Differential Expression Profiles of Tomato miRNAs Induced by Tobacco Mosaic Virus

A. Abdelkhalek¹,²*, and N. Sanan-Mishra²

ABSTRACT

Plant microRNAs (miRNAs) play important roles in plant development and responses to biotic and abiotic stress. Recently, there is clear evidence that miRNAs are involved in host-virus interactions. By using stem-loop RT-PCR, an expression levels change of thirteen miRNA belonging to six miRNA families targeting leaf development and morphogenesis were analyzed upon Tobacco Mosaic Virus (TMV)-tomato infection. Compared to mock plants, significant changes in relative expression levels of nine miRNAs were observed. The miR319c-5p showed the highest statistically significant increase in accumulation at 15 days post-inoculation. At all time points tested, miR159, miR164a-3p, miR164a-5p, miR166c-5p and miR319c-5p were up-regulated while miR160, miR319a, miR319b, miR319c-3p were down-regulated in most cases. Our data could provide new insights into the role of miRNAs in tomato-TMV interaction and in developing efficient strategies for improving tomato resistance against viral infection.

Keywords: Host-virus interaction, Stem-loop, RT-PCR, TMV, Viral infection.

INTRODUCTION

MicroRNAs (miRNAs) are approximately 22 nucleotides single strand, endogenous, non-coding RNA molecules that post-transcriptionally regulate gene expression by promoting cleavage or inhibiting translation of mRNAs coded by specific target genes (Bartel, 2004).

In plants, miRNAs play vital roles in regulation of multiple essential biological and metabolic processes such as leaf morphogenesis and polarity (Palatnik et al., 2003), floral organ identity (Aukerman and Sakai, 2003), flower development (Mallory et al., 2004), root and shoot development (Naqvi et al., 2010), seed germination (Sunkar et al., 2012) and interestingly are part of the response to biotic and abiotic stresses (Sunkar and Zhu, 2004; Sunkar et al., 2007; Bazzini et al., 2011; Luan et al., 2015; Sarkar et al., 2017). In addition, they are involved in plant development, expression of transcription factors, protein degradation, cell proliferation, signal transduction and metabolism (Xu et al., 2013).

miRNAs have been reported to possess antiviral capability (Khraiwesh et al., 2012). It was demonstrated that during and after virus infection, expression levels of numerous known and unknown miRNAs are changed and this alteration correlates with infection symptoms suggesting an important role of miRNAs in defense and manifestation of pathogen symptoms (Bazzini et al., 2007; Cillo et al., 2009; Guo et al., 2015). Plant viruses are notorious for causing various diseases and are responsible for huge losses of crop production all over the world. The annual global losses due to...
virus diseases in plants/crops reached close to $60$ billion (Srivastava and Prasad, 2014). Thus, viral diseases need to be controlled in order to maintain the quality and abundance of food, feed, and fiber produced by growers around the world.

Tomato (*Solanum lycopersicum*) is one of the most important vegetable crops that are cultivated worldwide. In addition, it is used as a model plant for the research and study of biological and metabolic processes like growth, fruit bearing, disease resistance, and environmental damage-resistance (Giovannoni, 2007).

*Tobacco Mosaic Virus* (TMV, genus *Tobamovirus*), a positive-sense ssRNA virus, has been voted as the most important plant virus in the poll of the plant virology community (Scholthof et al., 2011). TMV infects tomato plants systemically causing mosaic and chlorosis symptoms that are characterized by intermingled light and dark green regions and lead to alteration in plant structure and morphology. Consequently, identifying expression profiles of some relevant miRNAs related to leaf growth, patterning, and development are essential to better understanding of the role of these miRNAs in tomato-TMV interaction. In this study, TMV was inoculated on tomato seedlings and, at different times of infection, the relative expression levels of thirteen miRNAs were quantified.

**MATERIALS AND METHODS**

**Plants Growing Conditions, Viral Infections and Samples Collection**

Seeds of Heinz 1706 tomato cultivar, susceptible to TMV, were sterilized by 70% ethanol and washed thoroughly with sterile water and grown in vermiculite in green house under controlled conditions (28°C, 14 hours light/10 hours dark, 70% humidity). Two true leaves of 35 days old tomato seedlings were dusted with carborundum and 1 mL of semi-purified TMV Egyptian isolate (Acc# MG264131) in 10 mM sodium phosphate buffer (pH 7.0), containing 0.1% sodium sulfite was added and the surface of the leaf was gently abraded. Mock-treated plants inoculated with buffer only were used as control. Three biological replicates of each treatment as well as mock-inoculated plants were collected at 3, 6, 9, 12, and 15 days post-infection (dpi) and kept at -80°C until use.

**Extraction of Total RNA, cDNA Synthesis and Detection of TMV in Tomato Plants**

Tomato leaves total RNA was extracted using the guanidium isothiocyanate (GITC) extraction method (Chomczynski and Sacchi, 2006) with some modifications. Briefly, 1 g tomato tissue ground in liquid nitrogen. Three mL of GITC buffer with β-mercaptoethanol 7 μL mL⁻¹ was added along with phenol and chloroform/isoamyl alcohol. Then, the mixture was allowed to thaw slowly and centrifuged at 10,000 rpm for 15 minutes at 4°C. The obtained aqueous phase was extracted again with phenol: chloroform solution twice and kept overnight at -20°C with equal volume of isopropanol and 1/10 volume of 3M sodium acetate for precipitation. The RNA pellet was harvested by centrifugation at 10,000 rpm for 20 minutes at 4°C and washed by 75% DEPC-ethanol twice at 13,000 rpm. After RNA air dried, the pellet was dissolved in appropriate amount of DEPC-treated water. One μg of total RNA was used for oligo (dT) primed first-strand cDNA synthesis with reverse transcriptase enzyme of Super-Script II (Invitrogen, USA). For detection of tomato-TMV infection, the synthesized cDNA was subjected to PCR amplification using specific primer of TMV-Coat Protein (CP) gene, forward 5' - *ATTTAAGTGAGGGGAAAVCACT-3*', reverse 5' - *CGTCAGTGGCCGAAACAGAA-3' designed by Letscher et al. (2002). PCR reaction was performed as initial denaturation at 94°C for 3 minutes, followed
by 30 cycles at 94°C for 1 minute, 58°C for 1 minute and 72°C for 1 minute with final extension at 72°C for 7 minutes. The results were separated by 1.5% agarose gel electrophoresis in TAE buffer.

**Stem-Loop and Semi-Quantitative RT-PCR Amplification**

For miRNAs primer design, their mature sequences were downloaded from miRBase database (www.mirbase.org) and their stem-loop RT primers, forward primers, and reverse primers were designed according to Kramer (2011). One microgram of total RNA was used to synthesize cDNA using stem-loop miRNA primer with Superscript reverse transcriptase III (Invitrogen, USA) according to manufacturer’s instructions. Primers used in this study are listed in Table 1. A pulsed RT reaction was performed in a thermal cycler as follows: 30 minutes at 16°C, 60 cycles at 30°C for 30 seconds, 42°C for 30 seconds and 50°C for 1 second with final RT enzyme inactivation at 85°C for 5 minutes. The RT reaction mix without RT-enzyme was served as a negative control.

A 1 µL of cDNA was used for PCR amplification using miRNA specific forward primer and universal reverse primer. To normalize the transcript expression levels, actin gene forward 5’-ATGCCATTCTCCGTCTTGACTTG-3’, reverse 5’-GAGTTGTATGCTCGTGACTTG-3’ was used as an internal control. PCR products were visualized by electrophoresis on a 3% agarose gel along with 100 bp DNA ladder. Relative abundance was calculated as integrated density values by normalizing the obtained values with the internal control using Image J program.

**Statistical Analysis**

The relative expression values of three replicates for each set levels were analyzed by one-way ANOVA with P ≤ 0.05, using the CoStat software. The significant differences of the relative expression levels were plotted and Standard Deviation (±SD) is shown as a column bars. Relative expression values more than 1 demonstrate an increase of accumulation (up-regulation) and values lower than 1 mean a decrease in expression (down-regulation).

**RESULTS**

**Infectivity, Symptoms, and Viral Detection**

In tomato seedlings, the virus-infected plants displayed multiple morphological changes. TMV induced systemic mosaic with chlorosis of older tomato leaves, while the early symptoms were noticed at 9 dpi with mild mosaic leaves. At 14 dpi, all the inoculated plants showed severe TMV symptoms. TMV severely altered leaf morphogenesis resulting in leaf distortion and affected whole plant growth (Figure 1). The leaves were sampled at 3, 6, 9, 12 and 15 dpi for each treatment as well as mock-inoculated samples. Afterwards, total RNA extraction viral accumulation was checked by RT-PCR using TMV-CP-specific primer in the sampled leaves (Figure 2).

The virus started accumulating at 3 and by 12 dpi the virus accumulation in the infected leaves reached 93%. As indicated by the TMV-CP RNA levels shown in Figure 2, the level of CP accumulation was low (29%) at 3 dpi and increased slowly till 9 dpi (42%). Then, there was a dramatic increase in the CP levels and they accumulated to almost 100% maximum at 15 dpi. No virus was detected in mock-inoculated samples.

**miRNA Accumulation Levels are Differentially Altered in Virus Infected Tomato**

To investigate the changes in expression level of miRNAs during TMV infection, thirteen conserved microRNAs associated with leaf formation were selected. The
<table>
<thead>
<tr>
<th>miRNA</th>
<th>Mature Sequence</th>
<th>Reference</th>
<th>RT-Primer</th>
<th>Forward primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>sly-miR159</td>
<td>UUUGGAUUGAAGGGAGCUCUA</td>
<td>MIMAT0009141</td>
<td>GTCTATTCAGTGGAGGTCCGGAGG</td>
<td>GGATCTTTTGAGTTGAAGGG</td>
</tr>
<tr>
<td>sly-miR160a</td>
<td>UGCCUGCCUCCCUGUAUGCCA</td>
<td>MIMAT0007914</td>
<td>TATTCGACAGTGAGATCGAGCAG</td>
<td>GCTGCTGGCTCCCTGT</td>
</tr>
<tr>
<td>sly-miR164a-3p</td>
<td>CAUGUGCCUGUUUUCUCCCAUC</td>
<td>MIMAT003974</td>
<td>TATTCGACAGTGAGATCGAGCAG</td>
<td>GGATCCCATGTGCTCTGT</td>
</tr>
<tr>
<td>sly-miR164a-5p</td>
<td>UGGAGAACAGAGGCCAGUGCA</td>
<td>MIMAT003973</td>
<td>TATTCGACAGTGAGATCGAGCAG</td>
<td>GGATCTTGAGGAAGGAGCAG</td>
</tr>
<tr>
<td>sly-miR166a,b</td>
<td>UCGGACACAGCUCACUCUCUC</td>
<td>MIMAT0007915</td>
<td>TATTCGACAGTGAGATCGAGCAG</td>
<td>GCTGGAACAGGCTTCA</td>
</tr>
<tr>
<td>sly-miR166c-3p</td>
<td>UCGGACACAGCUCACUCUCUC</td>
<td>MIMAT0007944</td>
<td>TATTCGACAGTGAGATCGAGCAG</td>
<td>GCTGGAACAGGCTTCA</td>
</tr>
<tr>
<td>sly-miR166c-5p</td>
<td>GGGAGUUGUUGUGCGUCUCGA</td>
<td>MIMAT0007943</td>
<td>TATTCGACAGTGAGATCGAGCAG</td>
<td>GCGGAGATGTGTGCTG</td>
</tr>
<tr>
<td>sly-miR319a</td>
<td>CUUGGACUAGGAGGACUCUC</td>
<td>MIMAT0009145</td>
<td>TATTCGACAGTGAGATCGAGCAG</td>
<td>GGATCCCTTGAGACTGAAGG</td>
</tr>
<tr>
<td>sly-miR319b</td>
<td>UCGGACUAGGAGGACUCUCUC</td>
<td>MIMAT0007943</td>
<td>TATTCGACAGTGAGATCGAGCAG</td>
<td>GGATCCCTTGAGACTGAAGG</td>
</tr>
<tr>
<td>sly-miR319c-3p</td>
<td>UGGAGAUGAGGAGGACUCUCU</td>
<td>MIMAT0007943</td>
<td>TATTCGACAGTGAGATCGAGCAG</td>
<td>GCGGAGACGTAGAGGAG</td>
</tr>
<tr>
<td>sly-miR319c-5p</td>
<td>AGAGCUUCCUCUAGCCACUC</td>
<td>MIMAT0007943</td>
<td>TATTCGACAGTGAGATCGAGCAG</td>
<td>GCGGAGACGTACCCAGC</td>
</tr>
<tr>
<td>sly-miR396a-3p</td>
<td>GUUCAAUUAGCGGUGGAGAG</td>
<td>MIMAT0007943</td>
<td>TATTCGACAGTGAGATCGAGCAG</td>
<td>GGATCCGCTCAAATGGCTG</td>
</tr>
<tr>
<td>sly-miR396b</td>
<td>UUCCAGUACUUCUCUUGAAUU</td>
<td>MIMAT0007943</td>
<td>TATTCGACAGTGAGATCGAGCAG</td>
<td>GCGGAGACGTACCCAGC</td>
</tr>
</tbody>
</table>

**Universal reverse primer**

AGTGCAGGGTCCGAGGT
Figure 1. Effect of TMV infection on tomato growth at 12 dpi: (A) showing the mocked plant; (B) Infected plant, and (C, D) Characteristic symptoms of TMV on tomato showing leaf morphogenesis and leaf distortion.

Figure 2. Agarose gel electrophoresis (3%) in TAE buffer stained with ethidium bromide showing RT-PCR amplification of TMV-Coat Protein (CP), internal gene (Actin) and different sets of studied miRNAs at different time intervals of TMV-infected tomato compared with mock-inoculated plant.
Abdelkhalek and Sanan-Mishra

Figure 3. The relative expression levels of tomato miRNAs upon TMV infection at 3, 6, 9, 12 and 15 dpi showing up-regulation of miR159, miR164a-3p, miR164a-5p, miR166c-5p, and miR319c-5p were up-regulated, and miR160, miR319a, miR319b, miR319c-3p were down-regulated (Figures 2, 3, and 4). Although miR166a, miR166c-3p and miR396a showed slightly up-regulation and miR396b slightly down-regulation, there was no statistically significant difference between infected and mocked plants (Figures 2, 3, and 4). The accumulation and/or induction of miRNAs upon TMV infection (Figures 2 and 3) suggest that they may play important role in plant defense or in the manifestation of symptoms during viral infection.

The time kinetics analysis revealed that miR159 was up-regulated once tomato plant was infected by TMV (Figure 3). Its expression level induced 1.24-fold change during the first 3 dpi when compared to the mock-plants, and continued to increase dramatically to reach 2.75-fold change at 15 dpi. In addition, the expression level of miR164a-3p and 5p were also enhanced in response to the viral infection. At 3 dpi, the relative expression levels of these miRNAs were analyzed at different time intervals in infected and mock-inoculated plants, using stem-loop RT-PCR (Figure 2). According to the one-way ANOVA analysis, nine miRNAs had significant differences (Figures 3 and 4). Among these, miRNAs, miR159, miR164a-3p, miR164a-5p, miR166c-5p, and miR319c-5p were up-regulated, and miR160, miR319a, miR319b, miR319c-3p were down-regulated (Figures 2, 3, and 4). Although miR166a, miR166c-3p and miR396a showed slightly up-regulation and miR396b slightly down-regulation, there was no statistically significant difference between infected and mocked plants (Figures 2, 3, and 4). The accumulation and/or induction of miRNAs upon TMV infection (Figures 2 and 3) suggest that they may play important role in plant defense or in the manifestation of symptoms during viral infection.

The time kinetics analysis revealed that miR159 was up-regulated once tomato plant was infected by TMV (Figure 3). Its expression level induced 1.24-fold change during the first 3 dpi when compared to the mock-plants, and continued to increase dramatically to reach 2.75-fold change at 15 dpi. In addition, the expression level of miR164a-3p and 5p were also enhanced in response to the viral infection. At 3 dpi, the

Figure 3. The relative expression levels of tomato miRNAs upon TMV infection at 3, 6, 9, 12 and 15 dpi showing up-regulation of miR159, miR164a-3p, miR164a-5p, miR166c-5p, and miR319c-5p and miR396a. The mocked sample was normalized to 1. Columns represent mean value from three biological replicates and bars indicate Standard Deviation (±SD). Significant differences between samples were determined by one-way ANOVA using CoStat software. Means were separated by Least Significant Difference (LSD) test at P≤ 0.05 levels and indicated by small letters. Columns with the same letter means do not differ significantly.

480
increase in expression was 1.52-fold change and 1.03-fold change for miR164a-3p and miR164a-5p, respectively (Figure 3). The expression level continued to increase and miR164a-3p levels reached 3.16-fold change at 15 dpi, while miR164a-5p reached 3.18-fold change at 12 dpi (Figure 3). TMV infection also causes a rapid induction and very high accumulation levels of miR166c-5p and miR319c-5p. At 3 dpi, the expression level was 1.23-fold change for miR166c-5p and 2.27-fold change for miR319c-5p but at 15 dpi miR319c-5p expression levels increased to 6.53-fold change and that of

miR166c-5p expression levels increased to 5.91-fold change (Figure 3).

The data shown in Figure 3 revealed that the relative expression levels of miR166a, miR166c 3p and miR396a were slightly up-regulated after viral infection. The expression level of miR166a was 1.01-fold change at 5 dpi and this was retained until 12 dpi. The miR166c-3p expression level increased to 1.41-fold change by 12 dpi. Similarly, the expression level of miR396a was 1.06-fold change at 9 dpi and increased to 1.14-fold change at 15 dpi.

Among the down-regulated miRNAs,
miR160 and miR319b showed an initial increase in expression of 1.23-fold change and 1.05-fold change, respectively, at 3 dpi and decreased subsequently (Figure 4). In contrast, miR396b expression at 3 dpi was 0.87-fold change and was down-regulated at all other time points (Figure 4). The accumulation levels of miR319c-3p showed much fluctuation. It was rapidly up-regulated to 2.29-fold change at 3 dpi and then decreased to 0.81-fold change at 6 dpi. Its levels were up-regulated later at 9 and 12 dpi to 1.02-fold change and 1.42-fold change, respectively. However, at 15 dpi the relative expression levels decreased to reach 0.72-fold change (Figure 4).

DISCUSSION

Plant viral infections often display developmental abnormalities and produce a variety of disease symptoms including stunting, leaf curling and loss of pigmentation by altering cell division and/or expansion and symmetry (Padmanabhan et al., 2005; Bazzini et al., 2011). Many studies have reported that the importance of miRNAs in the regulation of plant’s immune system, gene regulation, and antiviral defense (Yi and Richards, 2007; Li et al., 2012; Huang et al., 2016). Identifying miRNAs and understanding their roles in host-virus interactions is very important for understanding disease resistance and host immune system to develop strategies for controlling viral-infection. In our present study, we identified the expression profiles of a set of tomato miRNAs under TMV infection.

The selected miRNAs are reported to play crucial role in leaf growth, leaf development, and leaf patterning. The miR160 targets auxin response factors that control hormone signaling and leaf development such as serration and curling (Mallory et al., 2005). The miR160 levels increased slightly at 3 dpi post TMV infection followed by decrease in expression at all other time intervals. These results are in agreement with those obtained by Tagami et al. (2007) who noticed the down-regulation in expression level of miR160 in TMV-Arabisopsis thaliana at 10 dpi and Bazzini et al. (2011) who reported down-regulation of N. tabacum-miR160 at 5 dpi.

The miR319 targets Teosinte branched1/cycloidea/PCF (TCP), Apetala2 (AP2) and bHLH TF factors that affect leaf patterning and flower morphogenesis (Palatnik et al., 2003; Chen, 2004). The relative expression levels of miR319 (a, b, c3p) were down-regulated at almost all sampling times after viral infection. This is in contrast to the observations of Havelda et al. (2008) that no significant alteration was detected in the level of miR319 after TMV infection in N. benthamiana. This suggests that TMV has the capability to shutdown the expression of this miRNA, probably as an effect of its RNAi suppression activity.

The miR164 targets NAC domain TFs, CUC1 and CUC2 that are important to leaf development and hormone signaling (Guo et al., 2005; Nikovics et al., 2006). The results showed highly expression levels of miR164a-3p and miR164a-5p. The miR396a,b targets GRL TFs and Rhodanase-like proteins and have important role in defense responses and leaf growth (Sunkar and Zhu, 2004; Debernardi et al., 2012). A slight up-regulation in its levels was observed after 5 dpi of TMV infection. This data is in agreement with earlier reports that TMV-infected tissues contained higher levels of miR164 (Bazzini et al., 2007; Tagami et al., 2007; Bazzini et al., 2011) and miR396 (Tagami et al., 2007).

The expression analysis clearly shows high accumulation of miR159 and miR166 in TMV infected tomato leaves after viral infection. The miR159 targets MYB TFs that have a well-established role in leaf development (Naqvi et al., 2010). The miR166 targets homeodomain-leucine zipper transcription factor (HD-ZIP-TF) family that is also important for leaf development (Juarez et al., 2004). The up-regulation of miR159 and miR166 in virus infected Arabidopsis and N. benthamiana
Expression of Tomato miRNAs under TMV Infection

has been reported earlier (Tagami et al., 2007; Bazzini et al., 2007). The leaf curling and deformation observed in TMV infected tomato leaves could be due to the altered levels of mir159 and mir166. The high levels of these miRNAs in infected samples could serve as an indicator of TMV infection. Naqvi et al. (2010) suggested that the accumulation mir159/319 and mir172 might be linked with Tomato leaf curl virus infection.

The study shows that TMV-induced alterations in expression levels of miRNAs may be the cause of the developmental abnormalities observed in the infected plants. The cause and effects of these alterations need to be analyzed in detail as they may also indicate an important role for the miRNAs in immune defense activity. This knowledge will also be useful in developing miRNA based strategies for improving resistance of tomato against viral infection.

ACKNOWLEDGEMENTS

Ahmed Abdelkhalek would like to thank Arturo Falaschi International Centre for Genetic Engineering and Biotechnology post-doctoral Fellowship. The authors thank International Centre for Genetic Engineering and Biotechnology (ICGEB, New Delhi, India) for providing the necessary facilities in carrying out this research work.

REFERENCES


پروفایل های بیانی تنکیکی مربوط به میکرو آر.آر.آن. "miRNAs" (miRNA) که گیاهی در شرایط آزمایشگاهی با ویروس موزائیکی تنباکو (TMV) ابتدا بسیار زوده و قبل از زندگی واکنش می‌بیند. این واکنش می‌تواند در طول زمان باعث افزایش کلیه miRNAs بوده و کاهش برخی از آنها نیز باشد. در این مطالعه، تغییر در سطح بیان miRNAs از ویروس موزائیکی تنباکو (TMV) با استفاده از روش‌های RT-PCR و مدل‌سازی سطح miRNAs از 6 فامیل miRNAs به شکل می‌آید. این اندازه‌گیری در موارد مربوط به ویروس موزائیکی تنباکو (TMV) در طول زمان نشان داد که از نظر میکرو آر.آر.آن. "miRNAs" (miRNA) و بیشترین "miR319c-5p" در حالی که "miR159"، "miR164a-3p" و "miR166a-5p" در موارد "up-regulated" (فرآیندهای کاهشی) پدید می‌آیند. در موارد "down-regulated" (فرآیندهای افزایشی) "miR319c-3p" و "miR166c-5p" پدید می‌آیند. این نتایج نشان دهنده کاهش تعداد miRNAs در بیشتر موارد می‌باشد و نیز به توسعه "积累" و "どの積" در سیستم میکرو آر.آر.آن. "miRNAs" و "累積" و "どの積" در برابر آلیه می‌باشد.