Discovery and Characterization of Differentially Expressed Soybean MiRNAs and Their Targets During Soybean Mosaic Virus Infection Unveils Novel Insight Into Soybean-SMV Interaction

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Abstract

Background: Soybean mosaic virus (SMV) is the most devastating pathogen of soybean. MicroRNAs (miRNAs) are a class of non-coding RNAs (21-24 nucleotides) and play important roles in regulating defense responses against pathogens. However, miRNA's response to SMV in soybean is not as well documented.

Result: In this study, we analyzed 18 miRNA libraries, including three biological replicates from two soybean lines (Resistant and susceptible lines to SMV strain SC3 selected from the near-isogenic lines of Qihuang No. 1× Nannong1138-2) after virus infection at three different time intervals (0 dpi, 7 dpi, and 14 dpi). A total of 1,092 miRNAs, including 608 known miRNAs and 484 novel miRNAs were detected. Differential expression analyses identified the miRNAs responded during soybean-SMV interaction. Then, miRNAs potential target genes were predicted via data mining, and functional annotation was done by Gene Ontology (GO) analysis. Eventually, the expression patterns of several miRNAs validated by quantitative real-time PCR analysis are consistent with sequencing results.

Conclusion: We have identified a large number of miRNAs and their target genes and also functional annotations. Our study provides additional information on soybean miRNAs and an insight into the role of miRNAs during SMV-infection in soybean.

Background

Soybean (*Glycine max* (L.) Merr.) is an important legume crop with numerous uses for food, feed, and industrial materials [1]. Soybean mosaic virus (SMV), a member of the genus *Potyvirus*, is a single-stranded sense RNA with about 9,600 nucleotides and encodes 11 putative proteins [2, 3]. It is one of the most economically upsetting pathogens of soybean worldwide, and 50–80% yield losses had been reported. This virus is predominantly found in many parts of the soybean-producing regions in China, and a total of 22 strains (designated as SC1-SC22) were identified nationwide [4]. Considering the impact of SMV on soybean production, deciphering the molecular mechanisms of the interaction between soybean and SMV is of prime importance and is necessary for soybean improvement programs to increase the yield levels.

MicroRNAs (miRNAs) are a class of genome-encoded, endogenous small non-coding RNAs that are 21–24 nucleotides (nt) long and have essential roles in regulating gene expression at the transcriptional and post-transcriptional level [5–9]. The miRNA encoding gene is initially transcribed by RNA polymerase II to produce a long primary-miRNA (pri-miRNA) molecule with a 5’ cap and 3’ poly-A tail. The original transcript is cleaved by RNAse III (Drosha and Dicer) in a single-stranded RNA precursor (pre-miRNA) with a hairpin structure of about 70–90 bases in size and then processed by Dicer to form a mature miRNA. Mature-miRNAs are assembled into the RNA-induced silencing complex (RISC) in the cell. In the RISC, base complementation guides the shearing of its target gene mRNA or inhibits its translation [5, 7, 10–12].

To date, thousands of miRNAs have been discovered in different plant species, and more are continuing to be discovered [13]. The role of miRNAs in regulating the expression of genes involved in various biological processes, including growth and development [14–16], phytohormone signaling [17, 18], immunity against pathogens and insect pests [19–21], and responses to environmental changes [22, 23], have been demonstrated. The majority of miRNAs have the ability to couple with several targets. Plant miRNAs and their putative target genes have been identified to be responsive to infection by viruses such as cucumber green mottle mosaic virus (CGMMV), mungbean yellow mosaic India virus (MYMIV), cowpea severe mosaic virus (CPSMV), sugarcane mosaic virus (SCMV) in cucumber, black gram, common bean, cowpea and maize[24–27].

With the support of high-throughput sequencing platforms and bioinformatics analysis, more than 700 miRNAs have been discovered in soybean. The number is much higher than other species in Fabaceae, such as common bean and cowpea, etc. Li et al.[28] constructed four small RNA libraries of two soybean varieties inoculated and uninoculated with soybean cyst nematode (SCN). A total of 364 known miRNAs and 21 novel miRNAs were detected, of which 101 miRNAs were induced by SCN stress, indicating that miRNAs play a vital role in soybean response to SCN stress. Cui et al. [29] detected gma-miR1510 by stem-loop qRT-PCR and found that the expression of gma-miR1510 was down-regulated after inoculation with *Phytophthora sojae*, and one of the target genes *Glyma.16G135500* of gma-miR1510 was identified by the 5’-RACE method, which encoded a typical TIR-NB-LRR plant disease resistance domain. Guo et al.[30] infected soybean varieties Willams82 (Resistant) and Harosoy (Susceptible) with *Phytophthora sojae* and found miR166, miR393, miR1507, miR2109, and miR3533 were up-regulated after infection, while miR168, miR319, and miR482 were down-regulated.

However, there are few studies conducted to known about miRNAs involvement in SMV-soybean interactions. For instance, several miRNAs have been discovered in soybean that responds to infection with SMV. Yin et al. [31] inoculated the SMV strain SC3 in susceptible soybean cultivar Nannong1138-2 and found 179 miRNAs, representing 52 families. Of these, five miRNAs belonging to three families were novel miRNAs in soybean. Besides, three miRNAs (miR160, miR393, and miR1510) involved in plant resistance were detected to respond to SMV infection. Chen et al. [32] identified that the miRNA168 expression level is highly elevated only in G7-infected PI96983 (incompatible interaction) but not in G2- and G7-infected Williams 82 (compatible interactions). The cleavage of miR168-mediated AGO1 mRNA and severe suppression of AGO1 protein accumulation occurs when miR168 is overexpressed. Chen et al. [33] performed small RNA (sRNA)-seq, degradome-seq and as well as a genome-wide transcriptome analysis and discovered a group of miRNAs and their mRNA targets that were differentially expressed in response to infections by different SMV isolates/strains and identified mutual miRNA-mRNA regulatory interactions during SMV infections. In another study, Bao et al. [34] inoculated mild and severe isolates (N1 and N3) into susceptible soybean genotype Hefeng25 by microarray analysis. Results showed that the up-regulated miRNAs, miR168a, miR403a, miR162b, and miR1515a likely regulate the expression of AGO1, AGO2, DCL1, and DCL2.

In the present study, we identified a set of miRNAs responsive to SMV infection in resistant and susceptible near-isogenic lines (NILs) from the cross between Qihuang No.1 and Nannong1138-2 at different time intervals and provided a synopsis of the soybean miRNA and SMV interaction system. This information adds toward a better understanding of the role of miRNAs during SMV-infection in soybean.

Methods

Plant genetic materials and virus source

All soybean materials used in this study are from the National Center for Soybean Improvement (NCSI), Nanjing, China. Our study complies with relevant institutional, national, and international guidelines and legislation. In a former study, we developed a set of NILs from the cross between Nannong1138-2 and Qihuang No.1 using the heterogeneous inbred family method for the R_{SC3Q} on chromosome 13 of soybean. Of these, two pairs of NILs with significantly contrasting responses to SMV (Resistant (R) and susceptible (S) lines) were used in this study. Formal confirmation of the plant materials has been done by phenotyping and genotyping. An SMV isolate belongs to the strain of SC3, also obtained from the NCSI, was used in this study.

Virus inoculation and tissue sampling

The inoculum of the virus was prepared from the leaves of the susceptible cultivar, Nannong1138-2. The inoculum preparation, inoculation, and phenotype evaluation were done following the method of Li et al. [4]. The resistant and susceptible lines were grown with three biological replicates in pots and kept in a growth chamber set at 28°C with 60-70 % relative humidity for a photoperiod of 16 /8 hours (Light/ Dark). After 10 days, when unifoliate leaves developed, resistant and susceptible lines were inoculated with SC3. The leaf samples were collected at 0 days post-inoculation (dpi), 7 dpi, and 14 dpi, immediately frozen in liquid nitrogen and kept at −80 ° C until processed.

Small RNA libraries preparation and deep sequencing

Total RNA was extracted from soybean leaves by miRcute Plant miRNA Isolation Kit (Tiangen, China). The concentration and integrity of RNA were detected by nanodrop (Thermo Fisher, USA) and Agilent 2100 (Agilent Technologies, USA). Then the sRNA library was constructed by using NEB Next Multiplex Small RNA Library Prep Set for Illumina ® (Set 1) (New England Biolabs, USA), including reverse transcription and PCR enrichment after adding adapters to 5’and 3’ of RNA. The PCR products were purified by VAHTSTM DNA Clean Beads (Vazyme, China), and then PAGE gel electrophoresis was performed, and the target fragment was selected for gel cutting and recycling. After confirming the quality and quantity of cDNA libraries, they were sequenced with the single-end read length of 50 bp using an Illumina HiSeq X-ten platform.

Small RNA analysis and miRNAs prediction

Raw sequencing reads were filtered to ensure the quality of the analysis by follows:(1) discard the low-quality reads, (2) discard the reads without 3’ and 5’ adaptor, (3) discard the reads with unknown base N (N specifies unidentified base) content that was larger than 10%, (4) discard sequences shorter than 18 nt or longer than 30 nt for further analysis. The fragments of the ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), small nucleolus RNA (snoRNA), other non-coding RNA (ncRNA), and repetitive

sequences were discarded by bowtie with the support of the Silva database, GtRNAdb database, Rfam database, and Repbase database, and unannotated reads were acquired, together with miRNAs. Further, the unannotated reads were mapped to the soybean reference genome (Wm82.a2.v1). Then, individually mapped reads were run through the miRDeep2 module to quantify known miRNAs (miRBaseV22) and discover novel miRNAs. The novel miRNAs were predicted following the criteria used by the Bayesian model.

Quantification and differential expression analysis of miRNAs

The expression level of miRNA was normalized as transcripts per million (TPM). The formula is: TPM=read count*1,000,000/mapped reads. DESeq2 was used to detect the differentially expressed miRNAs among the groups. To determine the significance of each miRNA expression difference, we used the value of |log2 fold change (FC)|≥1.00 and false discovery rate (FDR) ≤0.05 as the criterion. FC shows the ratio of expression levels between the two samples, and FDR is an indicator for differentially expressed miRNAs.

Prediction of miRNA-targeted genes and gene function analyses

Potential miRNA-targeted genes were predicted using the TargetFinder program by providing the sequences of all miRNAs. The functional annotation of the miRNA-targeted genes to GO terms was performed according to the Gene Ontology database (http://www.geneontology.org/). The obtained results were divided into three groups: biological process, molecular functions, and cellular components. Additionally, these annotated miRNA-targeted genes were further subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/) pathway enrichment analysis using KOBAS.

Expression analysis of the miRNA using stem-loop RT-PCR and qRT-PCR

The total RNA was isolated using TRIzol Reagent (Invitrogen, USA) following the user guidelines. For miRNAs, the cDNA was synthesized by RT using the miRNA 1st Strand cDNA Synthesis Kit (by stem-loop) (Vazyme, China) with a special stem-loop RT primer (Additional file 11：Table S5). The authenticity of miRNAs was verified by RT-PCR using Ex Taq (Takara, Japan), PCR procedure: Pre-denatured at 95 °C for 1 min; Reaction at 98 °C for 10 s, 57 °C for 15 s, 72 °C for the 20s, a total of 40 cycles. The cDNA was amplified by real-time RT-PCR using the SYBR green supermix (Vazyme, China) at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for the 60s, and 95 °C for 15s. The experiment included three biological replicates, and normalized expression levels were measured using the relative quantification ($2^{-\Delta\Delta Ct}$) method [61], and data were compared with U6 as the internal reference control.

Results

Post inoculation phenotype in resistant and susceptible lines

The phenotype was studied to define the SMV strain SC3-inoculated susceptible and resistant lines. As expected, SMV symptoms were not visible in mock-inoculated resistant and susceptible lines. The susceptible line resulted in yellow-green mosaic and slightly curling symptoms on the leaves. The onset of symptoms was observed 7 dpi onwards, and the mosaic and severe curling symptoms were at 14 dpi. In contrast, no visual disease symptoms were observed in the resistant line (Fig. 1). The plant height of the susceptible line was significantly lower than that of the control, while there was no significant difference seen in the resistant line and its control (Fig. 2A-B). The SMV-CP transcripts were quantified by qRT-PCR analysis. Resistant lines showed low accumulation of the SMV-CP transcripts at 7 and 14 dpi compared to that of the susceptible line. SMV inoculation in the susceptible line resulted in a rise in an SMV-CP transcripts accumulation from 7 dpi onwards, which is highly associated with the phenotype changes after virus infection. Further, it was confirmed by DAS-ELISA analysis (Fig. 2C-D). These results indicate that the virus can gradually expand to the upper leaves in the susceptible line and can inhibit the normal growth of the plant, while the development of the virus in the resistant line is blocked, allowing the plant to grow normally.

Summary of small RNA libraries data sets

This study constructed 18 small RNA libraries from resistant and susceptible lines at 0 dpi, 7 dpi, and 14 dpi of SMV and generated 231.41 million raw reads ranging from 10.46 to 18.73 million reads. After removing the low-quality reads, a total of 225.42 million clean reads were obtained. Each sample was not less than 10.26 million clean reads, and the Q30 value of all samples was >95% (Table 1). Next, we filtered out sRNAs such as rRNA, tRNA, snRNA, snoRNA, and other ncRNAs and repetitive sequences in the clean reads to obtain unannotated reads containing miRNAs. Among all the reads in each sample, the total amount of rRNA and unannotated reads accounts for about 95% or more. Notably, scRNAs were not detected in all the samples (Additional file 1: Figure S1). After comparing unannotated reads with the soybean reference genome (Wm82.a2. v1), it is found that the ratio of the sequence in the alignment ranges

from 27.83 to 53.80%, most of which are around 40%. The number of clean reads compared to the positive chain for each sample is higher than the number of clean reads compared to the negative chain (Additional file 2: Table S1, Additional file 3: Figure S2).

Identification of known and novel miRNA

A total of 1,092 miRNAs, including 608 known miRNAs and 484 novel miRNAs, were detected from all the libraries [35]. For known miRNAs, the number of miRNAs with a 21 nt length was the largest, followed by 22 nt and 24 nt. The proportion of miRNAs with a 20-23 nt length in which the first base of the 5' end was U exceeds 50% (Fig. 3A-C). For the novel miRNAs, the number of miRNAs with a length of 21 nt was the largest, followed by 24 nt and 20 nt. The preference for U at the 5'end of these novel miRNAs was weaker than that of known miRNAs (Fig. 3D-F). At the known miRNA level, 509 known miRNAs, which belong to 108 families. Of these, the MiR166 family having the highest number of miRNAs (18), followed by MiR169_2 (13), MiR159 (11), and MiR482 (11). At the novel miRNA level, 196 miRNAs in 80 families, of these, the MiR4352 family had the largest number of miRNAs (17), followed by MiR4378 (12) and MiR4374 (11) (Additional file 4: Table S2).

Differentially expressed miRNAs after SMV infection

To identify the differentially expressed miRNAs among the different groups, we analyzed the miRNAs expression by volcano plot. It revealed that the number of differentially expressed miRNAs in the susceptible line from 0 dpi to 7 dpi, 7 dpi to 14 dpi, and 0 dpi to 14 dpi was higher compared to the resistant line. When the two lines were compared at the same time point, the number of differentially expressed miRNAs at 7 dpi and 14 dpi was significantly higher than 0 dpi (Additional file 5: Figure S3). By comparing the two lines 0 dpi with 7 dpi with the Wayne diagram, we found that 4 miRNAs were individually up-regulated while 1 miRNA was individually down-regulated in the resistant line. On the other hand, 295 and 119 miRNAs were individually up-regulated and down-regulated, respectively, in the susceptible line. Of these, two up-regulated miRNAs (gma-miR5761b, novel-miR-173) and four down-regulated miRNAs (gma-miR5037c□gma-miR5371-3p□gma-miR5371-5p□novel-miR-297) were common in both lines (Fig. 4A-F). Next, we compared the two lines 0 dpi with 14 dpi. It revealed that 8 miRNAs were individually up-regulated in the resistant line, and 3 were individually down-regulated. In the susceptible line, 400 miRNAs were individually up-regulated, and 98 were individually down-regulated. One up-regulated miRNA (gma-miR391-5p) and four down-regulated (gma-miR5371-5p novel-miR-101□novel-miR-224□novel-miR-317) were common in both lines (Fig. 4A-F). Collectively, these results indicate that different miRNAs may be involved in the process of virus-plant interaction by responding to SMV infection at different stages of the resistant and susceptible lines.

Notably, among the 295 miRNAs individually up-regulated at 0 dpi to 7 dpi in the susceptible line, 7 miRNAs including novel-miR-50, novel-miR-70, novel-miR-449, novel-miR-466; gma-miR2118a-3p, gma-miR5041-5p, and gma-miR10440 were targeting the NBS-LRR genes. Besides, 1 miRNA (novel-miR-49) that was individually up-regulated from 0 dpi to 7 dpi in the resistant line also targeted the NBS-LRR gene. Moreover, among the 400 miRNAs were individually up-regulated from 0 dpi to 14 dpi in the susceptible line, 7 miRNAs were targeting NBS-LRR genes, 6 of which are the same as before, and the remaining one is gma-miR390d (Table 2), indicating that the up-regulation of miRNAs targeting NBS-LRR resistance genes in the susceptible line could lead to the down-regulation of corresponding target genes, which may potentially affect the disease resistance of plants.

Target gene prediction and annotation of all miRNAs and differentially expressed miRNAs

A total of 20925 potential target genes were obtained. Five hundred eighty-one known miRNAs predicted 9240 target genes, and 439 novel miRNAs predicted 15,256 target genes (Table 3, Additional file 6: Table S3). Out of 20925 target genes, 20,913 genes were annotated (Table 4). The number of target genes annotated by differentially expressed miRNAs between different samples were analysed, and it was found that the minimum was 373 target genes at 0 dpi between the resistant and susceptible lines, and the maximum was 15,522 target genes in susceptible line from 0dpi to 14 dpi (Additional file 7: Table S4). The Gene Ontology (GO) analysis showed that: (1) From 0 dpi to 7 dpi, the metabolic process, cell part, and binding were the most enriched terms in both lines under the biological process, cellular component, and molecular function categories (Fig. 5A, Additional file 8: Figure S4A). (2) From 0 dpi to 14 dpi, the metabolic process cell and catalytic activity were the most enriched terms in the resistant line, respectively, while metabolic process cell, cell part binding, and catalytic activity were the most enriched terms in the susceptible line (Fig. 5B, Additional file 8: Figure S4B). It revealed that genes associated with different pathways in the two lines are likely to contribute to plants' resistance and susceptibility.

The Kyoto Encyclopedia of Genes and Genomes□KEGG□analysis showed that under the cellular processes, environment information processing, genetic information processing, metabolism, organismal systems categories: (1) 0 dpi to 7 dpi, the number of genes

annotated to RNA degradation (11, 9.40 %) and biosynthesis of amino acids (12, 10.26 %) accounted for a higher proportion of the total annotated genes in the resistant line. The proportion of genes annotated to endocytosis (124, 5.09 %), plant hormone signal transduction (133, 5.46 %), and plant-pathogen interaction (132, 5.41 %) pathways were higher in the susceptible line (Fig. 6A, Additional file 9: Figure S5A). (2) From 0 dpi to 14 dpi, RNA degradation (5, 8.93%) and glutathione metabolism (5, 8.93%) annotated in the resistant line had a high proportion of genes in the pathway, while endocytosis (124, 5.09%), plant hormone signal transduction (133, 5.46%), biosynthesis of amino acids (109, 4.47%) and plant-pathogen interaction (132, 5.41%) annotated in the susceptible line had a high proportion of genes in the pathway (Fig. 6B, Additional file 9: Figure S5B). The results of the KEGG analysis also showed that the plant resistance response was a complex process. The genes involved in RNA degradation may play an important role in the resistant line, while the genes involved in endocytosis and plant hormone signal transduction may promote susceptibility in the susceptible line.

Validations of miRNA expressions by stem-loop RT-PCR and qRT-PCR

Two known miRNAs (gma-miR1507a and gma-miR390d) and two novel miRNAs (novel-miR49 and novel-miR70) have contrast expression patterns and targeting NBS-LRR genes in the resistant and susceptible lines were validated by stem-loop RT-PCR and qRT-PCR [36]. By stem-loop reverse transcription and RT-PCR, about 65 bp bands were found at 0 dpi, 7 dpi, and 14 dpi in both lines, indicating that these selected miRNAs were real in soybeans, and the figures showed the first six bands of each miRNA cropped from full-length gels (Fig. 7A-D, Additional file 10: Figure S6). Then qRT-PCR showed that these four miRNAs' expression patterns were almost the same as the sequencing results (Fig. 7E-F). From 0 dpi to 7 dpi, the expression of novel-miR49 was down-regulated in the resistant line but not significantly changed in the susceptible line. The novel-miR70 did not show significant changes in the resistant line. There was no significant difference of miR1507a in the resistant line, but it was significantly down-regulated in the susceptible line. From 0 dpi to 14 dpi, novel-miR49 showed no significant difference in the resistant line.

Discussion

In the present study, we offer a detailed snapshot of the miRNA expression pattern in host soybean after SMV infection that assisted in understanding the interaction among soybean and SMV and the mechanisms of SMV resistance related to miRNAs. We performed deep sequencing and compared the miRNA expression between the resistant and susceptible lines following virus infection at different time intervals.

Characteristics of miRNAs

Sequencing analysis revealed 1,092 miRNAs, including 608 known miRNAs and 484 novel miRNAs from resistant and susceptible lines. The maximum length of all known miRNAs and novel miRNAs was 21 nt, followed by 24 nt, which was consistent with the length characteristics of miRNAs in plants, including soybean. Studies described that miRNA's length might be related to factors such as the structure of pre-miRNA and the properties of DCL protein [37-39]. Lee et al. [40] showed that asymmetric bulges and mismatched miRNAs play an important role in the formation of 20 nt miRNAs in plants. MiRNAs with different lengths may have different regulatory effects on plants. For instance, 24 nt miRNAs regulate DNA methylation by binding to AGO4 proteins and ultimately play an important role in the transcriptional silencing of transposons and centromeres [41, 42]. At the same time, miRNAs with 21 and 22 nt in length are more likely to bind to AGO and cleave target genes by forming silencing complexes [43]. The first base of known miRNAs with 20-23 nt length was more inclined to U (Uracil), while the novel miRNAs with 20 nt, 21 nt, 23 nt, and 24 nt lengths were more inclined to U (Uracil) and A (Adenine). Previous studies have shown that the 5'-terminal Uracil of miRNAs was beneficial to the binding of miRNA and AGO1 protein.

Differentially expressed miRNAs in resistant and susceptible lines

We compared the miRNA expression of the resistant and susceptible lines following SMV infection at two time intervals: 0 dpi to 7 dpi and 0 dpi to14 dpi. From 0 dpi to 7 dpi, gma-miR1535a, gma-miR319g, gma-miR319q, and ptc-miR319f were significantly up-regulated, and novel-miR49 was significantly down-regulated in the resistant line. On the other hand, 295 miRNAs including gma-miR10405a, gma-miR10440, gma-miR1510a-5p, and gma-miR1510b-5p were up-regulated in the susceptible line, while 119 miRNAs such as gma-miR1507a, gma-miR1507b, gma-miR1535a, and gma-miR156d were significantly down-regulated. From 0 dpi to 14 dpi, gma-miR319g, gma-miR319j, gma-miR319k, gma-miR319q, novel-miR-237, novel-miR-374, novel-miR-397 and ptc-miR319f were up-regulated, while gma-miR5371-3p, novel-miR-206 and novel-miR-280 were down-regulated. There were 400 up-regulated miRNAs in the susceptible line from 0 dpi to 14 dpi, including gma-miR1510a-5p, gma-miR1512a-3p, gma-miR1513a-5p, gma-miR1514b-3p, and 98 down-regulated

miRNAs including gma-miR1535a, gma-miR169o, and gma-miR169w. Collectively, these results indicated that up-regulation or down-regulation of miRNAs might have positive or negative effects on soybean response to SMV infection.

Previously, Yin et al. [31] showed that miR160, miR393, and miR1510 were up-regulated in the response of susceptible soybean varieties to SMV infection by qRT-PCR, and the results were consistent with the sequencing results. Bao et al. [34] found that miRNAs such as miR1507a, miR1507c, and miR482a may have regulatory effects on the resistance genes of the NBS-LRR family, and thus jointly participate in the response process of soybean to SMV infection. Chen et al. [32] found that miR168 was highly expressed in PI96983 infected by G7 strain. Our study had similar conclusions to the previous ones and predicted more differentially expressed miRNAs. For gma-miR1507a, Yan et al. [44] found that its target gene may be *Glyma.04G137800*, a type of NBS-LRR disease resistance gene. Ma et al. [45] analyzed the promoter elements upstream of the transcription initiation site of the gma-miR1507a precursor and found some elements related to stress, pathogen infection, and light response, indicating that gma-miR1507a may be involved in the regulation process of adversity stress, pathogen infection and light response. Cui et al. [29] found that the expression of gma-miR1510 was down-regulated after infection with *P. sojae* in soybean, indicating that gma-miR1510 may be involved in the interaction of soybean and *P. sojae*.

Multiple pathways may be involved in soybean response to SMV infection

MiRNA participates in regulating its target genes by reducing the level of mRNA or inhibiting its translation, it is necessary to study the function of target genes [46]. In our study, 1020 known and novel miRNAs predicted the 20925 target genes. Of these, 20,913 genes were annotation information was obtained. Further, the differentially expressed miRNA target genes were classified by Gene Ontology (GO) analysis. It revealed that from 0 dpi to 7 dpi, the target genes corresponding to the differentially expressed miRNAs in the two lines were highly enriched in terms such as metabolic process, cellular process, cell part, cell, catalytic activity, and binding, and the enrichment of the two lines from 0 dpi to 14 dpi is similar to that from 0 dpi to 7 dpi. In addition, we noticed that with the change of time, the number of genes enriched in certain pathways had changed significantly. For instance, the degree of enrichment of resistant line in terms of detoxification, biological adhesion, etc., has been considerably reduced. The results indicate that these pathways, which are enriched differently at different time points, may play an important role in the process of SMV infecting soybeans.

KEGG analysis also showed that the number of genes annotated to different pathways of the target genes of the differentially expressed miRNAs in the two lines in different time intervals for differences in the total number of genes annotated. For instance, from 0 dpi to 7 dpi, the proportion of genes annotated to RNA degradation, biosynthesis of amino acids, and other pathways in resistant line are relatively high, while those in susceptible line are mainly annotated to pathways such as endocytosis, plant hormone signal transduction, and plant-pathogen interaction. It is speculated that this difference makes the number of genes that function in different pathways at different time points after the two lines are inoculated with the virus, and finally, the plants have different phenotypes for the virus.

Identification of candidate genes for disease resistance by miRNA-seq combined with previous mapping results

Plants are often attacked by a variety of pathogens during their growth and development. In the course of long-term evolution, they have acquired a series of complete and complex defense mechanisms, and the formation of disease resistance genes is one of them [47, 48]. The majority of plant disease resistance genes belong to the nucleotide-binding site leucine-rich repeat (NBS-LRR), receptor-like kinases (RLKs), and serine/threonine kinases (STKs) proteins and other types [49, 50]. In particular, soybean contains many NBS-LRR genes, but limited studies were only conducted to describe the role of the NBS-LRR gene's involvement in resistance against pathogens. Several SMV resistance genes, including $Rsv_{1,}$ R_{SC3Q}, R_{SC11} , $R_{SC14Q,}$ and R_{SC20} were located at 4.295 Mb genomic region between 27 656 895-31 951 960 bp on chromosome 13 [51-56]. This genomic region is rich in NBS-LRR genes [57]. In this study, we predicted the miRNAs potential target genes (i.e., *Glyma.13G184800*, *Glyma.13G187900*, *Glyma.13G188300*, *Glyma.13G190300*, *Glyma.13G190400, Glyma.13G190800, Glyma.13G193100*, and *Glyma.13G193300*), and they were present in in the same genomic region on chromosome 13. Wu et al. [58] mapped the resistance loci to bean common mosaic virus (BCMV) resistance genes mapped in the 58.1 kb interval between BARSOYSSR _13 _ 1114 and SNP-49 through positional cloning, including a CC-NBS-LRR type of resistance gene *Glyma.13G184800*, while *Rsv1-h* responsible to SMV was mapped to almost the same region as the previous SMV resistance allele, indicating that this gene may respond to both viruses. Previously, mapping and transcriptome sequencing analyses identified SC3 resistance gene candidates in chromosome 13 [52, 54, 59, 60]. Zheng et al. [54] found the SC3 resistance gene candidates involved in the process of disease resistance. Three genes, including *Glyma.13G25730*, *Glyma.13G*25750 (*Glyma.13G187900*), and *Glyma.13G25950* (*Glyma.13G190300*) were up-regulated in resistant line and down-regulated in susceptible

line after SMV infection. Besides, two genes *Glyma.13G25970* (*Glyma.13G190400*) and *Glyma.13G*26000 (*Glyma.13G190800*) were only expressed in the resistant line. In another study, Li et al. [59] showed that the SC3 resistance might be attributed by several candidate genes such as *Glyma.13G25920* (*Glyma.13G190000*), *Glyma.13G25950*, *Glyma.13G25970,* and *Glyma.13G26000*. We selected the candidate genes for SC3 resistance based on our miRNA sequencing results and the existing mapping studies [54,59, 60]. In future, we will test these candidate genes function by genetic transformation or virus induced gene silencing and provide a novel insight for understand the SMV resistance mechanism.

Conclusions

In the present study, miRNAs regulated by SMV in soybean were identified and characterized by deep sequencing analysis, and some miRNAs related to plant defense mechanisms were found to be responded after virus infection. The function of these miRNAs in soybean resistance to SMV infection may need supplementary study. Even so, our finding helps to advance the knowledge of SMV infection in host plants while also offering new sight for the development of management strategies for SMV infection in the future.

Abbreviations

miRNA: microRNA; nt: nucleotide; smv: soybean mosaic virus; NILs: near-isogenic lines;

dpi: day post-inoculation; pri-miRNA: primary miRNA; pre-miRNA: precursor miRNA; RISC: RNA-induced silencing complex; CP: coat protein; qRT-PCR: quantitative real-time PCR; sRNA: small RNA; rRNA: ribosomal RNA; tRNA: transfer RNA; snRNA: small

nuclear RNA; snoRNA: small nucleolar RNA; ncRNA: Non-coding RNA; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genome; NBS-LRR: nucleotide-binding site leucine-rich repeat

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The small RNA raw sequencing data have been deposited in the Sequence Read Archive (SRA) at the National Center for Biotechnology Information (NCBI), and the Bio project number is PRJNA743161(https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA743161).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JG, HZ, and BL designed the experiments. BL performed the experiments and analyzed the data. LW, JY, TJ, HL, and KL contributed reagents, materials, and analysis tools. BL, KA, and HZ wrote the manuscript. JG, HZ, and BL revised the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1 Statistical table of sequencing data

Samples	Raw reads	Low quality	Containing 'N'reads	Length <18	Length >30	Clean reads	Q30(%)
R-0-1	10,943,860	0	0	160,919	0	10,782,941	96.05
R-0-2	10,462,389	0	0	199,240	0	10,263,149	95.3
R-0-3	11,474,337	0	0	313,220	0	11,161,117	96.78
R-7-1	14,104,702	0	0	526,839	0	13,577,863	96.51
R-7-2	12,147,715	0	0	531,193	0	11,616,522	96.4
R-7-3	11,769,498	0	0	311,735	0	11,457,763	95.94
R-14-1	11,115,807	0	0	333,049	0	10,782,758	96.6
R-14-2	11,142,894	0	0	430,347	0	10,712,547	96.47
R-14-3	11,179,221	0	0	185,087	0	10,994,134	95.78
S-0-1	11,060,909	0	0	372,772	0	10,688,137	95.52
S-0-2	12,724,182	0	0	415,931	0	12,308,251	95.92
S-0-3	18,737,861	0	0	471,647	0	18,266,214	96.04
S-7-1	13,915,992	0	0	318,714	0	13,597,278	95.92
S-7-2	13,027,907	0	0	178,403	0	12,849,504	95.95
S-7-3	11,647,746	0	0	191,013	0	11,456,733	95.95
S-14-1	15,553,512	0	0	246,334	0	15,307,178	96.16
S-14-2	17,450,155	0	0	410,914	0	17,039,241	96.32
S-14-3	12,954,807	0	0	400,776	0	12,554,031	96.47

Note:

R-0-1, R-0-2, R-0-3 are samples taken from resistant line inoculated with SC3 at 0 dpi; S-0-1, S-0-2, S-0-3 are samples taken from susceptible line inoculated with SC3 at 0 dpi; R-7-1, R-7-2, R-7-3 are samples taken from resistant line inoculated with SC3 at 7 dpi;S-7-1, S-7-2, S-7-3 are samples taken from susceptible line inoculated with SC3 at 7 dpi; R-14-1, R-14-2, R-14-3 are samples taken from resistant line inoculated with SC3 at 14 dpi;S-14-1, S-14-2, S-14-3 are samples taken from susceptible line inoculated with SC3 at 14 dpi .

Table 2 Statistical table of miRNA targeting NBS-LRR genes

miRNA	miRNA Seq	Target gene	R-0-1_R-0-2_R-0-3 vs R-7-1_R-7-2_R-7-3	S-0-1_S-0-2_S-0-3 vs S-7-1_S-7-2_S-7-3	R-0-1_R-0-2_R-0-3 vs R-14-1_R-14-2_R-14-3	S-0-1_S-0-2_S-0-3 vs S-14-1_S-14-2_S-14-3
novel-miR-49	GAGAUUGGAGCAAUCAGAAUUUGUG	Glyma.18G287100.Wm82.a2.v1	down	normal	normal	normal
novel-miR-50	AAAUAAGAAGGAAUAAUGAA	Glyma.16G118600.Wm82.a2.v1	–	up	–	up
novel-miR-70	UGCGAGUGUCUUCGCCUCUG	Glyma.02G023800.Wm82.a2.v1	normal	up	normal	up
novel-miR-449	AAGAGGCUUGUAGAAGAAGUC	Glyma.15G168500.Wm82.a2.v1	normal	up	normal	up
novel-miR-466	AGCAUCUUAAAACACUUGAAU	Glyma.16G118600.Wm82.a2.v1	normal	up	–	up
gma-miR10440	UUGGGACAAUACUUUAGAUAU	Glyma.13G184800.Wm82.a2.v1	normal	up	normal	up
gma-miR10440	UUGGGACAAUACUUUAGAUAU	Glyma.13G188300.Wm82.a2.v1	normal	up	normal	up
gma-miR10440	UUGGGACAAUACUUUAGAUAU	Glyma.13G190400.Wm82.a2.v1	normal	up	normal	up
gma-miR10440	UUGGGACAAUACUUUAGAUAU	Glyma.13G190800.Wm82.a2.v1	normal	up	normal	up
gma-miR10440	UUGGGACAAUACUUUAGAUAU	Glyma.13G193300.Wm82.a2.v1	normal	up	normal	up
gma-miR1507a	UCUCAUUCCAUACAUCGUCUGA	Glyma.04G137800.Wm82.a2.v1	normal	down	normal	normal
gma-miR2118a-3p	UUGCCGAUUCCACCCAUUCCU	Glyma.13G184800.Wm82.a2.v1	normal	up	normal	up
gma-miR2118a-3p	UUGCCGAUUCCACCCAUUCCU	Glyma.13G187900.Wm82.a2.v1	normal	up	normal	up
gma-miR2118a-3p	UUGCCGAUUCCACCCAUUCCU	Glyma.13G188300.Wm82.a2.v1	normal	up	normal	up
gma-miR390d	AAGCUCAGGAGGGAUAGCACC	Glyma.20G100500.Wm82.a2.v1	normal	normal	normal	up
gma-miR5041-5p	UUUCAUCUUCAACUUGCUCAA	Glyma.13G190300.Wm82.a2.v1	normal	up	normal	normal
gma-miR5041-5p	UUUCAUCUUCAACUUGCUCAA	Glyma.13G190400.Wm82.a2.v1	normal	up	normal	normal
gma-miR5041-5p	UUUCAUCUUCAACUUGCUCAA	Glyma.13G193100.Wm82.a2.v1	normal	up	normal	normal
gma-miR5041-5p	UUUCAUCUUCAACUUGCUCAA	Glyma.13G193300.Wm82.a2.v1	normal	up	normal	normal

Note:

miRNA: Differentially expressed miRNAs targeting NBS-LRR genes.

miRNA Seq: The corresponding miRNA sequences.

Target gene: NBS-LRR genes predicted by corresponding miRNAs.

R-0-1, R-0-2, R-0-3 vs R-7-1, R-7-2, R-7-3: The variation trend of corresponding miRNAs from 7 dpi to 0 dpi in resistant line.

S-0-1, S-0-2, S-0-3 vs S-7-1, S-7-2, S-7-3: The variation trend of corresponding miRNAs from 7 dpi to 0 dpi in susceptible line.

R-0-1, R-0-2, R-0-3 vs R-14-1, R-14-2, R-14-3: The variation trend of corresponding miRNAs from 14 dpi to 0 dpi in resistant line.

S-0-1, S-0-2, S-0-3 vs S-14-1, S-14-2, S-14-3: The variation trend of corresponding miRNAs from 14 dpi to 0 dpi in susceptible line.

Table 3 Statistical details of the number of miRNA and their target genes

Types	All miRNA	miRNA with Target	Target gene
Known miRNA	608	581	9,240
Novel miRNA	484	439	15,256
Total	1,092	1,020	20,925

Note:

Types: The type of miRNA.

All miRNA: The total number of miRNAs.

miRNA with Target: Predict the number of miRNAs with target genes.

Target gene: Number of predicted target genes.

Table 4 Statistical details of target gene annotation results

Anno Database	Annotated Number	300<=length<1000	length>=1000
COG Annotation	8,292	1,424	6,842
GO Annotation	10,471	2,727	7,639
KEGG Annotation	7,734	1,931	5,701
KOG Annotation	11,459	2,554	8,791
Pfam Annotation	17,813	4,292	13,373
Swissprot Annotation	15,641	3,745	11,733
eggnog Annotation	18,666	4,679	13,789
nr Annotation	20,911	5,699	14,492
All Annotated	20,913	5,699	14,492

Note:

Anno Database: Each annotation database.

Annotated Number: Number of annotations in each database.
300<=length<1000: The number of mRNAs between 300 and 1000 in length.
Length>=1000: The number of mRNAs greater than or equal to 1000bp.

Figures

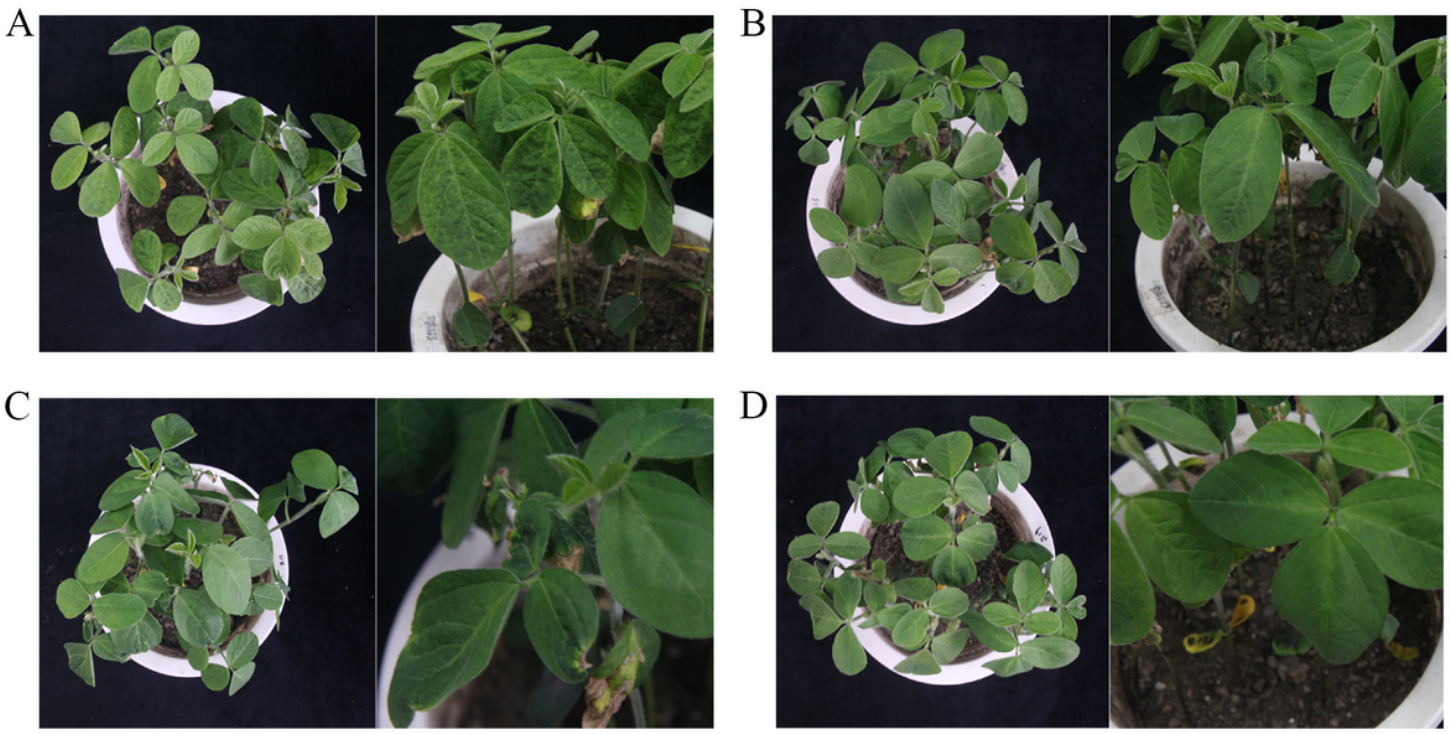

Figure 1

Phenotypic characteristics of resistant and susceptible lines inoculated with SC3 and PBS buffer. A-B: Leaves growth of susceptible line inoculated with SMV and PBS buffer at 14 dpi. C-D: Leaves growth of resistant line inoculated with SMV and PBS buffer at 14 dpi.

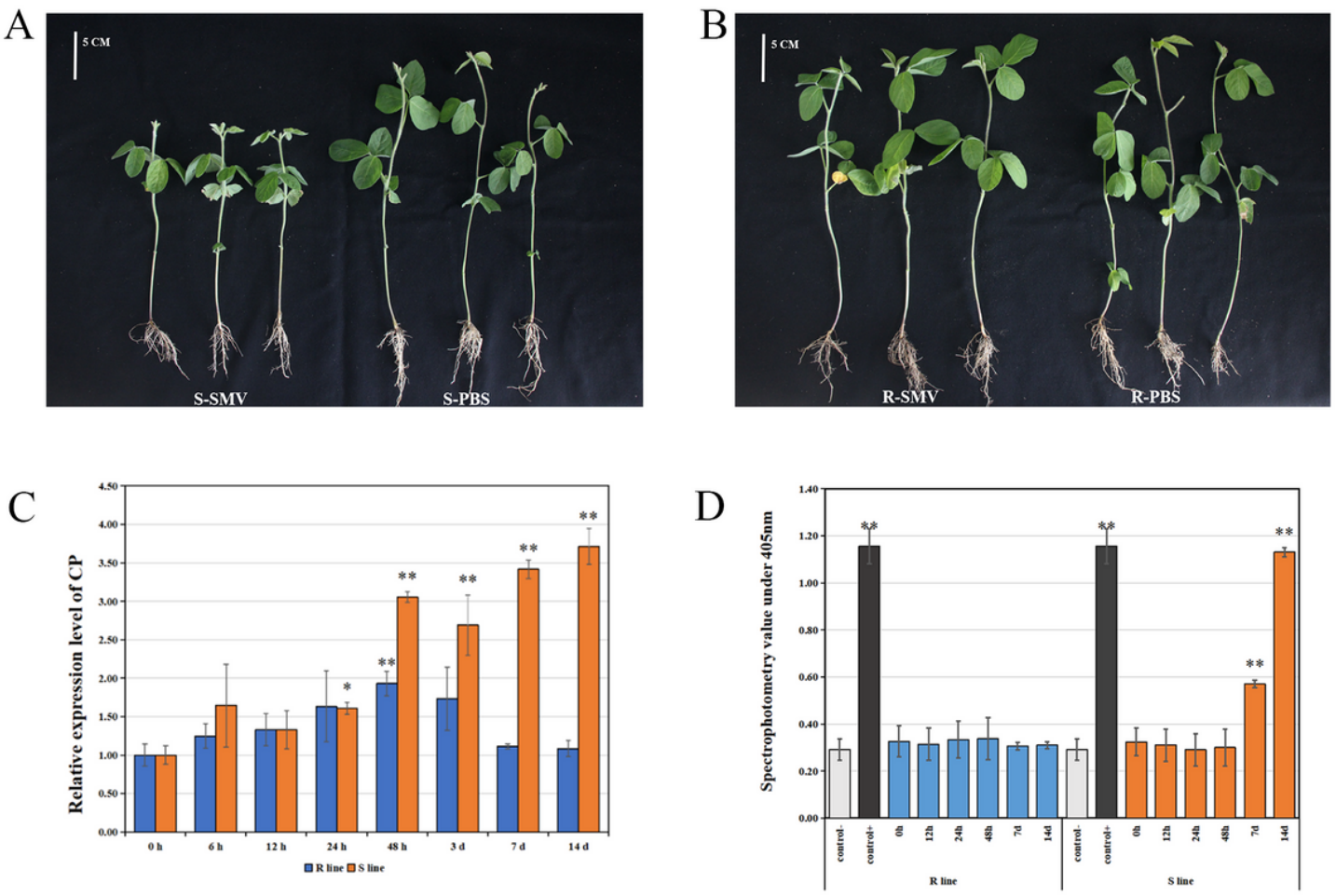

Figure 2

Phenotypic characteristics of plant height and detection of virus content after SC3 inoculation. A: Comparison of plant height of susceptible line inoculation with SMV and PBS buffer at 14 dpi. B: Comparison of plant height of resistant line inoculation with SMV and PBS buffer at 14 dpi. C: qRT-PCR detection of the variation trend of viral content in the two lines after inoculation with SMV (for CP gene). Three biological repeats were set and the reference gene was Tubulin. The expression level of CP gene was calculated by 2-ΔΔCT. D: DAS-ELISA detection of the viral content after SMV inoculation in two lines (for CP protein). Three biological replicates were set, control- was non-inoculated leaves of Nannong1138-2, and control+ was infected leaves of Nannong1138-2 after inoculation with SC3.

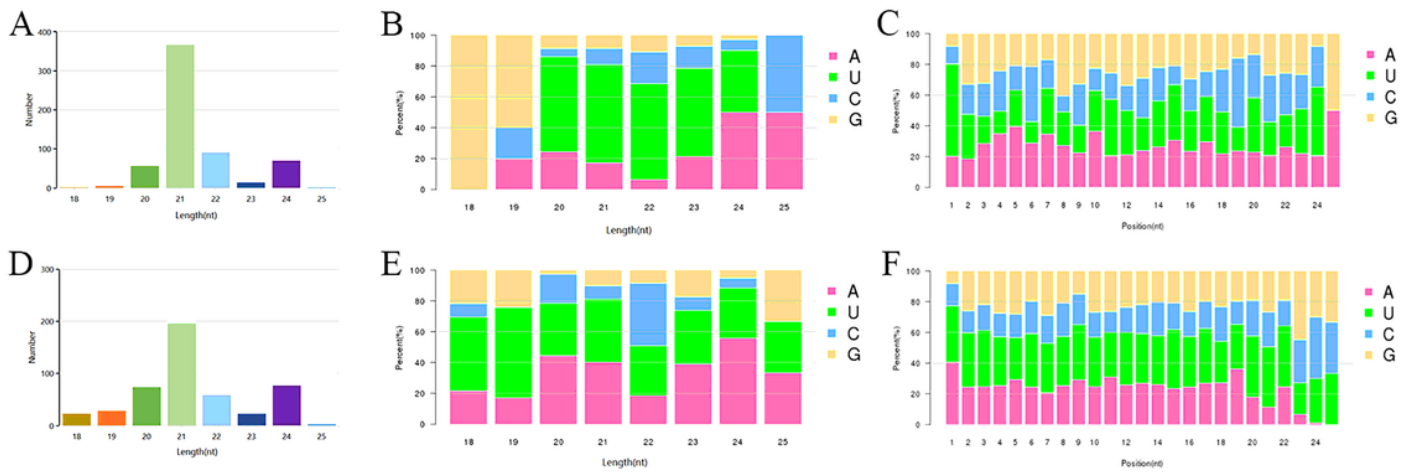

Figure 3

Identification of all miRNAs in the libraries. A-C: The length distribution, the base preference of the first nucleotide, and the base preference of each position of all known miRNAs. D-F: The length distribution, the base preference of the first nucleotide, and the base preference of each position of all novel miRNAs.

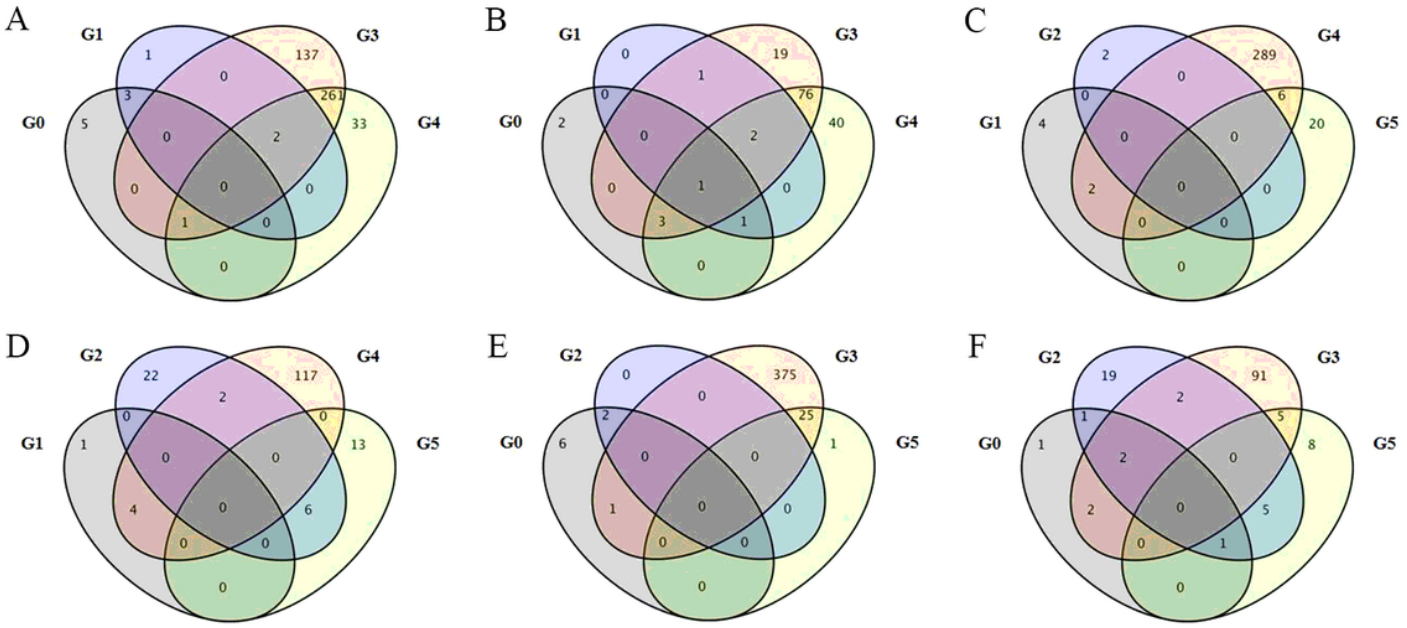

Figure 4

Comparative analysis of Wayne diagrams between different samples. A, C, E: Up-regulation analysis between different groups. B, D, F: Down-regulation analysis between different groups. Note: G0: R-0-1, R-0-2, R-0-3 vs R-14-1, R-14-2, R-14-3 G1: R-0-1, R-0-2, R-0-3 vs R-7-1, R-7-2, R-7-3 G2: R-7-1, R-7-2, R-7-3 vs R-14-1, R-14-2, R-14-3 G3: S-0-1, S-0-2, S-0-3 vs S-14-1, S-14-2, S-14-3 G4: S-0-1, S-0-2, S-0-3 vs S-7-1, S-7-2, S-7-3 G5: S-7-1, S-7-2, S-7-3 vs S-14-1, S-14-2, S-14-3

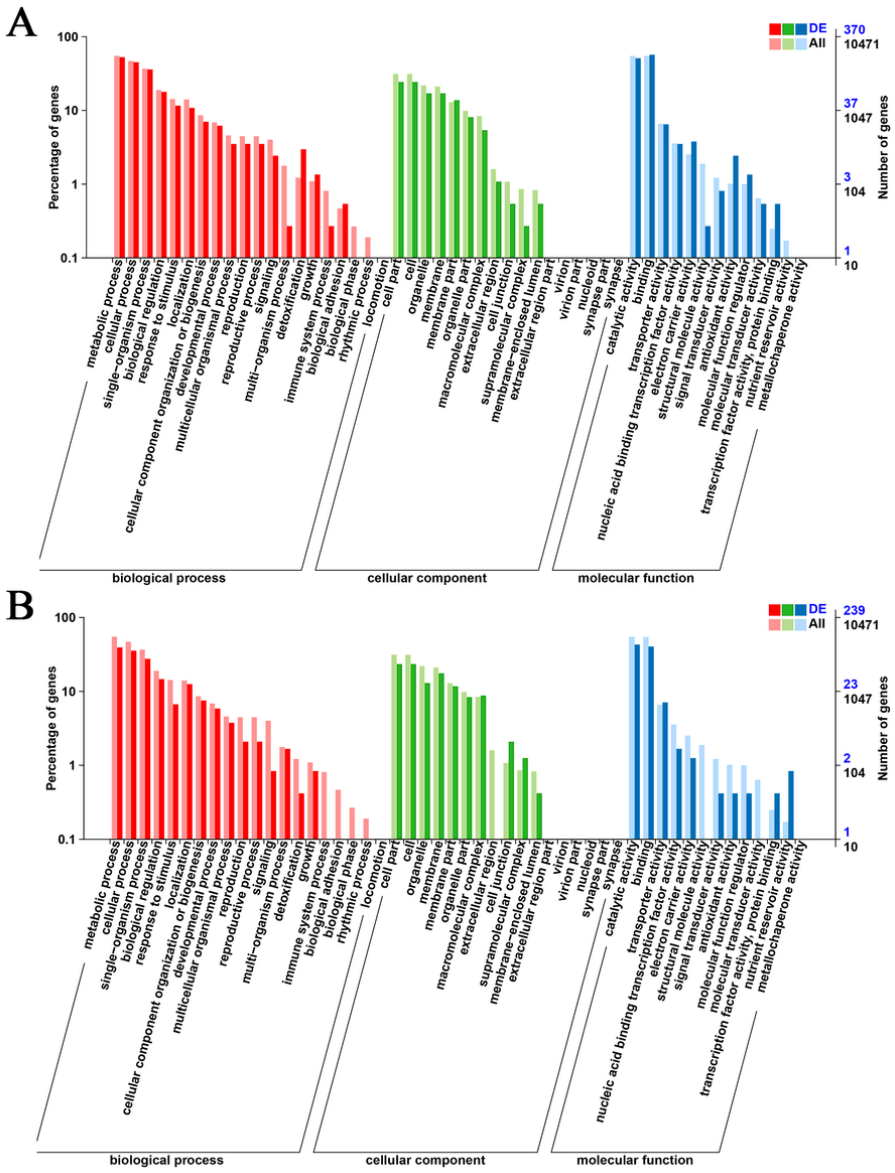

Figure 5

Gene ontology (GO) classification analysis of target genes of the differentially expressed miRNAs. A: GO annotation of the target genes of differentially expressed miRNAs for 7 dpi compared with 0 dpi in resistant line. B: GO annotation of target genes of differentially expressed miRNAs for 14 dpi compared with 0 dpi in resistant line. The x-axis represents three GO categories, the right y-axis represents the number of target genes in the category, and the left y-axis represents the percentage of target genes annotated with a specific term under the main category.

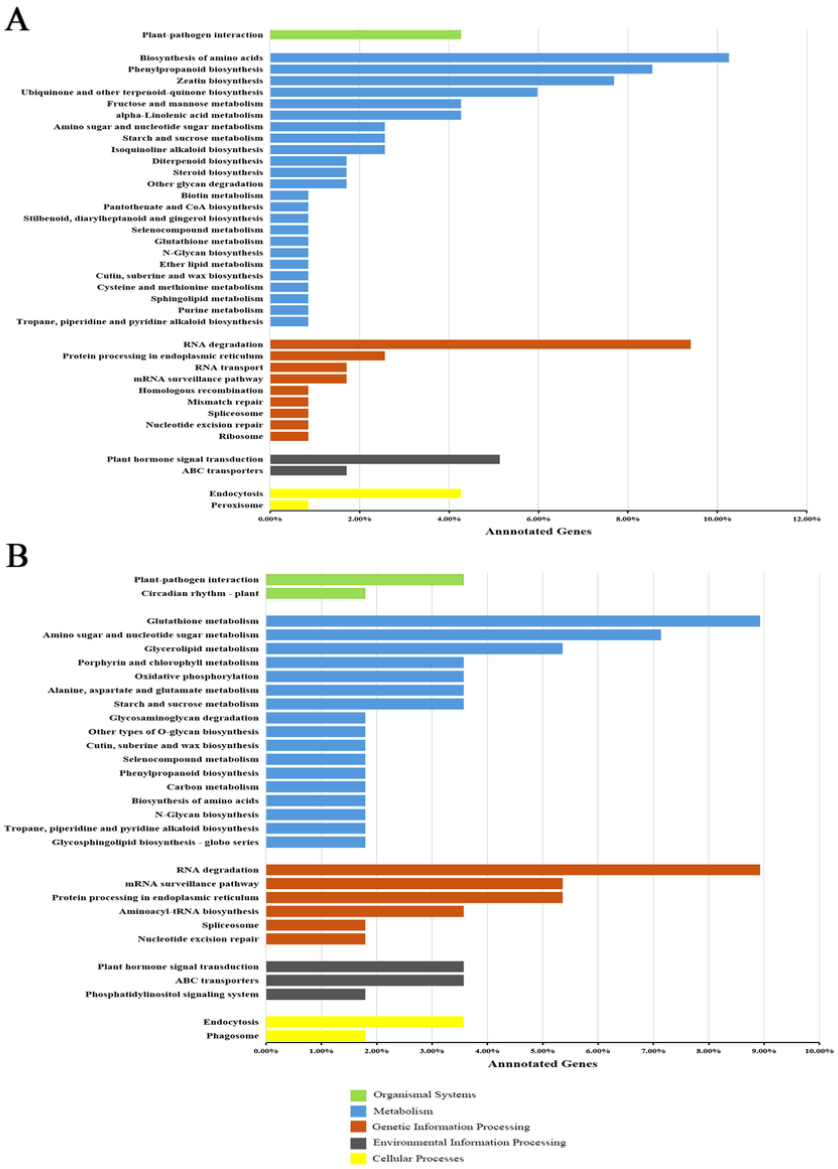

Figure 6

Kyoto encyclopedia of genes and genomes (KEGG) classification map of the target genes of the differentially expressed miRNAs. A: KEGG classification map of the target genes of the differentially expressed miRNAs for 7 dpi compared with 0 dpi in resistant line. B: KEGG classification map of the target genes of the differentially expressed miRNAs for 14 dpi compared with 0 dpi in resistant line. The left y-axis shows different KEGG metabolic pathways, and the x-axis represents the number of genes annotated to the pathway and their proportion to the total number of genes annotated.

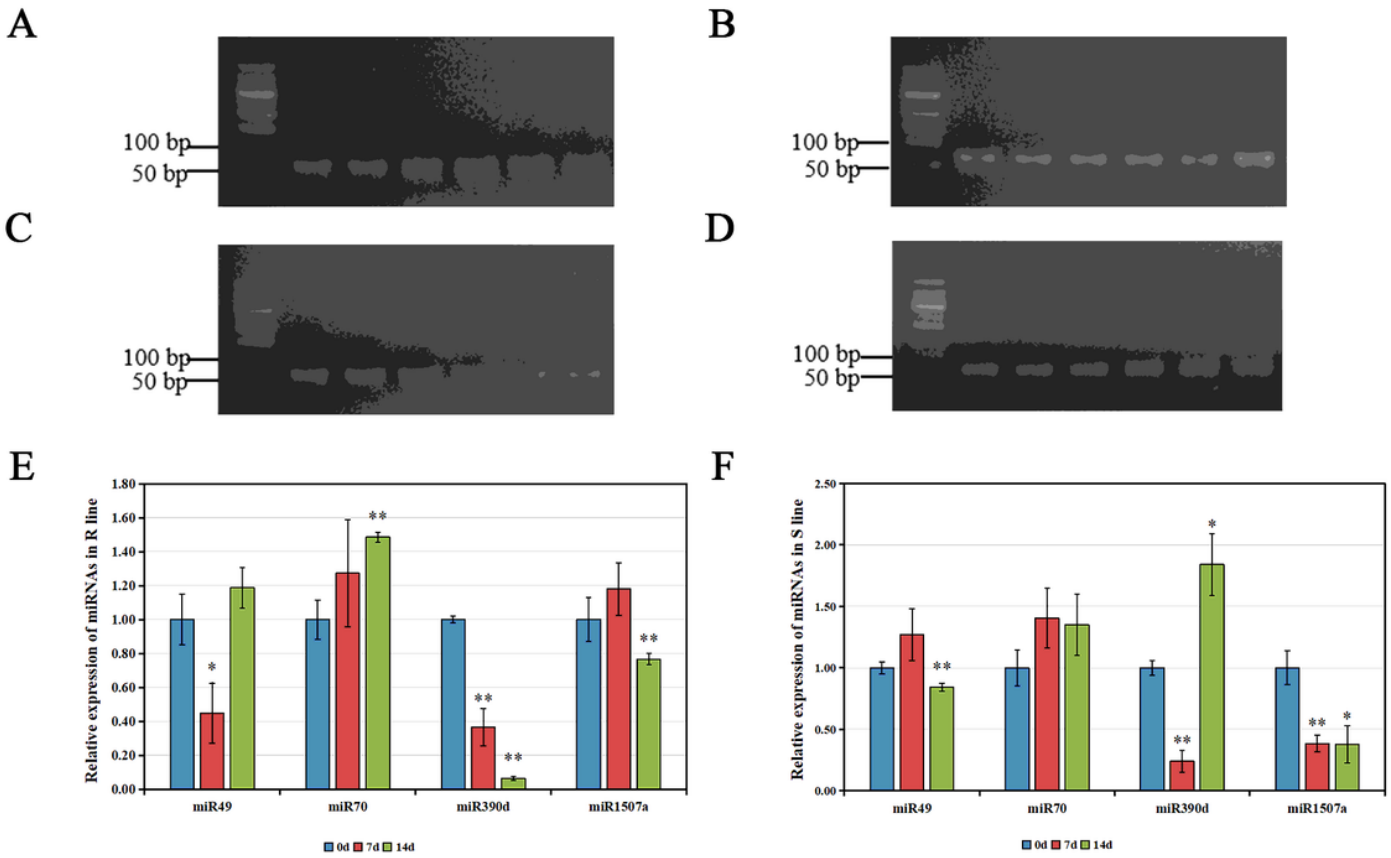

Figure 7

Common RT-PCR and qRT-PCR detection of selected miRNAs. A-D: RT-PCR detection of miR49 (novel), miR70 (novel), miR1507a(known) and miR390d(known) in leaves of two lines inoculated with SC3 at 0 dpi,7 dpi and 14 dpi (The first three bands are from resistant line, the last three bands are from susceptible line). A-D were cropped from the full-length gels which were presented in Additional file 10: Figure S6, the 4 rows of the full-length gels showed the 4 miRNAs, and 24 channels in each row represented 4 replicates of the same miRNA at different time points, respectively. A-D showed one set of the replicates, respectively. The selected marker was 50 bp DNA ladder and the target band was about 65 bp; E-F: For the qRT-PCR detection of the above 4 miRNAs in two lines, three biological replicates were set, the selected internal reference gene was U6, and 2-ΔΔCT was used to calculate the expression level of miRNAs.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1FigureS1.pdf
- Additionalfile2TableS1.xlsx
- Additionalfile3FigureS2.pdf
- Additionalfile4TableS2.xlsx
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