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Temporal expression of three conserved putative microRNAs in response of *Citrus × Limon* to *Xanthomonas citri* subsp. *citri* and *Xanthomonas fuscans* subsp. *Aurantifolii*

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Abstract

Citrus canker is a widespread bacterial disease that severely affects citrus production. *Xanthomonas* spp. are the causative agents of the disease, which manifests as necrotic pustule-like lesions on the plant organs including leaves, fruits, and stems. MicroRNAs (miRNAs) are well-established post-transcriptional gene expression regulators that modulate plant responses during many stress conditions. Accordingly, some bacteria-responsive miRNAs have been shown to have regulatory functions in the plant-pathogen interactions. In this study, we successfully assessed the expression patterns of three conserved miRNAs: miR159, miR167, and miR398, in citrus × limon (lemon) infiltrated with *Xanthomonas* strains A and C using stem-loop RT-qPCR. Our results showed that the expressions of miR159 and mir167 have nearly similar patterns upon inoculations, while the expression of miR398 remains constantly up-regulated after an early induction.

Key words: lemon, citrus canker, conserved miRNAs, stem-loop RT-PCR, temporal expression

Introduction

The citrus genus, consisting of diverse fruit crops, makes a significant contribution to the human diet (Rouseff and Nagy, 1994; Economos and Clay, 1999). Among the commercial citrus fruits, lemon (citrus × limon) is considered an important horticultural product worldwide (USDA, 2013). Unfortunately, the lemon yield and fruit quality are frequently influenced by a wide range of biotic stresses. *Xanthomonas citri* subsp *citri* strain A (Xc), with a broad range of hosts, causes citrus canker disease, which is the most destructive threat to lemon production (Brunings and Gabriel, 2003). Citrus canker disease is characterized by symptoms such as water-soaked eruptions, circular lesions, and pustule-like lesions that appear on all plant tissues (Brunings and Gabriel, 2003). The lesions seem to be formed by changes in cell enlargement (hypertrophy) and cell division (hyperplasia) (Schubert et al., 2001; Brunings and Gabriel, 2003; Lin et al., 2009). In addition to the lesions, *Xanthomonas fuscans* subsp. *aurantifolii* strain C (XaC) can induce a hypersensitive response (HR) in lemon tissues (Brunings and Gabriel, 2003). HR is a form of cell death induced by the plant defense system to limit the growth and spread of pathogens at the infection site (Morel and Dangl, 1997).

In 2006, Jones and Dangl presented a simple model for the plant immune system, called the "zigzag" model (Jones and Dangl, 2006). In the first phase, conserved molecules shared by many classes of microbes (pathogen-associated molecular patterns) are recognized by pattern recognition receptors and trigger pattern-triggered immunity (PTI). Only successful pathogens can overcome PTI by the secretion of virulence effectors that lead to effector-triggered immunity (ETI). In both PTI and ETI, the perception of pathogens by a host triggers a series of immune responses including reactive oxygen species (ROS) signaling, hormone activation, and gene expression reprogramming (Nicaise et al., 2009).

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Moreover, ETI is associated with the appearance of HR (Heidrich et al., 2012).

MicroRNAs (miRNAs), 20-24 nucleotides in length, are post-transcriptional regulators of gene expression that play crucial roles during developmental processes and responses to various biotic and abiotic stresses (Khraiwesh et al., 2012; Barciszewska-Pacak et al., 2015). Furthermore, miRNAs have been demonstrated to be critical regulators in gene expression reprogramming during both PTI and ETI establishment (Navarro et al., 2006; Li et al., 2010). Moreover, several bacteria-responsive miRNAs play regulatory roles in hormone signaling pathways and gene regulatory systems (Zhang et al., 2011). In recent years, almost all plant hormones have been recognized as key regulators of plant immunity (Denancé et al., 2013). Among many characterized miRNAs, miR159 and miR167 are known to be involved in various hormone signaling pathways through the down-regulation of the expression of the MYB and ARF transcription factors, respectively (Li et al., 2010; Zhang et al., 2011). The miR159 family members are specific to 3 *GAMYB-like* genes, *MYB33*, *MYB65*, and *MYB101* (Alonso-Peral et al., 2010). MiR167 negatively regulates the auxin signaling pathway by targeting auxin response factor 6 and 8 (ARF6 and ARF8) transcripts (Glazińska et al., 2014). MiR398 targets copper- and zinc-containing superoxide dismutase (SOD) transcripts (Sunkar et al., 2006). SOD enzymes predominantly act to decrease high levels of ROS that are triggered during a pathogen attack (Jagadeeswaran et al., 2009).

The isolation of mature miRNAs and an accurate determination of their expression levels may be technically challenging (Benes and Castoldi, 2010). The stem-loop quantitative reverse transcription polymerase chain reaction (qRT-PCR) method, which takes advantage of specific stem-loop primers during cDNA synthesis, serves as a sensitive and specific strategy to quantify the abundance of mature miRNAs (Varkonyi-Gasic et al., 2007).

In the present study, the stem-loop RT-PCR method was used for analyzing the temporal expression of the selected miRNAs (miR398, miR159, and miR167) upon Xc and XaC infections.

Materials and methods

Bacterial inoculum preparation

Xanthomonas fuscans subsp. *aurantifolii* strain 9181 and *Xanthomonas citri* subsp. *citri* strain 9322 were obtained from the National Institute of Genetic Engineering and Biotechnology (NIGEB, Iran). Inoculum was prepared from three-day-old colonies grown on YPGA agar plates (yeast extract 3 gl^{-1}, peptone 5 gl^{-1}, glucose 10 gl^{-1}, and agar 20 gl^{-1}; pH 6.5-7.0). Single colonies were transferred to 30 ml of a yeast extract peptone (YP) broth medium (3 gl^{-1} yeast extract and 5 gl^{-1} peptone; pH = 7.0), and bacterial growth was monitored spectrophotometrically (OD_{600}) at 1-h intervals for a 48-h period. To prepare the bacterial inoculum, suspensions of bacterial cells were grown for 15 h in a rotary shaker (180 rpm) at 28°C. When OD_{600} reached 0.3, bacterial suspensions were diluted to OD_{600} of 0.1 (corresponding to approximately 10^7 cfu/ml) by using a 0.075 M phosphate buffer (2.37 g NaH_2PO_4 and 9.54 g Na_2HPO_4) (pH = 7.0).

Plant material preparation and RNA extraction

Experiments were conducted on two-year-old lemon plants (citrus × limon) obtained from certified nurseries from the south of Iran and grown in a greenhouse at 30/28°C (day/night) under 16/8-h day/night conditions. To obtain new young leaves, plants were pruned a month before inoculation. Inoculation was performed using the handheld syringe method (Viloria et al., 2004). Leaves with 75% of the full expansion were used for inoculation by pressing the syringe tip against their abaxial surfaces. Inoculated regions of leaves were picked using a 0.5 mm cork borer and immediately snap-frozen in liquid nitrogen. Tissue sampling was done at 0.5, 3, 6, 12, 24, 48, and 72 h post-inoculation (hpi). Three biological replicates were considered for each treatment along with a control. The same number of leaves from the bottom, middle, and top of the plants were inoculated, and all the infected regions were pooled. Total RNA was extracted from the leaf tissues by using a TRIzol® reagent (Invitrogen, USA) and following the manufacturer's instructions. In brief, the frozen tissue was homogenized by Trizol, and the total RNA was precipitated with isopropanol and washed with 75% ethanol. Then, an RNA pellet was suspended with RNase-free water. Subsequently, RNA samples were treated by RNase-free DNaseI (Fermentas Life Sciences, Germany) according to the manufacturer's instructions. The integrity and quantification of the total RNA were evaluated by electrophoresis in a 2% agarose gel and using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific, USA).

Table 1. Primer sequences used for stem-loop RT-PCR and real-time PCR analysis. *RT* indicates stem-loop RT primer, *F* indicates a specific forward primer, and *R* indicates a universal RT primer

Primer name	Primer sequences (5′ → 3′)	T_m	Product size
miR159	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT AGA GC (**RT**) GCA GCG TTT GGA TTG AAG GGA (**F**)	59.1	60
miR167	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA AGA TC (**RT**) CAC GGT GAA GCT GCC AGC AT (**F**)	59.7	61
miR398	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA AGG GG (**RT**) GCA GCG TGT GTT CTC AGG TC (**F**)	58.8	58
F-Box	TTGGAAACTCTTTCGCCACT (**F**) CAGCAACAAAATACCCGTCT (**R**)	58.2 58.8	121
Universal reverse primer	GTG CAG GGT CCG AGG TAT TC	58.1	

Primer design and stem-loop RT-PCR

Primers were designed using mature miRNA sequences of citrus species (miR159, miR167, and miR398) retrieved from the Sanger Institute miRBase Sequence Database (Release 21, June 2014). The stem-loop reverse transcription primers and PCR primers were designed according to the criteria described in the stem-loop RT-PCR method (Chen et al., 2005; Varkonyi-Gasic et al., 2007). The 3′ primer was a universal reverse transcription (RT) primer (Kramer, 2011; Varkonyi-Gasic et al., 2007). In our experiment, cDNA synthesis was conducted using an F-box reverse specific primer as a reference gene (Mafra et al., 2012) in a mixture with other stem-loop primers.

The specific forward primers and a universal reverse primer were tested with Primer Express 3.0 (Applied Biosystems, Foster City, CA, USA) (Table 1). The stem-loop RT reactions were subjected to first-strand cDNA synthesis using the above-described primers and 400 ng of *DNaseI*-treated total RNA by M-MLV reverse transcriptase (Vivantis, Vivantis Technologies, Subang Jaya, Malaysia) according to the manufacturer's instructions. Each sample, containing 400 ng of RNA and a primer, in a volume of 12.5 µl was heated at 85°C for 5 min to denature RNA and placed on ice for 5 min to stabilize the denatured structures. The RT reaction mixture consisted of dNTPs (1 mM), *RNase* inhibitor (1 U/µl), and M-MLV reverse transcriptase (20 U/µl) in a final volume of 20 µl. The mixture was incubated for 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C, and held at 4°C.

PCR amplification was performed on cDNA with a profile of 2 min at 94°C, followed by 35 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 15 s, and a final extension at 72°C for 3 min. PCR products were analyzed on a 4% agarose gel.

Real-time PCR analysis

Real-time PCR reactions were performed on an Applied Biosystems 7500 instrument (Applied Biosystems). PCR mixture reactions consisted of SYBR green PCR master mix (AccuPower® 2X Greenstar qPCR Master Mix, Bioneer, Korea), 1 µl of the diluted (1:20) cDNA template (from the previous step), Rox reference dye (1X), and primers (0.5 µM) in a final volume of 10 µl. The real-time PCR amplification was carried out under the following conditions: pre-denaturation at 95°C for 10 min, followed by 40 cycles of denaturing at 95°C for 15 s, annealing, and extension at 60°C for 45 s. An additional cycle was carried out to obtain the melting curve in the temperature range of 65-95°C. Further, the PCR efficiency was estimated using the LinReg PCR software version 11.0 (Ruijter et al., 2009). The experiment was repeated thrice, and the reaction mixture with no template was included as a negative control to detect possible contaminations. The relative expression of miRNAs, normalized on the basis of the reference gene expression, was calculated using the $2^{-\Delta\Delta CT}$ comparative method (Livak and Schmittgen, 2001). $2^{-\Delta\Delta CT}$ is considered a fold change (FC), and data are presented as Log_2FC.

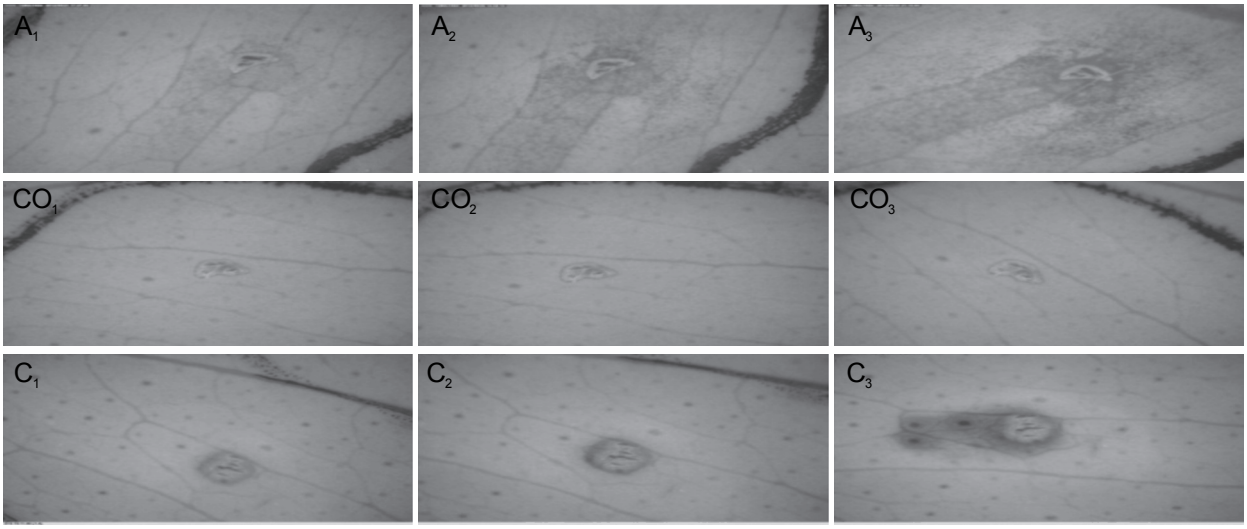

Fig. 1. Differential response of lemon leaves to *Xanthomonas* strains. Progression of disease symptoms during infection with Xc (A_1-A_3) as compared to the reaction when infected with XaC (C_1-C_3). As is shown in Figures $1C_1$, $1C_2$, and $1C_3$, progression of the bacterial infection in an incompatible reaction was inhibited by the induction of hypersensitive reactions in a host plant. It led to programmed cell death, which resulted in the formation of a necrotic layer to limit bacterial agents. Subscript numbers indicate day(s) after inoculation. CO indicates the control condition under which injury symptoms showed no changes throughout the experimental time series

Results

Analysis of temporal progression of disease and HR

A visual assessment of the plant-pathogen interaction for three days post inoculation revealed that the host plant differentially interacted with the Xc and XaC bacterial strains (Fig. 1). The plant–Xc pathogen interaction resulted in a progressive increase in the symptoms of the canker disease (Fig. $1A_1$-$1A_3$), while the pathogen XaC induced HR in the infected leaves and subsequently caused the appearance of necrotic rings around the inoculum regions (Fig. $1C_1$-$1C_3$). The injury symptoms under control conditions remained unchanged (Fig. $1CO_1$–$1CO_3$).

Detection and amplification of investigated miRNAs

According to the literature, three conserved miRNAs (miR159, miR167, and miR398), which are known to be involved in the biotic stress responses of plants, were considered in this survey. Accordingly, the expression of their mature forms in lemon leaves was investigated upon Xc and XaC infections. MiRNAs and F-Box PCR products were of the expected size, 60-65 bp and 114 bp in length, respectively. The investigated miRNAs were successfully detected and amplified using stem-loop RT-PCR in a non-model plant.

Expression patterns of miRNAs upon Xanthomonas infection

To investigate the temporal expression patterns of miR159, miR167, and miR398 upon inoculation with Xc and XaC, relative quantifications of the miRNA expression levels were performed using stem-loop RT-qPCR during seven post-infection time series. Except for the very early time point (i.e., 0.5 hpi), nearly similar expression patterns of miR159 and miR167 were noted in response to both bacterial strains (Fig. 2). A steep change in the relative expression of both miRNAs was observed during transition from 3 to 6 hpi, where we observed a change from down-regulation to up-regulation, and then, a decrease in the expression level at 12 hpi. Another increase in the expression of both miRNAs was observed (48 hpi). The relative expressions for XaC were significantly greater (in absolute values) than those for Xc, except at 6 and 48 hpi. At an early time point (0.5 hpi), miR159 was down-regulated (in contrast to miR167), with greater absolute values for XaC than for Xc.

Although the relative expression patterns of miR398 were distinct from those of miR159 and miR167, the trends of miR398 expressions were similar against both bacterial strains. The level of miR398 decreased at early infection times (0.5 and 3 hpi). A sharp increase (6 hpi) in the expression levels was observed for both strains,

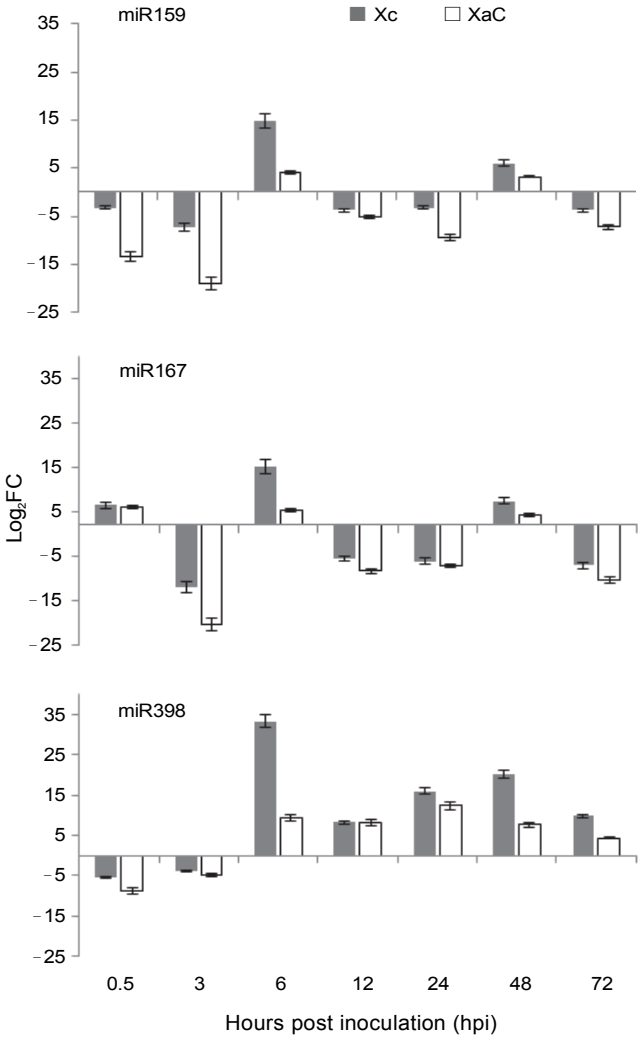

Fig. 2. Time-course expression pattern of selected miRNAs in lemon leaves upon infection with *Xanthomonas* Xc and XaC strains. The effect of both *Xanthomonas* strains on the expression levels of miR159, miR167, and miR398 was analyzed in the infected lemon leaves by using stem-loop qRT-PCR at 0.5, 3, 6, 12, 24, 48, and 72 h post infection (hpi). The vertical axis indicates $\log_2$ of the relative expression level or fold change (Log_2FC) where the relative expression (ratio) is the normalized expression level of miRNAs in the inoculated samples related to the control samples (non-inoculated). Data were presented as means of $Log_2FC \pm SD$

particularly upon Xc infection. Further, the increase in the miR398 level was considerably higher upon Xc inoculation than upon XaC infection. Up-regulation of miR398 was maintained throughout the remaining tested time points.

Discussion

Plants have an excellent capacity to recognize pathogens. Further, pathogens are capable of affecting the defense systems of plants. The pathogen *Xanthomonas citri* subsp *citri* strain A (Xc) induces citrus canker symptoms by triggering hypertrophy and hyperplasia (Schubert et al., 2001), while *Xanthomonas fuscans* subsp. *aurantifolii* strain C (XaC) induces HR in certain citrus species including sweet oranges and lemon tissues (Brunings and Gabriel, 2003). An expression analysis performed by Cernadas and colleagues (2008) revealed that XaC induces the expression of genes, which are involved in basal defense systems, whereas Xc induces the expression of genes associated with cell division and growth (Cernadas et al., 2008). A subsequent study performed by Cernadas and Benedetti (2009) demonstrated that cell-wall remodeling genes encoding cell-wall remodeling enzymes and proteins associated with cell-wall elongation, including the beta-1,3 (B1.3G) and beta-1,4 (B1.4G) endoglucanases, acidic and basic cellulases, pectinesterase, pectinacetylesterase, xyloglucan galactosyltransferase, and expansin, are induced by Xc inoculation and auxin and gibberellin exposures. Therefore, the auxin and gibberellin hormones may play key roles in contributing to the development of citrus canker symptoms. Like other biotrophic pathogens, *Xanthomonas* strains can synthesize active forms of auxins to promote disease symptoms in plants (Wang et al., 2007; Chen et al., 2007).

Many studies have clearly emphasized the regulatory roles of host miRNAs in plant-pathogen interactions (Chen and Cao, 2015; Navarro et al., 2006; Zhang et al., 2011). According to our results, expression patterns of the investigated miRNAs in response to different *Xanthomonas* strains followed a fluctuating pattern in lemon at the analyzed time points (Fig. 2). Previously, researchers have suggested that miR159 and miR167 contribute to PTI and ETI in the *Arabidopsis pseudomonas syringae* interaction (Rhoades et al., 2002; Zhang et al., 2011). Thus for, the up-regulation of miR159 and miR167 at 6 hpi and again at 48 hpi might represent important time points in the cases of the Xc and XaC infections in lemon. They have also been described during the identification and characterization of differentially expressed genes for several citrus species in response to *Xanthomonas* spp (Cernadas et al., 2008; Khalaf et al., 2011; Shi et al., 2015; Hu et al., 2016).

The modulation of phytohormone networks in the plant-pathogen interactions has been previously reported to play a key role in plant immunity (Zhang et al., 2011).

MiR159 and miR167, being highly conserved miRNAs involved in plant immune systems (Wu and Poethig, 2006; Reyes and Chua, 2007), have been reported to have key regulatory effects in gibberellin and auxin signaling pathways, respectively. Some *GAMYM-like* genes (*MYB33*, *MYB65*, and *MYB101*), which are considered to be miR159 regulatory targets, have been reported to be among the gibberellin signaling activators (Millar and Gubler, 2005; Reyes and Chua, 2007). During the events leading to an increase in the expression level of miR159 at 6 and 48 hpi upon a *Xanthomonas* infection in lemon, the plant defense system may suppress gibberellin signaling, which results in a reduction of the cell growth. As reported by other researchers, miR167 through targeting ARF transcription factor family members (ARF6 and ARF8) reduces auxin signaling upon bacterial infection and therefore, diminishes the hypertrophy resulting from high levels of auxin in the plant system (Hutchison et al., 1999; Zhang et al., 2011). Interestingly, we could observe nearly the same expression patterns for miR167 and miR159 upon infection by both *Xanthomonas* strains. Thus, we suggest that miR159 and miR167 may play roles in the lemon defense system through the regulation of the auxin and gibberellin pathways.

MiR398 is one of the bacteria-responsive miRNAs with a proven role in regulating ROS levels as it targets SOD transcripts (*CSD1* and *CSD2*) (Sunkar and Zhu, 2004; Bonnet et al., 2004). ROS, produced during the onset of a pathogen-induced HR, have been reported to trigger hypersensitive plant cell death (Zeier et al., 2004). SOD enzymes decrease the level of superoxide radicals by converting them into H_2O_2 and O_2 (Draper, 1997). The up-regulation of miR398 as a response to the infection of both *Xanthomonas* strains observed in our experiments was contradictory to the findings of other researchers who reported the down-regulation of miR398 under biotic stresses (Jagadeeswaran et al., 2009, Zhang et al., 2011, Pérez-Quintero et al., 2012, Kumar et al., 2011a; Kumar et al., 2011b). Further, the time-course expression of miRNAs in the compatible and incompatible interactions of poplar-foliar rust fungus indicated that miR398 was up-regulated during post-infection periods (Chen and Cao, 2015). Note that a comparative expression analysis between the disease-inducing and the HR-inducing *Xanthomonas* Xc and XaC strains led to the observation of a lower relative expression of miR398 in the case of the HR-inducing isolate at all time points longer than 3 hpi. These differences in the miR398 level may suggest its possible role in the incompatible interaction that resulted in HR.

Conclusions

The experimental results indicated that stem-loop RT-PCR can be used as an efficient method to detect highly conserved miRNAs in non-model plants. Moreover, the expression analysis clearly showed similar patterns for both miR159 and miR167 during infections with both bacterial strains. The expression of miR398 was differentially regulated during post-infection times. Further experiments are required to understand the *citrus-Xanthomonas* interactions. A global mRNA expression analysis at the tested time points is recommended to further confirm the observations made with respect to miRNAs.

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