns after CGMMV infection. For example, miR164b-3p and miR164d were significantly upregulated, whereas miR164b was downregulated in response to CGMMV infection (Table S5), providing further evidence of the complexity of miRNA-mediated regulation.

Target prediction and annotation of CGMMV-responsive miRNAs

Predicting the targets of these CGMMV-responsive miRNAs is important for elucidating their biological functions. In total, 1,024 genes were predicted to be targets of the 120 CGMMV-responsive known miRNAs (Table S6). The target genes encoded a variety of transcription factors, including zinc finger, ARF, eIF4, MYBs, ERFs, bZIPs, bHLH, and WD40, which might regulate the corresponding transcriptional processes in watermelon. Other predicted target genes encoded functional proteins such as laccase, pectinesterase, cellulose synthase, LRR, disease resistance protein RGA, cytokinin dehydrogenase, cinnamyl alcohol dehydrogenase, phenylalanine ammonia-lyase, flavanone 3-hydroxylase, dihydroflavonol-4-reductase, and long-chain-fatty-acid-CoA ligase. Therefore, these proteins and enzymes may be involved in the CGMMV response in watermelon.

The targets of CGMMV-responsive miRNAs were queried against the KEGG database (Fig. 3). The predominant pathways identified in the CGMMV response included fat digestion and absorption (ko04975), pancreatic secretion (ko04972), chloroalkane and chloroalkene degradation (ko00625), bile secretion (ko04976), atrazine degradation (ko00791), adipocytokine signaling pathway (ko04920), valine, leucine and isoleucine degradation (ko00280), thiamine metabolism (ko00730), ABC transporters (ko02010), basal transcription factors (ko03022), bacterial invasion of epithelial cells (ko05100), RNA degradation (ko03018), beta-alanine metabolism (ko00410), PPAR signaling pathway (ko03320), aminoacyl-tRNA biosynthesis (ko00970), ubiquitin mediated proteolysis (ko04120), Fc gamma R-mediated phagocytosis (ko04666), purine metabolism (ko00230), and ether lipid metabolism (ko00565).

RT-qPCR and 5'-RLM-RACE validation of CGMMV-responsive miRNAs and their targets

To confirm the high-throughput sequencing results and examine the expression patterns of selected CGMMV-responsive miRNAs in watermelon, six miRNAs (miR1222a, miR162a-5p, miR390a-5p, miR393a-3p, miR394a, and miR477b) were chosen for RT-qPCR validation (Fig. 4a). Among them, miR1222a, miR162a-5p, miR390a-5p, miR393a-3p, and miR394a showed upregulated expression after CGMMV infection, consistent with the high-throughput sequencing results. miR477b was downregulated after CGMMV infection according to the Solexa sequencing result, whereas the RT-qPCR result showed an upregulated expression pattern. Most miRNAs showed similar expression patterns in RT-qPCR and the high-throughput sequencing results, indicating that the sRNA sequencing data were reliable.

The dynamic expression patterns of the following six corresponding target genes were also validated by RT-qPCR (Fig. 4b): Cla010953 (auxin-induced protein) targeted by miR1222a, Cla002608 (LRR receptor-like serine) targeted by miR162a-5p, Cla002774 (LRR receptor-like protein) targeted by miR390a-5p, Cla015505 (pectinesterase 3) targeted by miR393a-3p, Cla003987 (dehydration-responsive protein) targeted by miR394a, and Cla019248

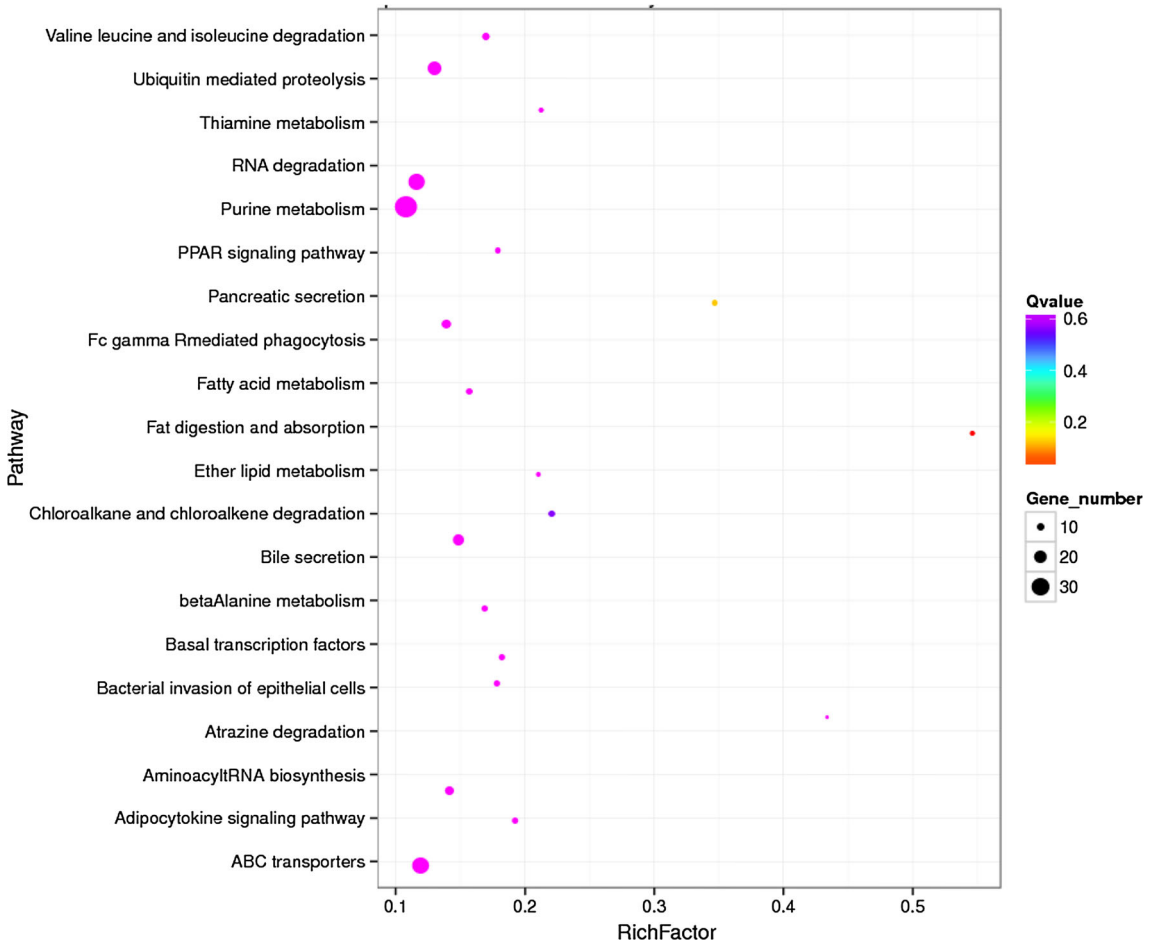


Fig. 3 Top 20 statistics pathway enrichment for different expressed miRNAs targets. The size of each circle represents the number of significantly differentially expressed genes enriched in the corresponding pathway. The enrichment factor was calculated using the number of enriched genes divided by the number of all background genes in the corresponding pathway. The Q-value was calculated using the Benjamini–Hochberg correction. A pathway with $Q < 0.05$ is considered significantly overrepresented

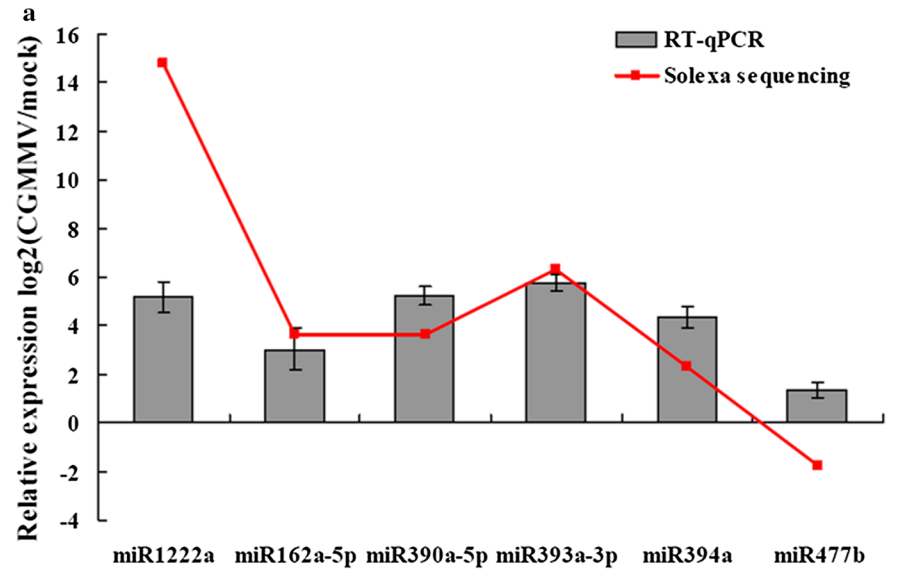
(DELLA protein GAI) targeted by miR477b. Four target genes, Cla010953, Cla002608, Cla002774, and Cla015505, showed downregulated expression after CGMMV infection. However, Cla003987 and Cla019248 were upregulated after CGMMV infection. Most miRNAs negatively regulate their corresponding target genes.

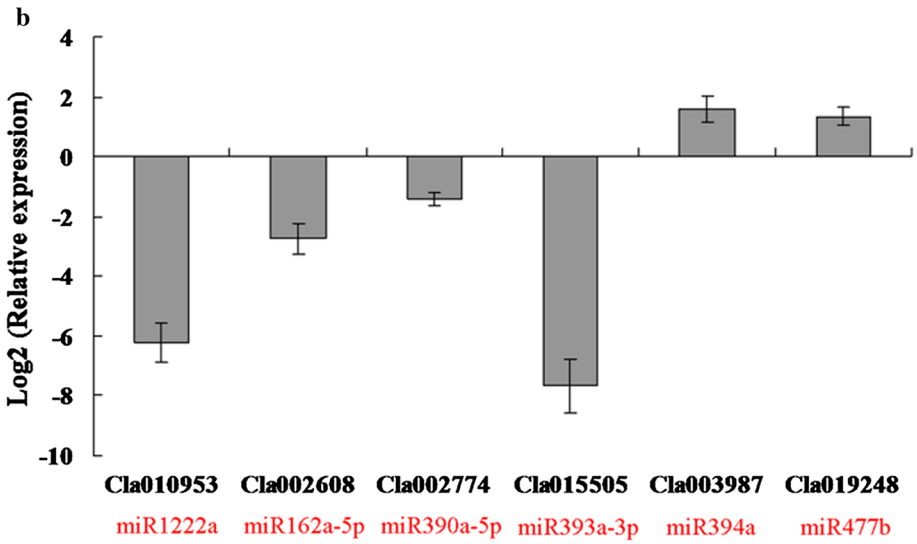
To confirm the predicted targets of miRNAs, several target genes of CGMMV-responsive miRNAs were selected for 5′-RLM-RACE analysis. One target gene for miR156a-5p, Cla018546 (squamosa promoter-binding-like protein 6, *SPL6*), was confirmed. The splicing site was located at the ninth and tenth bases of miR156a-5p (Fig. 5).

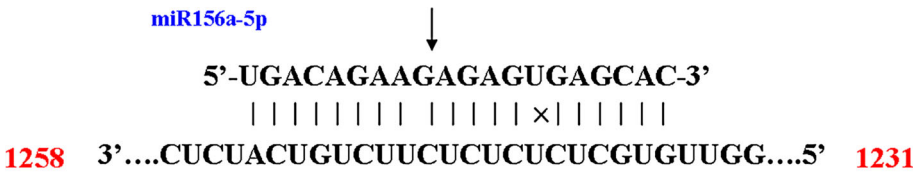
Discussion

miRNAs are well-known regulators of gene expression and play crucial roles in the plant response to viral infection [37]. Previous reports have demonstrated that miRNA regulation plays a significant role in the response to viral infection in *Arabidopsis* [17], *Brassica* [38], rice [21], and cucumber [2]. However, identification of virus-responsive miRNAs in watermelon has not been reported until now.

There was a notable difference in sRNA length distribution between the two libraries. The abundance of 21- and 22-nt sRNAs increased markedly, and that of 24-nt sRNAs decreased significantly during CGMMV infection (Fig. 2). Changes in the distribution of sRNA lengths after viral infection have also been reported in other studies [39, 40]. These highly accumulated 21- and 22-nt sRNAs might be virus-derived small interfering RNAs (vsiRNAs) yielded by Dicer-like proteins (DCLs), Argonaute (AGOs), and RNA-dependent RNA polymerase (RdRps) [39, 40]. These vsiRNAs share similar features with host siRNAs, and result in antiviral immunity via the RNA silencing mechanism [41]. There is increasing evidence that some of these vsiRNAs can guide the degradation of homologous transcripts to create suitable conditions for viral proliferation







Cla018546 (*Squamosa promoter-binding-like protein 6*)

Fig. 4 RT-qPCR analysis of several CGMMV-responsive miRNAs and their targets. **a.** Comparison of relative expression levels of miRNAs between RT-qPCR and Solexa sequencing in watermelon. **b**. RT-qPCR analysis of miRNA targets. Each bar shows the mean ± SD of triplicate assays

Fig. 5 Experimental confirmation of the predicted miRNA targets for miR156a-5p. Experimental validation of mRNA cleavage sites was performed using a modified 5'-RLM-RACE assay. Watson–Crick pairing (vertical dashes) and mismatched bases (cross) are indicated. Arrows indicate the 5' termini of mRNA fragments isolated from watermelon, as indentified by 5'-RLM-RACE. miR156a-5p and the target gene are indicated in blue. The cleavage position of the target gene is marked in red (color figure online)

[39, 40, 42]. Therefore, these vsiRNAs that mediate RNA silencing have the potential to provide specific antiviral immunity in plants.

In the present study, 377 known miRNAs were differentially expressed under CGMMV stress and were therefore considered as CGMMV-responsive miRNAs (Supplementary Table S5). Of the 22 miRNA families that were produced in cucumber plants infected with CGMMV [2], 11 families (miR156, miR164, miR167, miR171, miR172, miR390, miR395, miR437, miR812, miR827, and

miR838) showed significant changes in expression in response to CGMMV infection in watermelon, while the other families were not detected. This discrepancy suggested that miRNAs are potentially expressed in a species-specific manner during CGMMV infection. Additionally, some CGMMV-responsive miRNAs identified in this study might be involved in distinct biotic stresses. For instance, miR160, miR167, and miR393 were found to be highly induced after infection by the bacterium *P. syringae* pv. *tomato* DC3000 [43]. Similarly, miR156, miR160, and miR164 were induced after virus infection in tobacco [44]. miRNA families miR160, miR166, miR167, miR171, and miR396 accumulated in response to RSV infection in rice [21]. As expected, most of these previously identified biotic-responsive miRNAs also showed differential expression after CGMMV infection in watermelon. For instance, our results showed that miR156a-5p, miR159a-3p, miR164b, miR166u, and miR171a were downregulated, whereas miR166d-5p, miR167b-3p, miR171a-3p, miR393a-3p, and miR396a-3p were upregulated in response to CGMMV infection (Supplementary Table S5). This is likely because the genes regulated by these stress-related miRNAs are common to a variety of stress responses [18]. New miRNAs, such as miR158 and miR1885, were specifically induced in *Brassica* by TuMV infection [38]. In the present study, 114 known miRNAs, including miR164d, miR166d-5p, miR172d, and miR319a, were specifically detected in the CGMMV library (Table S5), indicating that they were induced in response to CGMMV infection in watermelon. However, further research is required to explore their regulatory roles.

Plant defense mechanisms consist of multiple defense layers that protect against pathogen invasion [45]. In this study, the targets of 120 CGMMV-responsive known miRNAs were predicted (Table S6). Many of the miRNAs might play roles in the CGMMV response by regulating their corresponding genes (Fig. 6). The cell wall is the first physical barrier encountered by the virus when it attacks the host. Therefore, host plants need to secrete various cell-wall-enhancing enzymes to strengthen the barrier against the invading virus. For example, miR408b-targeted laccase 3 is related to lignification and thickening of the cell wall, and was shown to play a role in alleviating virus attack [46]. miR1027a, miR2873c, miR393a-3p, and miR8578.1 target genes encoding pectinesterase/pectinesterase inhibitors. A cell-wall-associated pectinesterase was shown to be involved in host-cell receptor recognition for the TMV movement protein and to play an important role in the plant response to pathogen attack [47]. miR5303 targets the gene encoding cellulose synthase, which is involved in cellulose synthesis to reinforce the cell walls [48].

Pathogen invasion leads to changes in the levels of phytohormones [49]. The auxin-induced and auxin-responsive proteins targeted by miR9752, miR8578.1, miR2655b, and miR1222a in the present study are related to auxin signaling. Interactions between the virus and auxin-signaling proteins affect the transcriptional activation of auxin-responsive genes and produce disease symptoms in *Arabidopsis* and tomato [50]. The miR477b-targeted DELLA protein was shown to negatively regulate gibberellic acid signaling and control plant immune responses by modulating the salicylic-acid- and jasmonic-acid-dependent defense mechanisms in *Arabidopsis* [51]. miR1027a, miR1028a-5p, and miR5741 target genes encoding ethylene-responsive factors, which have been shown to play important roles in the defense response in *Arabidopsis* [52]. Furthermore, miR5741 targets the cytokinin dehydrogenase gene, thus playing a role in cytokinin signaling. Although the role of cytokinins in plant defense is poorly understood, these hormones have been shown to be associated with plant defense responses against some pathogens [53].

Resistance (*R*) genes regulate plant resistance to pathogens [54]. As expected, a set of *R* genes (encoding LRR, RGA, and defense-related protein) were predicted as miRNA targets in the present study and were found to be regulated by miR162a-5p, miR390a-5p, miR838-3p, and miR1027a. Eukaryotic translation initiation factors (eIFs) and their isoforms (isoeIFs) play important roles in several plant–virus interactions [55–57]. Among them, eIF4G and eIF(iso)4F (targeted by miR5303 and miR530b, respectively) were shown to affect viral RNA replication and participate in host resistance to virus attack [57–59].

Primary and secondary metabolites are believed to help plants fight microbial attack. In this study, several genes identified as miRNA targets were related to the phenylpropanoid biosynthetic pathway. These genes included *bHLH*, *WD40*, *MYB*, *SPL*, *PAL*, *F3H*, *CHI*, *DFR,* and *CAD,* which were regulated by miR838-3p, miR858, miR894, miR156a-5p, miR1435a, miR5257, miR5827, and miR5998a. The phenylpropanoid biosynthetic pathway generates secondary metabolites such as lignin, flavonoids, and anthocyanins. Many phenylpropanoids have been shown to have antimicrobial activity [60, 61]. SPL transcription factors are plant-specific and play vital regulatory roles in plants, such as plant growth and development [62], anthocyanin biosynthesis [63], and abiotic stress response [64]. The fatty acid (FA) signaling pathway is another important component of the plant response to invading microbial pathogens [65]. Long-chain-fatty-acid-CoA ligase (targeted by miR8051-5p), fatty acyl-CoA reductase (targeted by miR894), and lipoxygenase A (targeted by miR5303 and miR8578.1) participate in the FA signaling pathway and may play important roles in the response to CGMMV infection in watermelon.

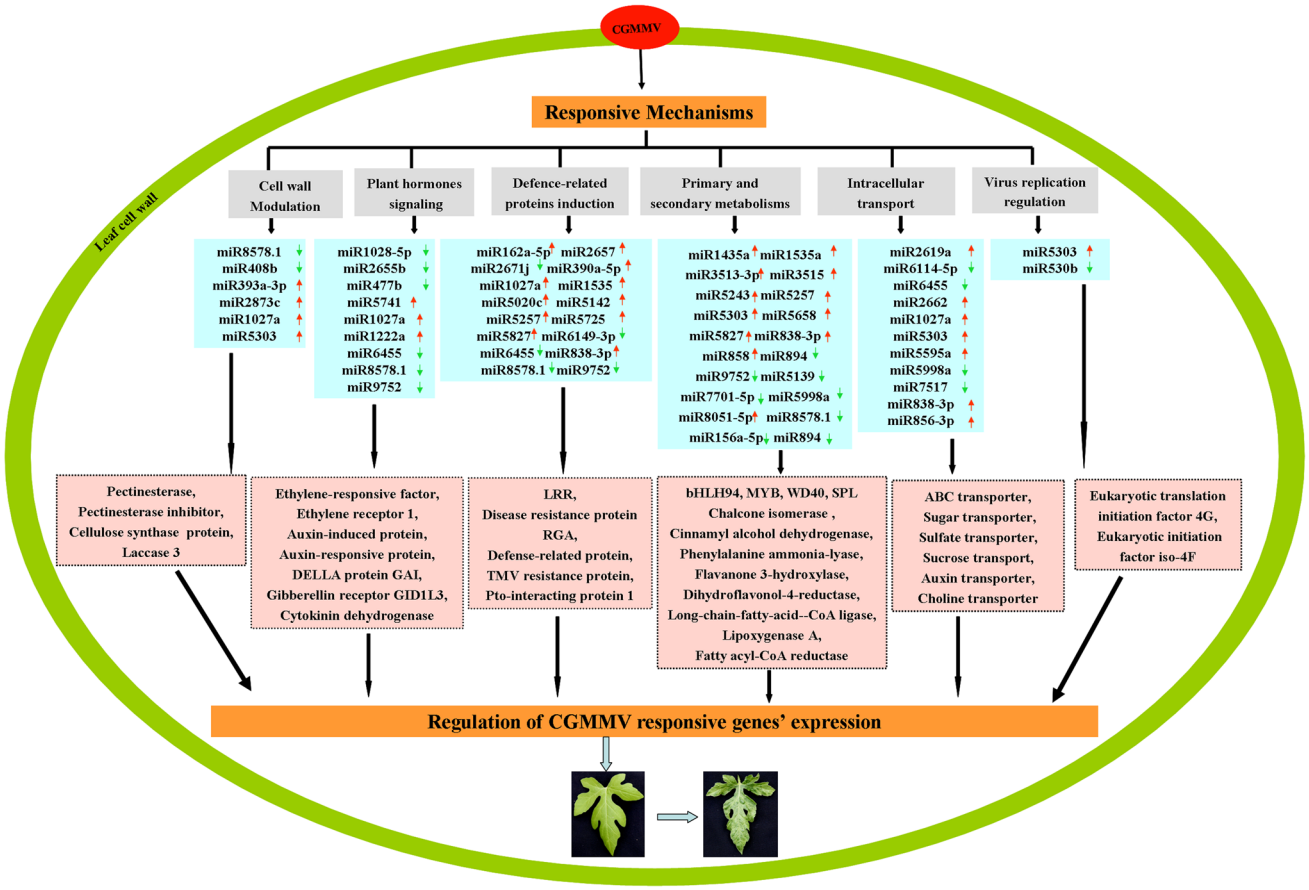


Fig. 6 The potential miRNA-mediated regulatory network in response to CGMMV infection in watermelon. Potential interactions between CGMMV-responsive miRNAs and their target genes are shown. The red and green arrows indicate miRNAs that are upregulated and downregulated of after CGMMV infection

Several genes encoding intracellular transporters were also identified as miRNA targets. For instance, ABC transporters (targeted by miR1027a, miR2619a, miR5303, miR6114-5p, and miR6455) play a significant role in mediating plant resistance to microbial attack [66]. The auxin transporter targeted by miR838-3p is associated with asymmetrical distribution of auxin and is responsive to abiotic stresses in plants [67]. The miR5998a-targeted sulfate transporter was shown to be involved in *Verticillium dahliae* resistance in tomato [68]. The sugar transporter (targeted by miR2662 and miR5998a) may contribute to pathogenicity by enhancing pathogen survival [69]. The miR7517-targeted sucrose transporter is essential for natural resistance against viruses, and its expression was shown to be correlated with inhibited virus replication and movement [70]. These results indicate that the miRNA-regulated target genes might play significant roles in the plant response to viral infection in watermelon. The next steps are to experimentally confirm the interactions between miRNAs and their targets, and to design bioengineering strategies based on miRNAs to improve plants' resistance to virus attack.

Conclusions

We have identified CGMMV-responsive miRNAs in watermelon at the genome-wide level using small RNA sequencing technology. Expression patterns of some differentially expressed miRNAs were shown to regulate the CGMMV response. The predicted target genes for the CGMMV-responsive known miRNAs were involved in diverse biological processes including cell-wall modulation, plant hormone signaling, defense-related protein induction, primary and secondary metabolism, regulation of virus replication, and intracellular transport. Furthermore, a putative miRNA-target module related to CGMMV response was proposed. These findings could advance our understanding of the functional characterization of miRNAs and their targets in regulating the response to viruses in plants.

Availability of sequencing data

Sequencing data from this article were deposited at the sequence read archive (SRA) of the National Center for Biotechnology Information (NCBI) under the accession

numbers SRR3318267 for the CGMMV-infected sample and SRR3318270 for the mock-inoculated sample.

Acknowledgements This work was funded by grants from the China Postdoctoral Science Foundation (2016M601973), the National Natural Science Foundation of China (31572145).

Compliance with ethical standards

Human and animal rights statement This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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