Expression Analysis of miR393 and Target Gene Ve of Solanum torvum Swartz. Infected by Verticillium dahliae Kleb.

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Abstract In this study, Solanum torvum Swartz. was used as material and B-Actin gene and U6 were used as internal references. First, the expression characteristics analysis of miR393 and target gene Ve were performed on the root, stem, leaf, flower and fruit of Solanum torvum Swartz. during flowering and fruiting stage. The strong Verticillium dahliae Kleb. were inoculated into Solanum torvum Swartz. during 5-6 leaf stage, and the control group was treated with ultrapure water. The Solanum torvum Swartz. roots were taken at 0 h, 3 h, 6 h, 12 h, 24 h, 36 h, 48 h and 72 h for expression analysis of infection by Verticillium dahliae Kleb.. The results showed that under normal growth conditions, miR393 and target gene Ve had specific expression patterns in different tissues of Solanum torvum Swartz., and the differential expression was significant. Both miR393 and target gene Ve had the highest expression levels in roots, followed by stems, and had the lowest levels in fruits. After infection by Verticillium dahliae Kleb., both miR393 and target gene Ve had certain expression in different infection periods. The expression of target gene Ve showed an upward tendency with the extension of infection time, and the highest expression level appeared at 48 h, followed by 72 h. While the expression of miR393 showed a downward tendency with the extension of infection time, and the highest expression level appeared at 6 h. The expression of miR393 and target gene Ve showed opposite tendencies in different infection periods. These results provided a foundation for further research on the interaction mechanism between miR393 and target gene Ve and the molecular mechanism of resistance to Verticillium wilt.

Keywords Solanum torvum Swartz.; Target gene; miR393; Target gene Ve; Verticillium wilt; qPCR

Solanum Torvum Swartz., also known as spiny tomato, belladonna, golden button and so on, is a related wild species of Solanum in Solanaceae, which has strong resistance to Verticillium wilt, Fusarium oxysporum, Meliodogyne spp. and other kinds of pests and diseases.

There are a large number of non-coding RNAs in organisms. These non-coding RNAs transcribed from the genome are not translated into proteins. They exercise their respective biological functions at RNA level, which is an important way to regulate gene expression during the growth and development of plants (Ambros, 2001; Zhang et al., 2006a). Since the first discovery of miRNA in nematode in 1993, the research on its regulation mechanism has been carried out continuously (Lee et al., 1993; Lee and Ambros, 2001). Studies have found that the same miRNA can inhibit the function of multiple target genes, and the same target gene can be regulated by multiple miRNAs. Moreover, miRNAs in plants participate in complex physiological processes, including growth and development, organ formation, metabolism, cell proliferation and apoptosis, adversity stress, signal transduction, and self-feedback regulation (Bartel and Chen, 2004; Kloosterman and Plasterk, 2006). In the process of exerting regulatory function, miRNA regulates the abundance and function of target mRNA by degrading target mRNA or inhibiting the translation of target mRNA (Lyu et al., 2013; Zhang and Zhang, 2014). At present, the research on the mechanism of plant response to biotic and abiotic stresses mostly focuses on the interaction mechanism between miRNA and target gene: Navarro et al. (2006, 2008) studied the defense mechanism of miRNA in plants and found that miR393 in Arabidopsis thaliana played a role in resisting bacterial diseases by regulating the auxin signal transduction pathway. Huang et al. (2010) conducted cadmium stress in Brassica napus, studied the expression regulation relationship between miR395 and its target gene ATP sulfurylase (APS) under cadmium stress, and initially concluded that miR395 responded to plant cadmium stress by regulating the expression of APS.
gene. Csukasi et al. (2012) found that miR159a and miR159b jointly responded to the change of gibberellin level by interacting with the target gene GA MYB when studying the growth of strawberry fruit trays. Similarly, Hu et al. (2014) studied the expression of miR475 and its target gene in Populus suaveolens under low temperature stress and found that with the extension of low temperature induction time, the expression of miR475 showed a downward tendency, while the expression of its target mRNA showed an opposite tendency. Therefore, it was believed that miR475 in Populus suaveolens may play a role in resisting low temperature by degrading target mRNA. The above studies showed that miRNA played an important regulatory mechanism by regulating the expression of target genes in the process of plant response to adversity stress, which was necessary for the normal growth of plants and the resistance to various biotic and abiotic stresses.

In the previous research, our group constructed a miRNA library of Solanum torvum Swartz. before and after infection by Verticillium dahliae Kleb. The expression of miR393 before and after infection by Verticillium dahliae Kleb. reached a very significant level. The binding site of miR393 and target gene Ve was predicted by rna hybrid, and the RLM-5'-RACE method was used to verify that Ve was the target gene of miR393. In this study, we mainly analyzed the tissue expression characteristics of miR393 and target gene Ve. By analyzing the tissue expression characteristics of miR393 and its target gene Ve in Solanum torvum Swartz., and the expression characteristics of infection by Verticillium dahliae Kleb., our group explored the interaction mechanism between miR393 and target gene Ve, which provided a foundation for the research on the molecular mechanism of resistance to Verticillium wilt.

1 Materials and Methods

1.1 Test materials
The test materials were taken from the Solanum torvum Swartz. cultivated in the greenhouse during the flowering and fruiting stage. Meanwhile, the root, stem, leaf, flower and fruit of Solanum torvum Swartz. were taken as samples. After sampling, they were quickly frozen in liquid nitrogen and stored in the refrigerator at -80°C. The Verticillium dahliae Kleb. strains were preserved in the refrigerator at 4°C. The Solanum torvum Swartz. seedlings were also cultivated in greenhouse and were selected during 4-5 leaf stage for the infection treatment with crude toxin of Verticillium dahliae Kleb. pathogen.

1.2 Test reagents
Trizol reagent was purchased from Vazyme Biotech Co., Ltd; dNTPs, 10×PCR buffer, Taq enzyme and pMD19-T Vector were purchased from Takara Biotechnology (Dalian) Co., Ltd.; Clontech's Mir-X™ miRNA First Strand Synthesis and SYBR® qRT-PCR User Manual (638313) kit and TaKaRa's SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (RR820A) kit were purchased from Takara Biotechnology (Dalian) Co., Ltd.; GoScript™ Reverse Transcription System (A5001) reverse transcription kit and GoTaq® qPCR Master Mix (A6001) kit were purchased from Promega (Beijing) Biotech Co., Ltd.; The qPCR primer synthesis of Ve gene was completed by Invitrogen Trading (SHANGHAI) Co., Ltd.; The qPCR specific primers of miR393 were completed by Beijing Dingguochangsheng Biotechnology Co., Ltd.; The sequencing was completed by Shanghai Majorbio Bio-pharm Technology Co., Ltd.. All reagents were prepared with 0.1% DEPC water overnight. The mortar, centrifuge tube and pipette tip were treated with 0.1% DEPC water overnight, autoclaved at 121°C for 30 min, and dried in an oven at 80°C for later use.

1.3 Cultivation of pathogen
1.3.1 Configuration of PDA medium
(1) Cut 200 g fresh peeled potatoes into small pieces, put them in a pot, add 1 L ultrapure water, boil for 20-30 minutes until the potatoes became soft but not rotten, and filter with 6 layers of gauze.

(2) Take the filtered juice into a beaker, and add ultrapure water to 1 L.

(3) Pour the mixture into the pot and heat it. Add 20 g agar while stirring until it was completely dissolved.

(4) Add 20 g glucose, stir well, and dispense it into erlenmeyer flasks while hot.
Method

1.3.2 Configuration of Czapek-Dox Medium
(1) Add about 500 mL ultrapure water into the beaker, successively add 3 g sodium nitrate, 1 g dipotassium hydrogen phosphate, 0.5 g magnesium sulfate (MgSO₄·7H₂O), 0.5 g potassium chloride, 0.01 g ferrous sulfate and 30 g sucrose, and stir while adding.

(2) Add ultrapure water to 1 L, boil and stir until it was completely dissolved. Dispense into erlenmeyer flasks, autoclave at 121°C for 20 minutes, take out and place at room temperature for later use.

1.3.3 Preparation of crude toxin of pathogen
(1) Pick out the Verticillium dahliae Kleb. strains stored in the 4°C refrigerator plate and connect them to the PDA plate. Culture them upside down in the 25°C incubator for one month to obtain a large number of the colonies of Verticillium dahliae Kleb. (Figure 1).

![Figure 1 The colonies of Verticillium dahliae Kleb. cultured on PDA agar plate](image)

(2) After the colonies grew, wash the PDA plate that cultured the colonies with Czapek-Dox Medium, pour the obtained bacterial solution into a erlenmeyer flask, cut a 1×1 cm bacteria block with a sterile knife, put it into the erlenmeyer flask of Czapek-Dox Medium, and then culture for two weeks at 25°C and 90 rpm in a shaker.

(3) Measure and record the OD value of the culture solution with an ultraviolet spectrophotometer at 600 nm. The OD value of the culture solution was 1.9155.

(4) Use six layers of gauze to filter the obtained bacterial solution of Verticillium dahliae Kleb., and dilute it twice with ultrapure water for infection.

1.4 Extraction of total RNA from different tissues of Solanum torvum Swartz.
Referring to the total RNA extraction method of Solanum torvum Swartz., the improved Trizol method was used to extract the total RNA of the tissues of Solanum torvum Swartz., and the integrity, purity and concentration of the extracted total RNA were tested and analyzed to meet the requirements of subsequent tests (Qin et al., 2016).

1.5 Synthesis of the first strand cDNA
1.5.1 Synthesis of Ve gene qPCR template cDNA
The expression of target gene Ve in different tissues of Solanum torvum Swartz. was studied, and the synthesis of required cDNA was performed by using Promega’s GoScript™ Reverse Transcription System (A5001) reverse transcription kit. According to the instructions, reverse transcription of the total RNA from different tissues of Solanum torvum Swartz. was performed to synthesize the first strand cDNA, and the obtained cDNA was used for the qPCR test of the target gene Ve. The specific reversal steps were as follows:
(1) Prepare the mixture according to Table 1:

| Reagent                                      | Volume (µL) |
|----------------------------------------------|-------------|
| Experimental RNA (up to 5µg/reaction)        | 2.0         |
| Primer [Oligo(dT)15 (0.5µg/reaction)         | 1.0         |
| Nuclease-Free Water                          | 2.0         |
| Final volume                                 | 5.0         |

(2) After gently mixing, centrifuge and then heat in a 70°C water bath for 5 min, and immediately cool on an ice box for more than 5 min.

(3) On the ice box, the following components were successively added to the above mixture (Table 2):

| Reagent                                      | Volume (µL) |
|----------------------------------------------|-------------|
| Nuclease-Free Water                          | 5.0         |
| GoScript™ 5X Reaction Buffer                 | 4.0         |
| MgCl2 (final concentration 1.5-5.0 mM)       | 3.5         |
| PCR Nucleotide Mix (final concentration 0.5 mM each dNTP)2 | 1.0         |
| Recombinant RNasin® Ribonuclease Inhibitor (optional) | 0.5         |
| GoScript™ Reverse Transcriptase              | 1.0         |
| Final volume                                 | 15.0        |

(4) After evenly mixing the above mixture, centrifuge and heat in a 25°C water bath for 5 min, 42°C water bath for 1 h and 70°C water bath for 15 min. And then add 60 µL ddH2O into each transcription extraction tube to dilute the cDNA by five times. Finally, end the reverse transcription process, and store it in the refrigerator at -20°C for later use.

1.5.2 Synthesis of miR393 qPCR template cDNA

The expression of miR393 in different tissues of *Solanum torvum* Swartz. was studied, and the synthesis of required cDNA was performed by using Clontech’s Mir-X™ miRNA FirstStrand Synthesis and SYBR® qRT-PCR User Manual (638313) kit. According to the instructions, reverse transcription of the total RNA from different tissues of *Solanum torvum* Swartz. was performed to synthesize the first strand cDNA, and the obtained cDNA was used for the qPCR test. The specific reversal steps were as follows:

(1) RNA purification (Table 3):

| Reagent            | Volume (µL) |
|--------------------|-------------|
| RNA sample (5 µg)  | ≤44.0       |
| DNase I Buffer (10x)| 5.0         |
| DNase I (5 U/µL)   | 0.5         |
| RNase-free ddH2O   | Add to 50.0 |
| Total volume       | 50.0        |

(2) After gently mixing, centrifuge and then heat in 37°C water bath for 30 min. And add 1 µL 0.5 M EDTA, and then heat in 80°C water bath for 2 min.

(3) RNA reversal (Table 4):
Table 4 RNA reversal

| Reagent                  | Volume (µL) |
|--------------------------|-------------|
| mRQ Buffer (2x)          | 4.0         |
| RNA sample               | 5.0         |
| mRQ Enzyme               | 1.0         |
| Total volume             | 10.0        |

(4) After evenly mixing the above mixture, centrifuge and heat in a 37°C water bath for 1 h, 85°C water bath for 5 min to inactivate the enzyme. And then add 40 µL ddH₂O into each transcription extraction tube to dilute the cDNA by five times. Finally, end the reverse transcription process, and store it in the refrigerator at -20°C for later use.

1.6 Expression analysis of target gene Ve and miR393

1.6.1 Expression analysis of target gene Ve

According to the sequence information of target gene Ve, the generic specific primers Ve-L (5’-GCAATCTGAAAGGGAACA-3’) and Ve-R(5’-CTCGGAACACTACCCTA-3’) were designed. B-Actin was used as the internal standard gene (A-L:5’ACTGAGGCCACCCCCCTATCCC-3’) and (A-R:5’ACACCATCACCAGTCCCAACAC 3’), and the cDNA of different tissues of Solanum torvum Swartz. was used as template for fluorescence quantitative qPCR amplification. And then 2⁻ΔΔCt method was used to calculate the relative expression of gene and detect the expression of target gene Ve in different tissues. The reaction was carried out on the Jena qTOWER2.2 fluorescent quantitative PCR instrument, and the reaction system was 20 µL. The method was based on the instructions of Promega’s GoTaq® qPCR Master Mix (A6001) kit. The specific steps were as follows (Table 5):

Table 5 Preparation for Ve gene qPCR reaction solution

| Reagent                          | Volume (µL) |
|----------------------------------|-------------|
| GoTaq® qPCR Master Mix, 2X       | 10.0        |
| Nuclease-Free Water              | 7.2         |
| upstream PCR primers             | 0.4         |
| downstream PCR primers           | 0.4         |
| cDNA template                    | 2.0         |
| Final volume                     | 20.0        |

PCR reaction procedure was as follows: pre-denaturation at 95°C for 5 min, denaturation at 95°C for 15 s, annealing at 56°C for 15 s, extension at 72°C for 20 s, and for 40 cycles. And three repetitions were set for each sample.

1.6.2 Expression analysis of miR393

When studying the expression of miR393 in different tissues of Solanum torvum Swartz., synthesize the specific primers of miR393: TCCAAAGGGATCGATTGATCC. The mRQ 3’Primer and U6 included in the Clontech Mir-X™ miRNA First Strand Synthesis and SYBR® qRT-PCR User Manual (638313) kit were used as universal primers and internal reference primers. The cDNA of different tissues of Solanum torvum Swartz. was used as template for fluorescence quantitative qPCR amplification. And then 2⁻ΔΔCt method was used to calculate the relative expression of miR393 and detect the expression of miR393 in different infection periods. The reaction was carried out on the Jena qTOWER2.2 fluorescent quantitative PCR instrument, and the reaction system was 25 µL. The method was based on the instructions of TaKaRa’s SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (RR820A) kit. The specific steps were as follows:

(1) For miR393 qPCR reaction, prepare the PCR reaction solution according to the following components (Table 6) (the reaction solution should be prepared on ice):
Table 6 Preparation for miR393 qPCR reaction solution

| Reagent                                                                 | Volume (μL) |
|------------------------------------------------------------------------|-------------|
| SYBR Premix Ex Taq II(Tli RNaseH Plus)(2×)                            | 12.5        |
| PCR Forward Primer/miRNA-specific Primer(10 μM)                        | 0.5         |
| PCR Reverse Primer/miRQ 3′Primer(10 μM)                               | 0.5         |
| ROX Dye(50×)                                                           | 0.5         |
| cDNA (<100 ng)                                                        | 2.0         |
| ddH2O                                                                  | 9.0         |
| Total volume                                                           | 25.0        |

(2) For U6 qPCR reaction, prepare the PCR reaction solution according to the following components (Table 7) (the reaction solution should be prepared on ice):

Table 7 Preparation for U6 qPCR reaction solution

| Reagent                                                                 | Volume (μL) |
|------------------------------------------------------------------------|-------------|
| SYBR Premix Ex Taq II(Tli RNaseH Plus)(2×)                            | 12.5        |
| U6 Forward Primer(10 μM)                                               | 0.5         |
| U6 Reverse Primer(10 μM)                                               | 0.5         |
| ROX Dye(50×)                                                           | 0.5         |
| cDNA (<100 ng)                                                        | 2.0         |
| ddH2O                                                                  | 9.0         |
| Total volume                                                           | 25.0        |

(3) Carry out qPCR reaction, and the reaction procedure was as follows: Pre-denaturation at 95°C for 5 min, denaturation at 95°C for 15 s, annealing at 56°C for 15 s, extension at 72°C for 20 s, and for 40 cycles. And three repetitions were set for each sample.

1.7 Expression analysis of infection by Verticillium dahliae Kleb.

1.7.1 Infection by Verticillium dahliae Kleb.

(1) Take the bacterial solution of Verticillium dahliae Kleb. mentioned in 1.3.3 to water the seedlings, pour the bacterial solution into the cultivation medium of Solanum torvum Swartz. with the same growth, and then use a knife to treat the roots. The control group was treated with ultrapure water.

(2) The roots of plants were taken respectively after being treated for 0 h, 3 h, 6 h, 12 h, 24 h, 36 h, 48 h and 72 h. The plants treated with ultrapure water for 0 h were used as the control. After the samples were quickly frozen with liquid nitrogen, they were stored in a refrigerator at -80°C for later use.

1.7.2 Extraction of total RNA in different infection periods
The improved Trizol method was used to extract the total RNA in different periods of Solanum torvum Swartz. after infection by Verticillium dahliae Kleb., and the integrity, purity and concentration of the extracted total RNA were tested and analyzed to meet the requirements of subsequent tests.

1.8 cDNA synthesis and expression analysis in different infection periods
Same as steps 1.5 and 1.6, synthesize the first strand cDNA of miR393 and target gene Ve in different periods of Solanum torvum Swartz. after infection by Verticillium dahliae Kleb., and then analyze the expression pattern.

2 Results and Analysis

2.1 The disease symptom of Solanum torvum Swartz. before and after infection of Verticillium dahliae Kleb.
The Solanum torvum Swartz. seedlings were infected by bacterial solution of Verticillium dahliae Kleb. Before infection, the seedlings grew healthily without wilting. After being treated with bacterial solution for 72 h, the leaf edge of Solanum torvum Swartz., a highly resistant variety to Verticillium wilt, was slightly withered and yellow (Figure 2).
2.2 Expression patterns of miR393 and Ve gene in different tissues of Solanum torvum Swartz.

Total RNA was extracted from the root, stem, leaf, flower and fruit of Solanum torvum Swartz. cultivated in greenhouse, and then reversed into cDNA. B-Actin gene and U6 were used as internal references, and the expression differences of miR393 and target gene Ve in different tissues of Solanum torvum Swartz. were analyzed by qPCR (Figure 3; Figure 4). The results showed that under normal growth conditions, both miR393 and target gene Ve had the highest expression levels in roots, followed by stems, and had the lowest levels in fruits. The expression in different tissues showed a downward tendency, and the differential expression was significant.
2.3 Expression patterns of miR393 and Ve gene of *Solanum torvum* Swartz. infected by *Verticillium dahliae* Kleb.

*B-Actin* gene and U6 were used as internal references, and the expression differences of miR393 and target gene *Ve* of *Solanum torvum* Swartz. after infection by *Verticillium dahliae* Kleb. were analyzed by qPCR (Figure 5; Figure 6). The results showed that after infection by *Verticillium dahliae* Kleb., both miR393 and target gene *Ve* had certain expression in different infection periods. The expression of target gene *Ve* showed an upward tendency with the extension of infection time, and the highest expression level appeared at 48 h, followed by 72 h. While the expression of miR393 showed a downward tendency with the extension of infection time, and the highest expression level appeared at 6 h. The expression of miR393 and target gene *Ve* in roots showed opposite tendencies in different infection periods.

![Figure 5](image-url)  
**Figure 5** Expression patterns of *Ve* gene in different periods of *Solanum torvum* Swartz. after infection by *Verticillium dahliae* Kleb.

![Figure 6](image-url)  
**Figure 6** Expression patterns of miR393 in different periods of *Solanum torvum* Swartz. after infection by *Verticillium dahliae* Kleb.

3 Discussion

At present, new miRNAs and their target genes in plants have been found and identified by a variety of technical means. While the way miRNA regulates target genes, interactions between genes, and interactions between genes and proteins are also the main research directions (Ambros, 2008). People are trying to study multiple levels of regulation mechanism in plants, and have an overall understanding of the expression and regulation of miRNAs and the target genes in plants. Multiple studies have shown that plant miRNAs play a key role in the growth and development of plants and the process of stress resistance, mainly by mediating the cleavage of target genes or inhibiting the translation of target genes (Jones-Rhoades and Bartel, 2004; Mallory and Vaucheret, 2004; Axtell et al., 2007; Brodersen et al., 2008; Zhang et al., 2008).
In order to study the expression differences of miR393 and its target gene \textit{Ve} in different tissues of \textit{Solanum torvum} Swartz., the quantitative expression analysis of miR393 and target gene \textit{Ve} in different tissues of \textit{Solanum torvum} Swartz. was carried out in this study. The results showed that both miR393 and target gene \textit{Ve} had certain expression in different tissues of \textit{Solanum torvum} Swartz., and the expression levels were different. Both miR393 and target gene \textit{Ve} had the highest expression levels in roots and had the lowest levels in fruits, indicating that miR393 and target gene \textit{Ve} had specific expression patterns in different tissues, and the differential expression was significant. Besides, \textit{Verticillium dahliae} Kleb. mostly infects plants from roots, which may be the reason why both miR393 and target gene \textit{Ve} had the highest expression levels in roots. Therefore, in the expression tests of miR393 and target gene \textit{Ve} of \textit{Solanum torvum} Swartz. after infection by \textit{Verticillium dahliae} Kleb., the roots of \textit{Solanum torvum} Swartz. were selected for subsequent tests.

Through the expression analysis of miR393 and target gene \textit{Ve} of \textit{Solanum torvum} Swartz. after infection by \textit{Verticillium dahliae} Kleb., we found that the expression of target gene \textit{Ve} showed an upward tendency and the highest expression level appeared at 48 h, while the expression of miR393 showed a downward tendency and the highest expression level appeared at 6 h. The expression of miR393 and target gene \textit{Ve} showed opposite tendencies, which was consistent with the reports of miRNA regulating target genes in other plant studies. In the study of miRNA related to rice drought resistance, Kong (2010) analyzed three predicted target genes in osa-miR408 overexpressing rice by RT-PCR, and found that the relative expression of target genes was lower than the negative control in most strains, and the overexpressing osa-miR408 negatively regulated its target genes. Wang (2012) found in grape research that the expression levels of VV miRNAs and their target genes showed opposite tendencies in eight grape tissues. Hu et al. (2014) used \textit{Populus suaveolens} seedlings treated at 0°C in different periods as test materials and found that with the extension of low temperature induction time, the expression of miR475 showed a downward tendency, while the expression of its target mRNA showed an opposite tendency.

In summary, the expression of miR393 and its target gene \textit{Ve} of \textit{Solanum torvum} Swartz. showed an opposite trend during infection by \textit{Verticillium dahliae} Kleb.. The expression of target gene \textit{Ve} showed an upward tendency, while the expression of miR393 showed a downward trend. It was speculated that miR393 of \textit{Solanum torvum} Swartz. can improve disease resistance by regulating the expression of target gene \textit{Ve} during infection by \textit{Verticillium dahliae} Kleb.. The results will help us to fully understand the function and regulation mechanism of miR393 and its target gene \textit{Ve}.

Authors’ contributions
QYL and WZ designed and carried out this study. QYL, JZH and SXC completed the statistical analysis and drafted the manuscript. ZWB participated in the design of the study and performed the statistical analysis. PF conceived of the study and directed its design and statistical analysis and helped to draft and modify the manuscript. All authors read and approved the final manuscript.

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