

Differential Expression Profiles of Tomato miRNAs Induced by Tobacco Mosaic Virus

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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 (Khraiweh *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

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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, 1g tomato tissue grounded in liquid nitrogen. Three mL of GITC buffer with β -mercaptoethanol 7 μ L 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 μ g 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'-ATTTAAGTGGASGGAAA VCACT-3', reverse 5'-CGGTCAGTGCCGAACAAGAA-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'-GAGTTGTATGTAGTCTCGTGGATT-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 $P \leq 0.05$, using the CoStat software. The significant

differences of the relative expression levels were plotted and Standard Deviation ($\pm$ 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 plants. 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 1. List of miRNAs and primers used for stem-loop RT-PCR analysis.

miRNA	Mature Sequence	Reference	RT-Primer	Forward primer
sly-miR159	UUUGGAUUGAAGGGAGCUCUA	MIMAT0009141	GTCGTATCCAGTGCAGGGTCCGAGG TAATCGCACTGGATACGACTAGAGC	GGATCCTTTGGATTGAAGGGA
sly-miR160a	UGCCUGGCUCCUGUAUGCCA	MIMAT0007914	GTCGTATCCAGTGCAGGGTCCGAGG TAATCGCACTGGATACGACTGGCAT	GCTGCCTGGCTCCCTGT
sly-miR164a-3p	CAUGUGCCUGUUUCCCAUC	MIMAT0033974	GTCGTATCCAGTGCAGGGTCCGAGG TAATCGCACTGGATACGACGATGGG	GGATCCCATGTGCCCTGTTTTC
sly-miR164a-5p	UGGAGAAAGCAGGGCAGGUGCA	MIMAT0033973	GTCGTATCCAGTGCAGGGTCCGAGG TAATCGCACTGGATACGACTGCAAG	GGATCCTGGAGAACGAGGGA
sly-miR166a,b	UCGGACCAAGGCUUCAUUCCCC	MIMAT0007915	GTCGTATCCAGTGCAGGGTCCGAGG TAATCGCACTGGATACGACGGGAA	GCTCGGACCAAGGCTTCA
sly-miR166c-3p	UCGGACCAAGGCUUCAUUCUC	MIMAT0035444	GTCGTATCCAGTGCAGGGTCCGAGG TAATCGCACTGGATACGACGAGAA	GCTCGGACCAAGGCTTCA
sly-miR166c-5p	GGGAUGUUGUCUGGCUUGACA	MIMAT0035443	GTCGTATCCAGTGCAGGGTCCGAGG TAATCGCACTGGATACGACTGCGA	GCGGGATGTTGTCTGGC
sly-miR319a	CUUGGACUGAAGGGAGCUCU	MIMAT0009145	GTCGTATCCAGTGCAGGGTCCGAGG TAATCGCACTGGATACGACGGAGCT	GGATCCCTTGGACTGAAGGG
sly-miR319b	UUGGACUGAAGGGAGCUCU	MIMAT0035430	GTCGTATCCAGTGCAGGGTCCGAGG TAATCGCACTGGATACGACAGGAG	GGATCCTTGGACTGAAGGGAG
sly-miR319c-3p	UUGGACUGAAGGGAGCUCU	MIMAT0035432	GTCGTATCCAGTGCAGGGTCCGAGG TAATCGCACTGGATACGACAGGAG	GCGTTGGACTGAAGGGAG
sly-miR319c-5p	AGAGCUUCCUUCAGCCACUC	MIMAT0035431	GTCGTATCCAGTGCAGGGTCCGAGG TAATCGCACTGGATACGACGATGG	GCGAGAGCTTCCTTCAGC
sly-miR396a-3p	GUUCAAUAAAGCUGUGGGAAG	MIMAT0035456	GTCGTATCCAGTGCAGGGTCCGAGG TAATCGCACTGGATACGACCTTCCC	GGATCCGTTCAATAAAGCTGT
sly-miR396b	UUCACAGCUUUCUUGAACUU	MIMAT0035481	GTCGTATCCAGTGCAGGGTCCGAGG TAATCGCACTGGATACGACAAGTTC	GCGCTTCCACAGCTTCTT
Universal reverse primer				AGTGCAGGGTCCGAGGT

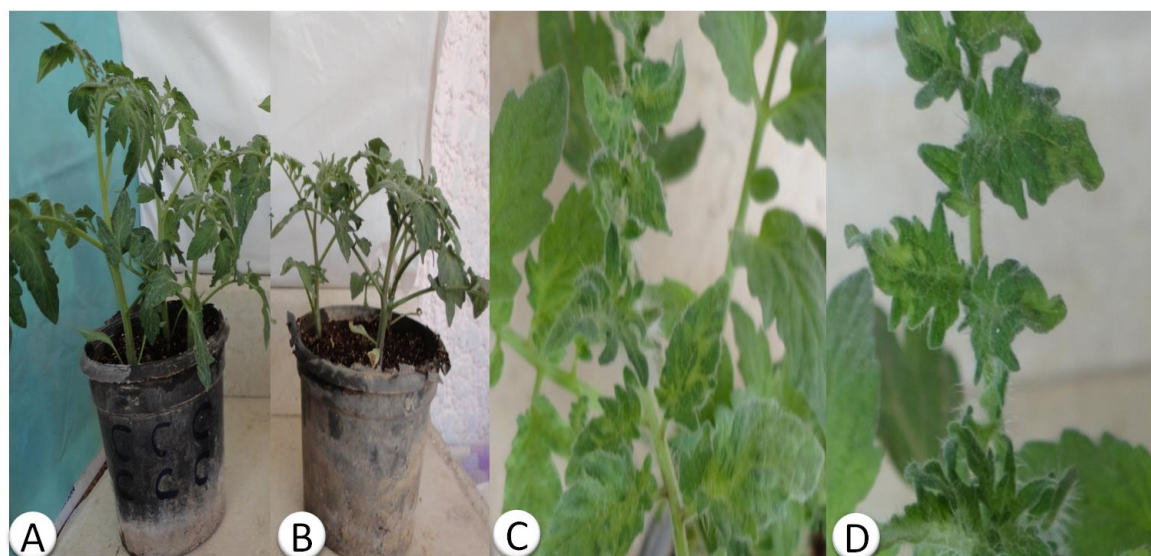


Figure 1. Effect of TMV infection on tomato growth at 12 dpi: (A) showing the mocked plant; (B) Infected plant, and (C, D) Characteristic symptoms of TMV on tomato showing leaf morphogenesis and leaf distortion.

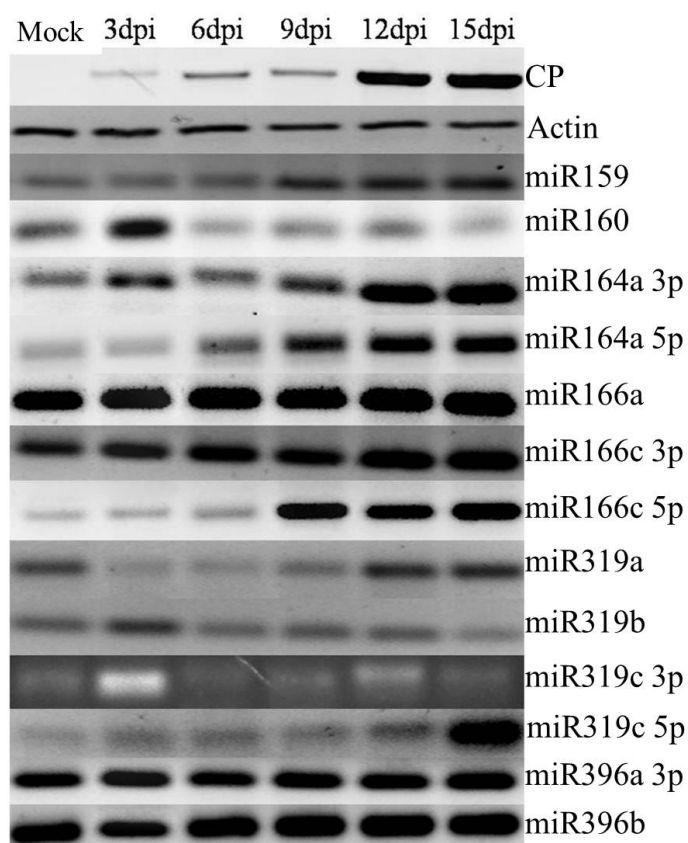


Figure 2. Agarose gel electrophoresis (3%) in TAE buffer stained with ethidium bromide showing RT-PCR amplification of TMV-Coat Protein (CP), internal gene (Actin) and different sets of studied miRNAs at different time intervals of TMV-infected tomato compared with mock-inoculated plant.



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

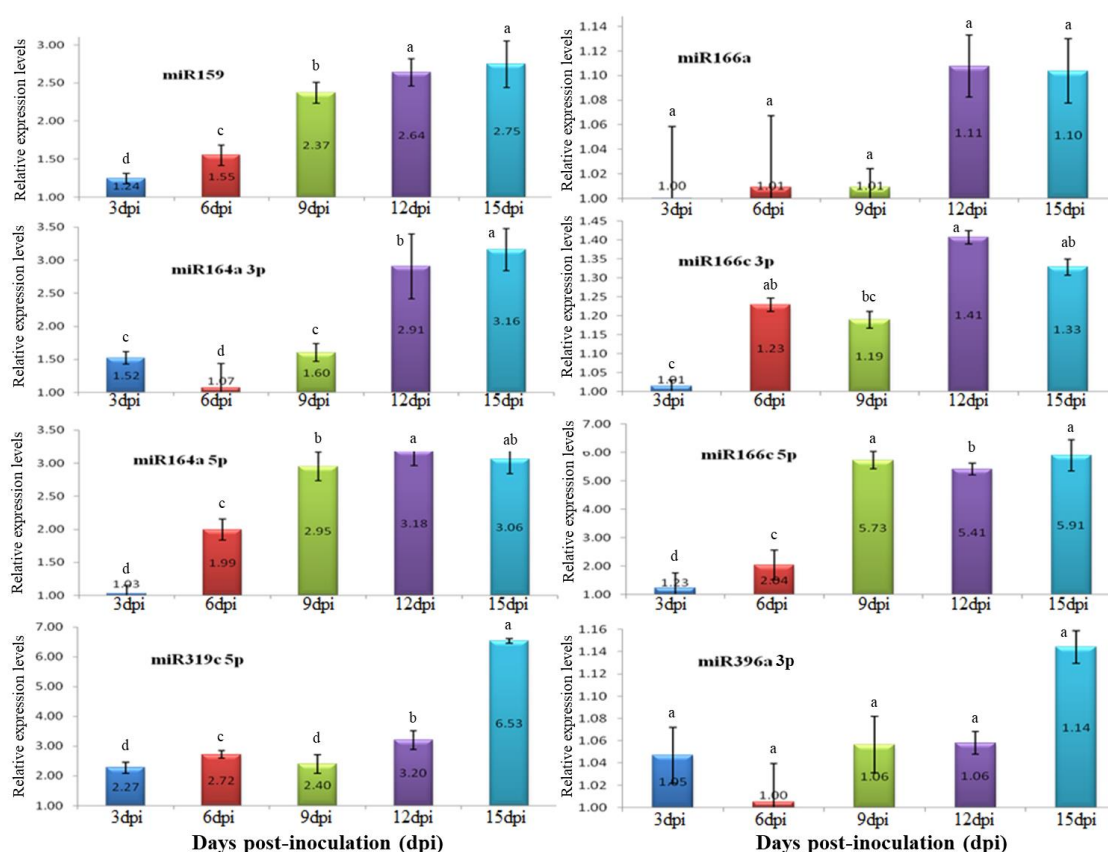


Figure 3. The relative expression levels of tomato miRNAs upon TMV infection at 3, 6, 9, 12 and 15 dpi showing up-regulation of miR159, miR164a 3p, miR164a 5p, miR166a, miR166c 3p, miR166c 5p, miR319c 5p and miR396a. The mocked sample was normalized to 1. Columns represent mean value from three biological replicates and bars indicate Standard Deviation ($\pm$ SD). Significant differences between samples were determined by one-way ANOVA using CoStat software. Means were separated by Least Significant Difference (LSD) test at $P \leq 0.05$ levels and indicated by small letters. Columns with the same letter means do not differ significantly.

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 miR164a5p 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,

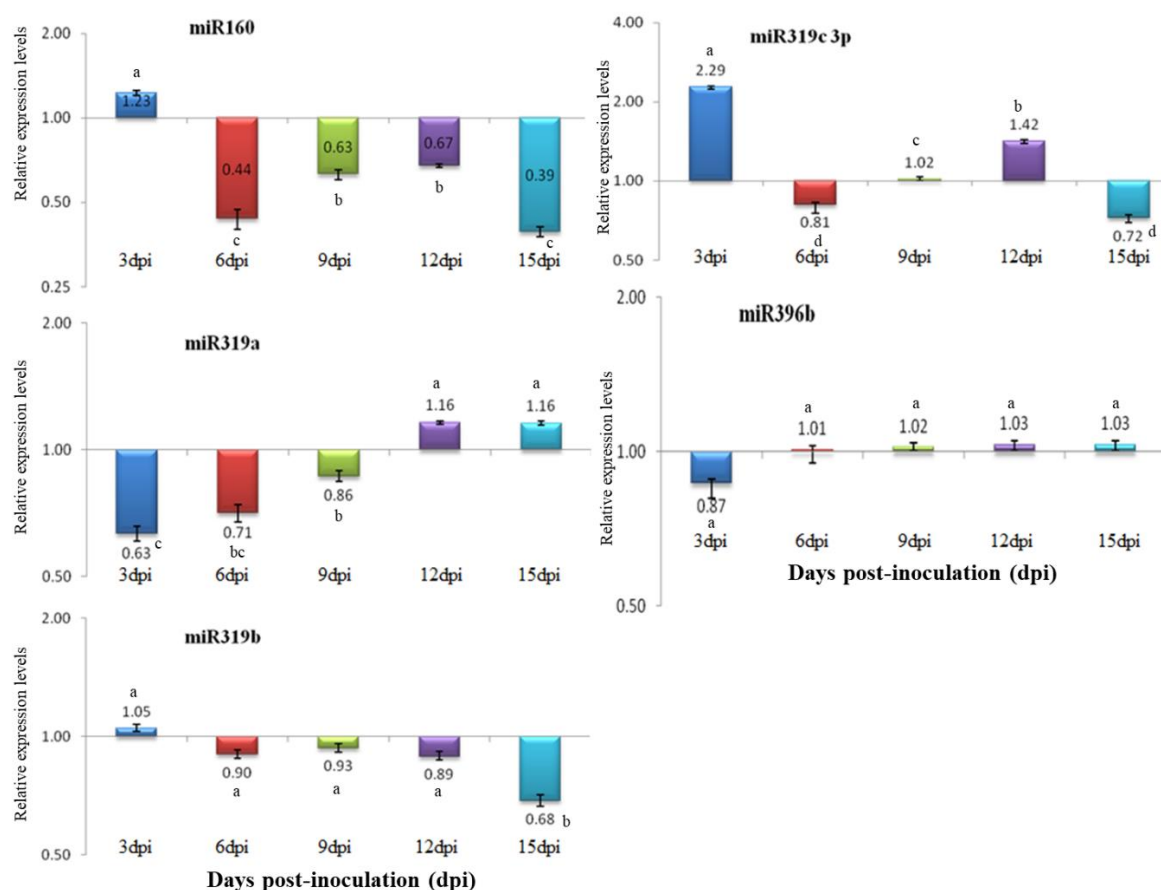


Figure 4. The relative expression levels of tomato miRNAs upon TMV infection at 3, 6, 9, 12 and 15 dpi showing down-regulation of miR160, miR319a, miR319b, miR319c-3p and miR396b. The mocked sample was normalized to 1. Columns represent mean value from three biological replicates and bars indicate Standard Deviation ($\pm$ SD). Significant differences between samples were determined by one-way ANOVA using CoStat software. Means were separated by Least Significant Difference (LSD) test at $P \leq 0.05$ levels and indicated by small letters. Columns with the same letter means do not differ significantly.



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-*Arabidopsis 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*

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.

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پروفايل های بيانی تفکیکی مربوط به میکرو آر.ان. ا (miRNAs) القاشده به وسیله ویروس موزائیکی تنباکو

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چکیده

میکرو آر.ان. ا (miRNA) های گیاهی در رشد گیاه و واکنش آن به تنش های زنده و غیر زنده نقش مهمی دارند. اخیراً، شواهد روشنی به دست آمده که نشان میدهد miRNAs در تعامل بین میزبان و ویروس دخالت دارند. در این زمینه، در پی آلودگی گوجه فرنگی با ویروس موزائیکی تنباکو (TMV)، با استفاده از روش stem-loop RT-PCR، تغییر در سطح بیان (expression levels change) سیزده miRNAs از ۶ فامیل miRNAs که رشد و مورفوزنز برگ را هدف قرار میدهند بررسی شد. تغییرات در سطح بیان نسبی ۹ میکرو آر.ان. ا (miRNAs) در مقایسه با گیاهان شاهد (mock plants) معنادار بود. ۱۵ روز بعد از آلوده شدن، miR319c-5p بیشترین افزایش در انباشت (accumulation) را نشان داد که از نظر آماری معنادار بود. در همه زمان های اندازه گیری، miR159، miR164a-3p، miR164a-5p، miR166c-5p و miR319c-5p فرا-تنظیم شده (up-regulated) بودند در حالی که miR160، miR319a، miR319b و miR319c-3p در بیشتر موارد فرو-تنظیم شده (down-regulated) بودند. داده های این آزمایش نگرش جدیدی در مورد نقش miRNAs در تعامل بین گوجه فرنگی و TMV و نیز برای توسعه راهبردهای کارآمد در زمینه بهبود مقاومت گوجه فرنگی در برابر آلودگی ویروسی فراهم می کند.