Isolation, Target Site Prediction and Validation of miR393 Prediction Target Gene Ve in Solanum torvum

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Abstract In this study, total RNA was extracted by modified Trizol method and the full-length sequence of Verticillium wilt resistance gene Ve was obtained by homologous cloning. The binding site of miR393 to Ve was predicted by rna hybrid and validated by RLM-5'-RACE. The results showed that Ve gene contains 3302 nucleotides, encodes 904 amino acids, and has 85.63% homology with tomato, potato, tobacco, etc. The predicted results of binding sites showed that miR393 and Ve were almost completely paired from site 263 in Solanum torvum. With the help of RLM-5'-RACE, we found that the miR393 and target gene Ve were cut between the 10th and 11th base sites of the complementary binding sequence. Through the verification of PCR and sequencing, Ve was finally confirmed as the target gene of miR393 in Solanum torvum. It provides theoretical basis for further research on the molecular mechanism of miR393 mediated Ve gene participating in Verticillium wilt resistance.

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

Solanum torvum Swartz., also known as spiny tomato, belladonna, golden button and spiny solanum, is a wild relative species of Solanum in Solanaceae. Solanum torvum has become the preferred material for researchers to explore the resistance genes of Solanum because of its strong resistance to Verticillium wilt, wilt disease, bacterial wilt, root knot nematode and other diseases and insect pests (Adeniji et al., 2012; Kumchai et al., 2013).

Ve is the most typical Verticillium wilt resistance gene. As early as 1994, Kawchuk et al. proposed that the cloning of Ve gene and its gene family could provide help for improving other susceptible crop varieties. At present, Ve or Ve homologous genes have been cloned from tomato, cotton, potato, wild eggplant and other plants, among which Solanaceae plants are the majority (Kawchuk et al., 2001; de Jonge et al., 2012). Liu et al. (2014) isolated Ve homologous gene from a Verticillium wilt resistant wild eggplant, and it was used to construct a recombinant vector TRV2-S1Ve. Then the seedlings were inoculated by Agrobacterium, and the results showed that the TRV2-S1Ve inhibited the expression of Ve gene, and the infected plants expressed symptoms of Verticillium wilt.

In the functional research of miRNA, it is necessary to study and analyze its target genes at the same time. In plants, miRNA regulate their target genes mainly by cutting mRNA. The target gene and cleavage site of miRNA are mainly predicted by prediction software, and then the cleavage site is verified with RLM-5'-RACE, transgene, qPCR combined with Western blot, luciferase reporter gene, etc. Several research teams validated miRNA target genes using RLM-5'-RACE and determined that shear action usually occurred at the 10th or 11th base of miRNA (Zeng et al., 2009; Liu et al., 2010).

In our previous study, the miRNA library before and after Verticillium wilt infection with Solanum torvum was constructed. A total of 70 miRNA sequences were obtained, including 16 new miRNA sequences, among which the expression level of miR393 was significantly different before and after Verticillium wilt infection. In order to study whether the Verticillium wilt resistance gene Ve is the target gene of miR393 and the possible target site of its action, we first extracted high-quality Solanum torvum RNA for the isolation and analysis of Ve genes in this study. Then, the binding site of miR393 and its target gene Ve was predicted by software, and RLM-5'-RACE was
used to verify whether miR393 cut its target gene Ve and its cutting site in *Solanum torvum*.

1 Results and Analysis

1.1 Total RNA integrity detection analysis

The modified Trizol method was used to extract the total RNA of *Solanum torvum* by non-denaturing gel electrophoresis on 1.0% (Figure 1), and the bright 28S, 18S and dark 5S bands were obtained. And 28S band is almost twice as bright as 18S, there is no obvious dispersion or DNA contamination, which indicated that the RNA extracted has good integrity and less degradation.

![Electrophoresis profile of total RNA isolated by modified Trizol method from Solanum torvum Swartz](image)

**Figure 1** Electrophoresis profile of total RNA isolated by modified Trizol method from *Solanum torvum* Swartz.

Note: M: 2000 DNA marke; 1-3: RNA of *Solanum torvum* Swartz

1.2 Total RNA purity and concentration analysis

Total RNA samples were detected by One Drop OD-2000+ ultramicro spectrophotometer, and the concentration of total RNA samples, OD$_{260nm}$/OD$_{280nm}$ and OD$_{260nm}$/OD$_{230nm}$ were obtained. We found that the total RNA extracted by modified Trizol method was very good in purity with high concentration, and it was less affected by protein and polysaccharides. It was consistent with gel electrophoresis and RNA quality was better (Table 1).

<table>
<thead>
<tr>
<th>Sample</th>
<th>OD$<em>{260nm}$/OD$</em>{280nm}$</th>
<th>OD$<em>{260nm}$/OD$</em>{230nm}$</th>
<th>RNA concentration (ng/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA1</td>
<td>2.02</td>
<td>2.26</td>
<td>1006.93</td>
</tr>
<tr>
<td>RNA2</td>
<td>1.87</td>
<td>2.40</td>
<td>741.35</td>
</tr>
<tr>
<td>RNA3</td>
<td>2.04</td>
<td>2.02</td>
<td>960.4</td>
</tr>
</tbody>
</table>

**Table 1** Comparison of RNA purity and concentration isolated by modified Trizol method from *Solanum torvum* Swartz

1.3 RT-PCR test detection

The gel electrophoresis of PCR products showed that the size of the bands was as expected (about 3 250 bp) (Figure 2). The PCR product was recovered with glue and transferred to *E. coli*. The PCR results were consistent with the expected results (Figure 3).

![Electrophoresis profile of RT-PCR amplification of Ve gene from Solanum torvum Swartz.](image)

**Figure 2** Electrophoresis profile of RT-PCR amplification of Ve gene from *Solanum torvum* Swartz.

Note: M: 2000 DNA marke; 1: Non-specific amplification of Ve gene; 2-7: Specific amplification of Ve gene
1.4 Homology analysis of amino acids encoded by *Solanum torvum* *Ve* gene

ORF finder was used to find the open reading frame of the sequence, and 904 amino acids encoded by *Ve* gene were obtained (Figure 4). Compared with the published *Ve* amino acid sequence, it was proved that the cloned gene was *Solanum torvum* *Ve* gene (Figure 5).

![Figure 3](image-url)  
Figure 3 Electrophoresis profile of bacterial PCR of *Ve* gene from *Solanum torvum* Swartz.

Note: M: 2000 DNA marke; 1-4: Electrophoresis profile of bacterial PCR of *Ve* gene

![Figure 4](image-url)  
Figure 4 The full-length cDNA and corresponding amino acid sequences of *Ve* gene

Note: *: Stop condon
Figure 5 Homologous alignment analysis of amino acid alignment of Ve gene from Solanum torvum Swartz

1.5 Phylogenetic analysis of the gene encoding protein of Ve gene from Solanum torvum

The amino acid sequence of Ve was analyzed by ExPASY software (http://web.expasy.org/protparam/). The results showed that the coding protein contained 904 amino acid residues, with a relative molecular weight of 100597.8 and pI of 5.86. In order to further analyze the relationship between Ve gene encoding proteins isolated from Solanum torvum and Ve encoding proteins of other species of plants, the phylogenetic tree was constructed (Figure 6). The results showed that the gene coding protein of Ve gene from Solanum torvum is closely related to ALK26502.1, which is in the same evolutionary branch.

Figure 6 Phylogenetic analysis of Ve encoding protein between Solanum torvum Swartz. and other plants
1.6 MiR393 and target site prediction of target gene Ve

In previous studies, the expression level of miR393 was significantly different before and after Verticillium wilt infection. The results of high-throughput sequencing showed that the pathogen treatment significantly inhibited the expression of miR393 (Figure 7). The binding site of miR393 to the predicted target gene Ve was obtained by using rna hybrid online prediction software. The results showed that miR393 and Ve in Solanum torvum were completely paired from site 263 (Figure 8), and the absolute value of mfe reached 31.7 kcal/mol.

![Figure 7 The expression of miR393 after V. dahliae inoculation in Solanum torvum Swartz](image1)

![Figure 8 The cleavate site prognosis of miR393 and the target gene Ve](image2)

1.7 Verification of miR393 and Ve cleavage site of target gene by RLM-5′-RACE

Through RLM-5′-RACE, miR393 and Ve were verified cleaved between the 10th and 11th base sites of the complementary binding sequence (Figure 9), which is consistent with the reports of Llave et al. (2002b) and Sunkar (2005). Through PCR verification, the fragment size obtained by gel electrophoresis was consistent with the target fragment size (Figure 10). The sequence obtained by cloning was recovered and sequenced (Figure 11), and Ve was finally confirmed as the target gene of miR393 in Solanum torvum, which was consistent with the predicted results.

![Figure 9 Identification of cleavate site prognosis of miR393 and the target gene Ve](image3)

![Figure 10 Gel electrophoresis analysis of RLM-5′-RACE product](image4)
2 Discussion

With the development of molecular plant breeding technology, people try to isolate disease-resistant genes of plant target and cultivate disease-resistant plants by means of genetic engineering. Fei et al. (2004) cloned the StVe gene with a total length of 3 640 bp from Solanum torvum by RACE technology. Bioinformatics analysis showed that StVe was highly homologous with Ve1 and Ve2 at nucleic acid level, encoding a leucine rich LRRs type transmembrane cell surface receptor protein, suggesting that StVe may have the function of anti-Verticillium wilt. Yin (2005) successfully cloned StoVel, which is homologous to Verticillium wilt resistant genes Ve1 in tomato, from Solanum torvum in the process of studying the disease resistance gene, which is of great significance for the research on the mechanism of plant resistance to Verticillium wilt and the cultivation of excellent germplasm with resistance to Verticillium wilt by genetic engineering. Vining and Davis (2009) first studied the homologous gene of Ve in Non-Solanum plant Mentha haplocalyx. In this study, anti-Verticillium wilt gene Ve was successfully cloned from the anti-Verticillium wilt Solanum torvum. Through gel electrophoresis, transformation and sequencing, it was shown to contain 3 302 nucleotides and encode 904 amino acids. Through homology comparison with tomato, potato and tobacco, it was found that its homology was as high as 85.63%. This result provided a theoretical basis for further study of miR393 and its target gene.

At present, the prediction of miRNA target genes in plants is mainly carried out through software. Rhoades et al. (2002) first proposed the prediction of miRNA target genes in Arabidopsis thaliana. With the continuous improvement of prediction software, software prediction target genes are widely used in plant miRNA target gene prediction (Allen et al., 2005). General sequence alignment software such as BLAST can also be used for target gene prediction, but the prediction results may not be reliable due to the lack of adherence to plant site-specific scoring rules.

MiRNA in plants are mostly verified by the combination of software target gene prediction and RLM-5’-RACE. Due to the high degree of complementary pairing between miRNA and target gene shear sites in plants, or even complete complementary pairing, the success rate of target gene prediction by informatics method is high, and the prediction is verified by RLM-5’-RACE. Therefore, this method has become a widely used method to identify miRNA target genes in plants. In this study, RLM-5’-RACE was used to detect the cutting of miR393 to predict target gene Ve. PCR product gel electrophoresis showed that the size of the fragment was consistent with the size of the target fragment. The results showed that miR393 and Ve were cleaved between the 10th and 11th base sites of complementary binding sequence, which provided theoretical basis for further study on the molecular mechanism of Ve gene involved in Verticillium wilt resistance mediated by miR393.

3 Materials and Methods

3.1 Experimental materials

The experimental Solanum torvum tissue materials were taken from the experimental site of Institute of Botany, Jiangsu Province and Chinese Academy of Sciences. It was quick-frozen in liquid nitrogen and stored in a -80°C refrigerator in the laboratory.
3.2 Experimental reagent
Trizol reagent was purchased from Vazyme Biotech Co., Ltd. CTAB, EDTA, β-Mercaptoethanol, and chloroform were purchased from Nanjing Boquan Technology Co., Ltd. dNTPs, 10 × PCR buffer, Taq enzyme and pMD19-T Vector were purchased from TaKaRa Bio Co., Ltd. GeneRacer Kit (Invitrogen) was purchased from Thermo Fisher Scientific China. All reagent preparation was treated overnight with 0.1% DEPC water. Mortar, centrifuge tube and tips were treated with 0.1% DEPC water overnight, autoclaved at 121°C for 30 min, and dried in an oven at 80°C for standby.

3.3 Isolation of target gene Ve predicted by miR93
3.3.1 Total RNA extraction and first strand cDNA synthesis of Solanum torvum
Referring to the total RNA extraction method of Solanum torvum, the modified Trizol method was used to extract total RNA from Solanum torvum tissue, and the extracted RNA was reverse-transcribed to synthesize the first strand cDNA (Qin et al., 2016).

3.3.2 RT-PCR detection
Primers are designed according to the Ve sequence of anti-Verticillium wilt gene registered in NCBI GeneBank, and the primer sequence is as follows: Ve_S: 5’-ACAAGTTGCATGAAAATGT-3’, Ve_A: 5’-GCTTATCTAATAAAGAGCAGT-3’.
The PCR reaction system is as follows: primer 1.5 μL each, 10×PCR buffer (Mg2+ plus) 2.5 μL, 2.5 mM dNTPs 2.0 μL, cDNA 0.5 μL, Taq enzyme 0.25 μL, ddH2O was used to add to 25 μL.
The PCR reaction procedure is as follows: 95°C for 5 min. 35 cycles were performed at 95°C for 30 s, 52°C for 40 s, and 72°C for 3 min. 72°C for 10 min. And 4°C preservation.
After the reaction, 7 μL PCR amplification products were added with 1.0 μL 10×loading Buffer and detected by electrophoresis on 1% agarose gel. Then photographed under the gel imaging analysis system.

3.3.3 Glue cutting recovery
The amplified DNA fragment was digested, purified and recovered, then linked to pMD19-T vector, and transferred to E.coli. The clones were selected for PCR detection, and the positive recombinants were sent to Shanghai Majorbio Co., Ltd. for sequencing.
Connecting T carrier: Solution I was 5.0 μL, pMD™19-T Vector™ was 0.5 μL, purified product was 4.5 μL, with a total of 10.0 μL. After mixing, the mixture was centrifuged and connected overnight at 16°C.
E. coli transform: 1. Took 100 μL of competent cells and melt them on an ice box. 2. Added 10 μL of the product, mix well, and placed on an ice bath for 30 min. 3. The centrifuge tube was placed in a water bath at 42°C for 70 s, and then quickly returned to the box for 2 min. 4. 1 mL LB medium was added, then the centrifuge tube was transferred to a shaking table at 37°C and incubated at 120 rpm for 1 h to resuscitate the bacteria and express the antibiotic resistance gene encoded by the plasmid. 5. Centrifuged the tube at 7000 rpm for 3 min, discard the supernatant of about 950 μL, and mixed the remaining supernatant with the bacteria. 6. The bacterial suspension was coated on LB ampicillin plate (100 mL LB containing 100 μL Amp) and incubated upside down in 37°C overnight.
Screening and sequencing of recombinant: 1. Single colonies were randomly selected from plate medium and inoculated in LB liquid medium (100 mL LB containing 100 μL Amp) for more than 6 h with 200 rpm at 37°C. Ve gene primers were used for bacterial liquid PCR verification to detect the inserted fragment size. 2. According to the PCR results, the positive clones were sent to the company for sequencing.

3.3.4 Analysis of amino acid homology of Ve gene in Solanum torvum
BLASTp program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of NCBI website was selected for sequence comparison analysis. DNAman was used to perform multiple comparisons of the amino acid sequences encoded
by the Ve genes of LaTMT and other plants homologous to LaTMT.

3.3.5 Phylogenetic analysis of Ve gene coding proteins in Solanum torvum
ExPaSy (http://web.expasy.org/protparam/) was used to calculate the protein isoelectric point, molecular weight, and hydrophobicity. DNAman was used for phylogenetic tree analysis of Ve gene encoding proteins and Ve gene encoding proteins of other plants with homologous genes.

3.4 Prediction and validation of Ve target site by miR93
3.4.1 cDNA synthesis
The connection of RNA Oligo: 1. 7 μL concentrated RNA samples were added into a 0.25 μg RNA Oligo tube. The pipetors was used to repeatedly blown and mixed. The liquid was centrifuged to collect at the bottom of the tube. 2. The samples were incubated at 65 °C for 5 min (evaporation of about 1 μL), then placed in ice bath for 2 min immediately and centrifuged. 3. Mixing solution: added Ligase Buffer(10×)1 μL, ATP(10 mM)1 μL, RNaseOut®(40U/μL) 1 μL, T4 RNA ligase(5U/μL) 1 μL to RNA samples, with a total of 10 μL. Incubated at 37 °C for 1 h, centrifuged and placed on ice.

RNA suspension: 1. 90 μL RNase-free H2O and 100 μL phenol/chloroform/isoamyl alcohol (25:24:1) were added, and the vortex oscillation lasted 30 s. 2. At room temperature, centrifuged at 14 000 rpm for 5 min, and then added into a 1.5 mL new centrifuge tube (about 100 μL). 3. Added the following ingredients to mix: Mussel glycogen (10 mg/mL) 2 μL, Sodium acetate (pH5.2, 3 M)10 μL, 95% ethanol 220 μL, with a total of 332 μL. 4. After rapid vortex, the samples were placed on dry ice for 10 min, centrifuged at 4 °C at 14 000 rpm for 20 min, and then centrifuged. 5. Washed with 500 μL 75% ethanol, dried and dissolved with 10 μL DEPC water.

Reverse transcription: 1. The following components were added to the connection products obtained in the previous step: Oligo dT Primer 1 μL, dNTP Mix 1 μL, RNase-Free H2O 1 μL, with a total of 13 μL. Incubated at 65 °C for 5 min, then cooled on ice immediately for 2 min, centrifuged. 2. Added the following components: First Strand Buffer (5×) 4 μL, DTT(0.1M) 1 μL, RNaseOut® (40U/μL) 1 μL, Superscript III RT(200U/μL) 1 μL, with a total of 20 μL, mixed and centrifuged. 3. Incubated at 50°C for 60 min, 70°C for 15 min, iced for 2 min, and centrifuged. 4. 1 μL RNase H(2U) was added and incubated at 37°C for 20 min. The samples were then stored at -20°C.

3.4.2 Primer design
The primer used for miR393 to detect the cutting sites of target gene Ve was as follows: 5′-AAGTACACAGCT CAAGACGGTGAA-3′, performed PCR reaction.

The reaction system was: Ex Taq Buffer(10×) 2.00 μL, Hot start EX Taq polymerase(5U/μL) 0.15 μL, dNTP Mix(10 mM) 1.60 μL, miR393 primer (10 μM) 1.20 μL, GSP(10 μM) 0.40 μL, cDNA (10 x diluted product) 2.00 μL, and RNase-Free H2O, with a total of 20.00 μL.

touchdown PCR reaction procedure: 94 °C for 30 s, 72 °C for 30 s, with 5 cycles. 94 °C for 30 s, 70 °C for 30 s, 72 °C for 30 s, with 5 cycles. 94 °C for 30 s, 68 °C for 30 s, 72 °C for 30 s, with 25 cycles. 94 °C for 30 s, 66 °C for 30 s, 72 °C for 30 s, with 5 cycles.

3.4.3 Cloning, transformation, sequencing of PCR products
Connected the PCR products to the pEASY-T3 clone vector, then carried out Trans-T1 receptor cell transformation, selected positive clones for bacterial liquid culture, and then sequenced to Invitrogen. The details are as follows:

Connection reaction system: PCR products 0.5-4.0 μL, pEASY-T3 Cloning Vector 1.0 μL, added ddH2O, with a total of 5 μL. Gently mixed and reacted at 25°C for 10 min. After the reaction, the centrifuge tube was placed on the ice.

Transformation and sequencing: Took 50 μL Trans-T1 competent cells, melt them in the ice bath on the ice box, added in the connection products, mixed them gently, and ice bath for 30 min. Heat shock 30 s, then immediately placed on the ice for 2 min. 1 mL LB liquid medium was added and incubated at 150 rpm at 37°C for 1 h. After
centrifugation at 4000 rpm for 2 min, 600 μL supernatant was removed, mixed and coated with LB Ampicillin plate, and cultured overnight at 37°C. Monoclones were selected and cultured in 1 mL liquid LB medium supplemented with ampicillin for 200 rpm for 3 h. The samples that meet the expected fragment size after PCR verification will be sent to Invitrogen for sequencing.

Authors’ contributions
QYL and WZ designed and carried out this study. QYL and SXC completed the statistical analysis and drafted the manuscript. ZWB and ZFJ 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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