



Article

Protein Extract of Tobacco Expressing *Solanum torvum* PP5-Encoding Gene Inhibits *Verticillium dahliae* Proliferation

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Abstract: Verticillium wilt, a soilborne disease caused by *Verticillium dahliae* (*V. dahliae*), can severely affect the yields of Solanaceae crops. In a previous study, it was observed in *Solanum torvum* (*S. torvum*) that protein phosphatase 5 (PP5) was induced by *V. dahliae* infection. To elucidate the function of PP5 more clearly, this study cloned an *StPP5* cDNA from *S. torvum* by PCR. The cDNA contained an ORF of 1458 bp long encoding a putative protein of 485 amino acid residues with a predicted molecular mass of 54.63 kDa and a theoretical isoelectric point of 5.66. *StPP5* protein contained a conserved PP domain and showed high similarity to other homologous members of the PP5 family from various plant species. The expression of *StPP5* gene was upregulated after *V.* infection and reached its maximum value at 24 h in leaves. In order to clarify the role of *StPP5*, four transgenic tobacco plants expressing *StPP5* were generated through *Agrobacterium*-mediated transformation and identified by PCR. In vitro culture assay showed that the growth of *V. dahliae* in PDA medium containing proteins extracted from the leaves of transgenic tobacco line P6 was inhibited, whose inhibition rate was 55.1%, higher than the non-transgenic control. These results indicated that *StPP5* might be involved in plant defense against *V. dahliae* infection.

Keywords: *Solanum torvum*; *StPP5*; gene cloning; function analysis



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1. Introduction

Eggplant (*Solanum melongena* L.), also known as aubergine or brinjal, has been widely cultivated in the world for centuries and is currently an important crop species [1]. However, the yields of eggplant are severely affected by certain soilborne diseases such as Verticillium wilt (VW), which is caused by the soil-inhabiting fungus *Verticillium dahliae* Kleb (*V. dahliae*) that has a worldwide distribution [2]. Once the plants are infected with *V. dahliae*, there are no effective available fungicides currently [3]. The effective methods are introducing genes from resistant germplasm into the eggplant cultivars to develop tolerant plants by breeding [4]. Therefore, transgenesis is utilized to achieve plants resistant to *V. dahliae*, but the effects have been varied [5,6]. Presently, some genes related to resistance to VW have been isolated in plants, but only a few genes have been analyzed in function. *Ve1* and *Ve2* are two resistance genes first isolated from tomato (*Solanum lycopersicum* L.) [7–9], but only *Ve1* was valuable for application in breeding according to its resistance [8]. *StoVe1*, the homolog of *Ve1*, is a gene with higher resistance, which was cloned from *Solanum torvum* (*Solanaceae*), and the expression of the gene in potato enhanced resistance to *V. dahliae* [2,10]. In recent years, cotton transformed respectively with *hpa1_{Xoo}* or *GbVe* genes conferred an improved resistance to *V. dahliae* [11,12].

Earlier studies discovered that alteration of the serine/threonine dephosphorylation state has significant effects on plant growth, which is induced by different types of mitogens [13]. Consequently, researchers cloned the gene encoding protein phosphatase from many plants such as Arabidopsis and Alfalfa, and certain proteins were purified [14,15]. In comparison with protein kinases, there are fewer studies about protein phosphatases of

plants, and most of the cloned protein phosphatase genes have not been studied extensively. Protein phosphatase 5 (PP5), a member of the serine/threonine-specific protein phosphatase family, is widely distributed and highly conserved among eukaryotes. Subcellular localization showed that PP5 is expressed in both the nucleus and the cytoplasm [16].

Wild eggplant has a rich genetic resource of genes with high resistance to VW [17]. In the previous study, we established a *V. dahliae*-induced *S. torvum* cDNA library, including 118 related defense transcript-derived fragments (TDFs) [18]. The gene *StPP5* was selected from this cDNA library. Here, we cloned and analyzed the *StPP5* sequence from *S. torvum* and the inhibitory effect of the protein extract of tobacco, including StPP5 to *V. dahliae* proliferation, which indicates that StPP5 might be involved in plant defense against *V. dahliae* infection.

2. Materials and Methods

2.1. Plant Materials

S. torvum was grown in a greenhouse without temperature and light control. Young leaves and stems were collected from well-developed plants, frozen in liquid nitrogen immediately, and stored at -70°C for gene cloning and expression analysis.

The seedlings of tobacco (*Nicotiana tabacum* L. cv. NC89) were produced by germinating sterilized seeds on Murashige and Skoog (MS) medium under conditions previously reported [19].

2.2. Isolation of Full-Length cDNA of *StPP5*

Total RNA was extracted from 0.1 g of fresh leaves with total RNA isolation reagent following the manufacturer's instructions. First-strand cDNA was synthesized with M-MLV-reverse transcriptase from TaKaRa Biotech Company (Dalian, China) according to the manufacturer's instructions.

To clone the conserved region of *StPP5* cDNA, a pair of primers, P1 (5'-AAAGATG CACTCAAGGATTTTCAACAGGT-3') and P2 (5'-GTTTACTAGGTCCTCTACCAGGCTGA GG-3') (Figure 1A), were designed according to the conserved region of PP5 gene from other plants using the DNAssist 2.0 software. Polymerase chain reaction (PCR) was carried out according to the following program: firstly, incubation at 94°C for 4 min, and then followed by 35 cycles (94°C for 40 s, 60°C for 40 s, 72°C for 1 min), with a final extension step at 72°C for 10 min. The PCR product was about 800 bp long.

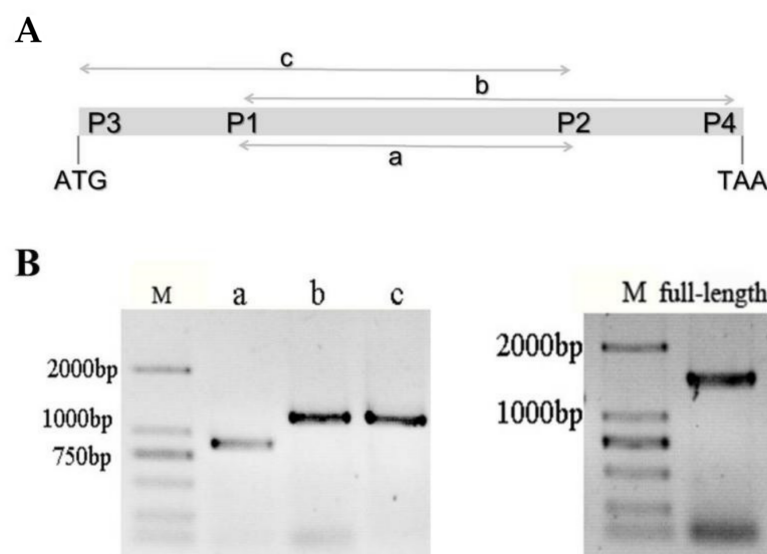


Figure 1. The schematic diagram (A) and agarose gel electrophoresis (B) of PCR amplification products of *StPP5*: P1,P2,P3,P4, the primers used for *StPP5* cloning; M, DL 2000 marker; a, middle fragment; b, 3' end fragment; c, 5' end fragment; full-length, full-length cDNA of *StPP5*.

To obtain 5' end sequence, a primer P3 (5'-ATGCCCCGGTATGGAAGCTGAG-3') was designed according to 5' end sequence of *PP5* (accession no. AY182777) (Figure 1A). The PCR was carried out using P3 and P2 under the following PCR condition: firstly, incubation at 94 °C for 4 min, then followed by 35 cycles (94 °C for 30 s, 62 °C for 50 s, 72 °C for 40 s) and by extension at 72 °C for 10 min.

To amplify the 3' end of *StPP5*, a primer P4 (5'-TTAAGAAAACATGCGAAGGAAG-TTGTTGG-3') was designed according to 3' end sequence of *PP5* (accession no. AY182777) (Figure 1A). The PCR was carried out using P1 and P4 under the following PCR condition: 94 °C for 4 min, followed by 35 cycles (94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s) and by extension at 72 °C for 10 min.

The full length of the gene cDNA was amplified with gene-specific primers P3 and P4 after assembling the full-length sequence of *StPP5* (Figure 1A). PCR conditions were: 94 °C for 5 min, followed by 30 cycles (94 °C for 50 s, 60 °C for 40 s, 72 °C for 40 s) and with a final extension step at 72 °C for 10 min.

The volume of PCR amplification reactions was 25 µL, containing 10 × PCR Buffer, 2.5 µL; 10 mmol/L dNTP, 1 µL; forward and reverse primer, 1 µL; cDNA template, 0.5 µL; 5 U/µL Taq polymerase, 0.25 µL; double-distilled water, 16.75 µL.

PCR products were separated on 1% agarose gels and recovered by gel extraction. Then, the target products were introduced into pMD18-T vector from TaKaRa Biotech, and finally transformed into competent cells of *Escherichia coli* strain DH5α. The positive colonies were identified by PCR and sequenced.

2.3. Bioinformatics Analysis

The protein similarity of *StPP5* was analyzed using DNAssist program. GenBank ORF finder (<http://www.ncbi.nih.gov/gorf/gorf.html>, accessed on 15 May 2019) was used for prediction of the open reading frame (ORF) of the sequence; phylogenetic analysis was performed using Mega 5.0. ProtScale (<http://us.expasy.org/cgi-bin/protscale.pl>, accessed on 27 January 2022) of ExPASy was used for prediction of protein hydrophobicity, and transmembrane domain was analyzed by TMHMM-2.0 (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>, accessed on 27 January 2022).

2.4. Expression Analysis of *StPP5* Gene

2.4.1. RT-PCR Analysis of *StPP5* Expression

To study the expression of *StPP5* gene in *S. torvum* under the stress of *V. dahliae* infection, *S. torvum* plants were treated with *V. dahliae* under greenhouse conditions. For *V. dahliae* infection test, we collected spore suspension and a fungus slice and then mixed them. After mixing, we filtered out the debris and infected the plant material with the filtrate. The stems and leaves of plants treated were harvested at 0 h, 12 h, 24 h, 48 h, and 72 h after treatment. The materials were frozen immediately using liquid nitrogen and stored at −70 °C.

Total RNA was extracted, and cDNA was synthesized using the same method as described above. RT-PCR was performed using specific primers: *PP5*-F (forward primer, 5'-TCAAATAGTACTGCAAACAAGA-3') and *PP5*-R (reverse primer, 5'-ATG CACTTGAAGG CAAATAATG-3'). The *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) gene was used as an internal control with specific primers (forward primer, CAAGGACTGGAGAG-GTGG; reverse primer, TTCACTCGTT GTCGTACC). The reaction system consisted of a 5-min denaturation at 94 °C, followed by 26 cycles of 40 s at 94 °C, 40 s at 53 °C, and 40 s at 72 °C.

2.4.2. RT-qPCR Analysis of *StPP5* Expression

The program included 94 °C for 1 min, 40 cycles at 95 °C for 15 s, and 54 °C for 20 s according to the reaction condition: 10 µL 2 × SYBR premix Ex Taq™ (TaKaRa Biotech), 0.5 µL forward primer (10 µM), 0.5 µL reverse primer (10 µM), 2 µL cDNA, and 7 µL nuclease-free water.

2.5. Construction of Overexpression Vector

The target gene cDNA fragment was added with *Nco*I and *Spe*I restriction sites at its ends. Then, it was connected with vector pCAMBIA1304, double-digested, and the connected product was transformed into *E. coli* DH5 α .

2.6. Genetic Transformation and Identification of Transgenic Plants

The *Agrobacterium*-mediated method was used for tobacco (*N. tabacum* ‘NC89’) transformation. Fully expanded leaves from sterile plants were cut into 0.5 cm² segments in Petri dishes. *A. tumefaciens* strain LBA4404 carrying recombinant plasmid was grown at 28 °C to OD₆₀₀ = 0.5–0.7 in LB medium with kanamycin (50 mg/L). The leaf segments were immersed in bacterial suspension of LBA4404 for 10 min. The leaf segments were blotted dry on aseptic filter paper and transferred to a solidified Murashige and Skoog medium (MS) for 2 days of cocultivation (MS + 1.0 mg/L 6-benzylaminopurine (6-BA) + 0.1 mg/L α -naphthalene acetic acid (NAA)). After that, the leaf segments were transferred to the medium (MS + 2 mg/L 6-BA + 0.5 mg/L NAA + 250 mg/L Cefotaxime (Cef) + 20 mg/L Hygromycin B (Hyg B)). When shoots appeared in callus parts, the explants were placed on shoot differentiation medium (MS + 3 mg/L 6-BA + 0.2 mg/L NAA + 250 mg/L Cef + 20 mg/L Hyg B). Approximately 1 month later, shoots (1 cm long) were chosen and rooted on MS medium supplemented with 2 mg/L NAA, 250 mg/L Cef, and 25 mg/L Hyg B. Surviving plantlets were propagated on MS medium.

After the screening, the resistant ones were then PCR-identified. The PCR positive transformants were grown in tissue culture room for rooting at 25 \pm 1 °C with 16 h light/8 h dark photoperiods.

2.7. Antifungal Assay

One gram of tobacco leaf was ground in phosphate buffer solution (PBS). After centrifugation at 12,000 \times g for 15 min, the supernatant liquid-containing proteins were collected as crude protein solution. Then, the concentrations of the crude protein were measured by the Coomassie brilliant blue method, and 36 μ g crude protein of each sample was used for the following antifungal assay in vitro.

Cake with the fungus method [5,20] was applied for analyzing the inhibitory activity of transgenic tobacco protein extracts to *V. dahliae* in the in vitro antifungal assay. During the experiment, *V. dahliae* was cultured at 25 °C for 7–10 days. The experiment consisted of three treatments, blank control (water), wild-type tobacco, and transgenic plants, with three biological repetitions, respectively. Inhibition zone diameter was measured, and inhibitory rate was calculated using the following formula: Inhibitory rate = (control colony diameter (mm) – treatment colony diameter (mm))/control colony diameter (mm) \times 100%. Difference in the significance between treatments was determined by *t*-test.

3. Results

3.1. Isolation of Full-Length cDNA of StPP5 Gene

A full-length cDNA of StPP5 was isolated from *S. torvum* by PCR with a pair of specific primers designed according to the assembled sequence of three fragment sequences of the gene. The cDNA fragment was about 1500 bp long (Figure 1).

DNAssist analysis showed that the *StPP5* gene contained an open reading frame of 1458 bp long and encoded a protein of 485 amino acids (Figure 2). The cDNA sequence was submitted to the NCBI; the accession number is JF421126. Alignment analysis showed that StPP5 shared with *Solanum lycopersicum*, *Zea. mays*, *Arabidopsis thaliana*, and *Oryza sativa Japonica Group* had a higher homology of 84%, 82%, 81%, and 80%, respectively (Figure 2).

| | | | | | | | | | | |
|---------|-----|---------|-------------|-----------|---------------|-----------|---------|---------|--------|-----|
| StPP5 | 1 | MPGMEAE | NSNTSRAGEL | KQLANEAFK | MYSCAIDLYTQ | ALIELNGEN | NFVYYAN | RAFAHTK | LEEYVG | 65 |
| LePP5 | 1 | MPGMEAE | NSNARSAREEL | KQLANEAFK | GHKYSQAIDLYTQ | ALIELNGEN | NFVYYAN | RAFAHTK | LEEYVG | 65 |
| AtPP5 | 1 | METKNE | NSDVSRAREEF | KSQANEAFK | GHKYSQAIDLYTQ | ALIELNGEN | NFVYYAN | RAFAHTK | LEEYVG | 64 |
| ZmPP5 | 1 | MDATA | NSDVQRAEEF | KLKANDAFK | GHKYSQAIDLYTQ | ALIELNGEN | NFVYYAN | RAFAHTK | LEEYVG | 63 |
| OsJGPP5 | 1 | MDANS | SLNECKSEEL | KLKANDAFK | GHKYSQAIDLYTQ | ALIELNGEN | NFVYYAN | RAFAHTK | LEEYVG | 63 |
| StPP5 | 66 | SAIQCG | TRAIEID | FRYSKGY | RRGAAYL | AMGKFK | DAIKDF | QCVRKLC | FNDP | 130 |
| LePP5 | 66 | SAIQCG | TRAIEID | FRYSKGY | RRGAAYL | AMGKFK | DAIKDF | QCVRKLC | FNDP | 130 |
| AtPP5 | 65 | SAIQCG | TRAKAIE | VDYSKGY | RRGAAYL | AMGKFK | DAIKDF | QCVRKLC | FNDP | 129 |
| ZmPP5 | 64 | SAVQ | QATKAIE | IDSRYKGY | RRGAAYL | AMGKFK | DAIKDF | QCVRKLC | FNDP | 128 |
| OsJGPP5 | 64 | SAVQ | QATKAIE | IDSRYKGY | RRGAAYL | AMGKFK | DAIKDF | QCVRKLC | FNDP | 128 |
| StPP5 | 131 | KLKEEA | ISVPSQ | RRVADS | SDIYH | SVIEVE | PQVAG | ARIEGD | VTLDE | 195 |
| LePP5 | 131 | KLKEEA | ISVPSQ | RRVADS | SDIYH | SVIEVE | PQVAG | ARIEGD | VTLDE | 195 |
| AtPP5 | 130 | KLKEEA | ISVPSQ | RRVADS | SDIYH | SVIEVE | PQVAG | ARIEGD | VTLDE | 194 |
| ZmPP5 | 129 | KIRFEE | ASAGD | DEARR | SVADS | SDIYH | SVIEVE | PQVAG | ARIEGD | 193 |
| OsJGPP5 | 129 | KIRFEE | ASAGD | DEARR | SVADS | SDIYH | SVIEVE | PQVAG | ARIEGD | 193 |
| StPP5 | 196 | AYQIVL | QTRM | LRAL | FSIVDT | VVPEG | KHIT | VC | GI | 260 |
| LePP5 | 196 | AYQIVL | QTRM | LRAL | FSIVDT | VVPEG | KHIT | VC | GI | 260 |
| AtPP5 | 195 | AYQIVL | QTRM | LRAL | FSIVDT | VVPEG | KHIT | VC | GI | 259 |
| ZmPP5 | 194 | AYQIVL | QTRM | LRAL | FSIVDT | VVPEG | KHIT | VC | GI | 258 |
| OsJGPP5 | 194 | AYQIVL | QTRM | LRAL | FSIVDT | VVPEG | KHIT | VC | GI | 258 |
| StPP5 | 261 | CRGSFS | LEVIL | TLFAF | WCMP | CSAT | HLARG | NHESK | SMNK | 325 |
| LePP5 | 261 | CRGSFS | LEVIL | TLFAF | WCMP | CSAT | HLARG | NHESK | SMNK | 325 |
| AtPP5 | 260 | CRGSFS | LEVIL | TLFAF | WCMP | CSAT | HLARG | NHESK | SMNK | 324 |
| ZmPP5 | 259 | CRGSFS | LEVIL | TLFAF | WCMP | CSAT | HLARG | NHESK | SMNK | 323 |
| OsJGPP5 | 259 | CRGSFS | LEVIL | TLFAF | WCMP | CSAT | HLARG | NHESK | SMNK | 323 |
| StPP5 | 326 | FLAFV | INR | VFV | VHGG | LF | SV | DGV | KLST | 390 |
| LePP5 | 326 | FLAFV | INR | VFV | VHGG | LF | SV | DGV | KLST | 390 |
| AtPP5 | 325 | FLAFV | INR | VFV | VHGG | LF | SV | DGV | KLST | 389 |
| ZmPP5 | 324 | FLAFV | INR | VFV | VHGG | LF | SV | DGV | KLST | 388 |
| OsJGPP5 | 324 | FLAFV | INR | VFV | VHGG | LF | SV | DGV | KLST | 388 |
| StPP5 | 391 | LSFGG | IVTK | RF | LQ | NNLD | LV | RS | HEV | 455 |
| LePP5 | 391 | LSFGG | IVTK | RF | LQ | NNLD | LV | RS | HEV | 455 |
| AtPP5 | 390 | LSFGG | IVTK | RF | LQ | NNLD | LV | RS | HEV | 454 |
| ZmPP5 | 389 | LSFGG | IVTK | RF | LQ | NNLD | LV | RS | HEV | 453 |
| OsJGPP5 | 389 | LSFGG | IVTK | RF | LQ | NNLD | LV | RS | HEV | 453 |
| StPP5 | 456 | KKPN | IVT | FS | VP | HH | IV | KPM | AYANN | 485 |
| LePP5 | 456 | KKPN | IVT | FS | VP | HH | IV | KPM | AYANN | 485 |
| AtPP5 | 455 | KKPN | IVT | FS | VP | HH | IV | KPM | AYANN | 484 |
| ZmPP5 | 454 | KKPN | IVT | FS | VP | HH | IV | KPM | AYANN | 483 |
| OsJGPP5 | 454 | KKPN | IVT | FS | VP | HH | IV | KPM | AYANN | 483 |

Figure 2. Alignment of the amino acid sequence of StPP5 with that of other homologous: LePP5 (AAN64317), AtPP5 (NP-565985), ZmPP5 (NP-001150042), and OsJGPP5 (NP-001054892).

The StPP5 protein had a TPR domain in the N terminal and a PP-type specific domain in the C terminal (Figure 3A). Furthermore, the hydrophobicity of the StPP5 protein was predicted using the ProtScale program. The result showed that the maximum value of hydrophobicity was 2.300, and the minimum value was −2.900. Hydrophobic amino acids were distributed in the entire polypeptide chain, and the result suggests that StPP5 is a hydrophobic protein (Figure 3B). In addition, the protein transmembrane domain was predicted with the TMHMM Server v.2.0., and the result indicated that StPP5 was not a transmembrane protein (Figure 3C).

3.2. Phylogenetic Analysis of StPP5

To determine the phylogenetic relationship of StPP5 with those from other species, a phylogenetic tree was constructed using the sequences of PP5 proteins from different species downloaded from GenBank. The phylogenetic tree analysis revealed that StPP5 was clustered to SIPP5 (Figure 4).

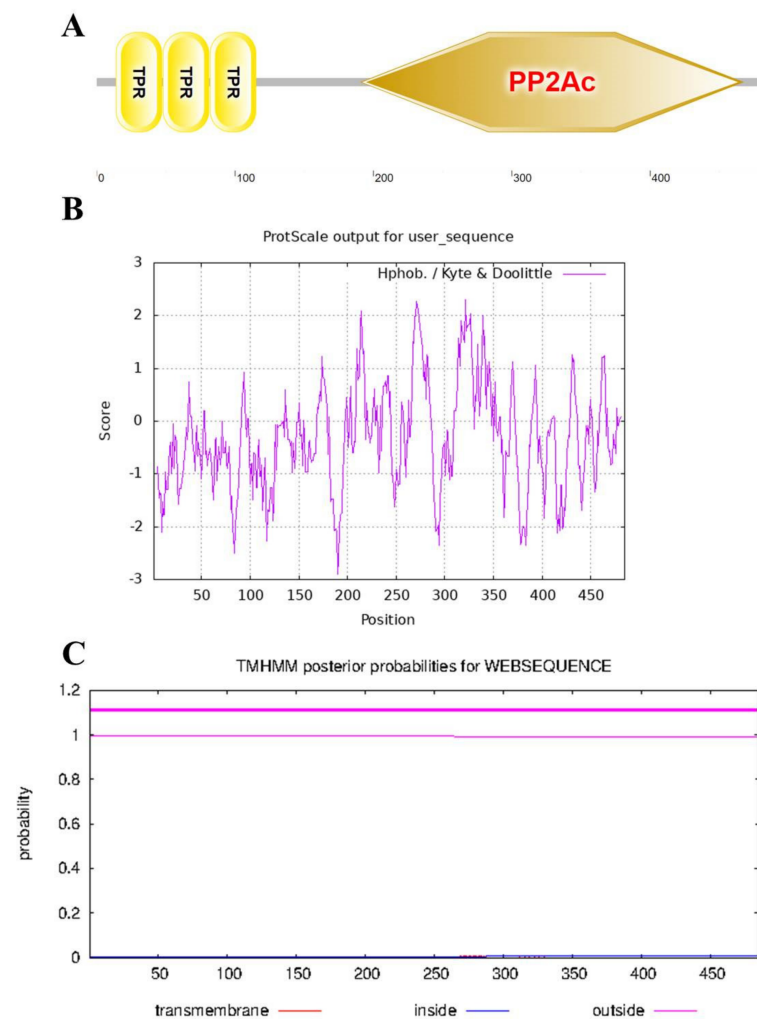


Figure 3. Structural analysis of StPP5: (A) structural domain; (B) hydrophobicity analysis of StPP5; (C) transmembrane domain prediction of StPP5.

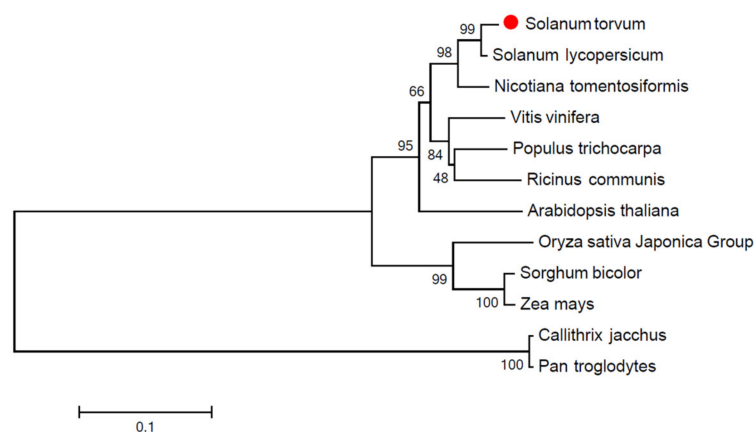


Figure 4. Phylogenetic analysis of StPP5. The Stpp5 were highlighted with the red circle. Source and accession number of PP5: *Vitis vinifera* (XP_002280111), *Zea mays* (NP_001150042), *Arabidopsis thaliana* (NP_001031534), *Callithrix jacchus* (XP_002762302), *Pan troglodytes* (XP_512768), *Ricinus communis* (XP_002520400), *Populus trichocarpa* (XP_024441018), *Sorghum bicolor* (XP_002439438), *Oryza sativa Japonica Group* (XP_015637335), *Nicotiana tomentosiformis* (XP_009603851), *Solanum lycopersicum* (AAN64317), and *Solanum torvum* (AEM45799).

3.3. Expression of *StPP5* Gene under *V. dahliae* Infection

The expression of *StPP5* under *V. dahliae* infection is shown in Figure 5. The expression patterns were different between stem and leaf. In stem, the maximal value of transcript appeared 12 h after *V. dahliae* inoculation and was maintained up to 24 h. In leaf, the maximal value of transcript was observed 24 h after inoculation; even the expression was raised from 12 h after infection.

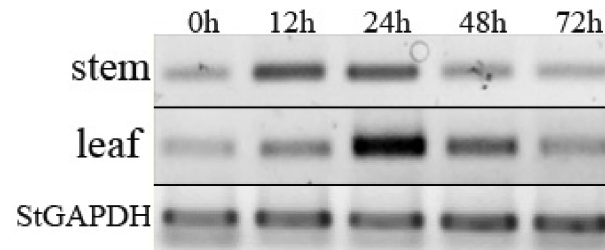


Figure 5. Expression analysis of *StPP5* at different times after *V. dahliae* infection.

3.4. Genetic Transformation and Plant Regeneration

For transformation, pCambia1304-*StPP5* plasmid (Figure 6A) was transferred to competent *Agrobacterium* GV3101. PCR amplification produced a sequence of about 500 bp, which was consistent with the expected size (Figure 6B).

The transformation of tobacco was performed by the *Agrobacterium*-mediated method. Leaf fragment explants were cultured on the medium for callus induction, and the callus appeared 10 days later, after which it was transferred to the shoot induction medium. Shoots emerged from about 20 days of culture on the shoot induction medium (Figure 6C). Next, the shoots were excised and rooted on the rooting medium containing antibiotics for selection (Figure 6C). Surviving plantlets were propagated on the MS medium. In total, nine Hyg B positive plants were obtained and four transgenic lines were identified by RT-PCR, which are P3, P5, P6, and P7 (Figure 6D).

In order to confirm the expression of the *StPP5* gene in transgenic lines, the lines P6 and P7 were selected for RT-qPCR analysis. The results showed that the level of *StPP5* mRNA in lines P6 and P7 was much higher than the control, and P6 was 23 times of the control (Figure 6E).

3.5. Inhibitory Effect of Transgenic Tobacco Protein Extracts on *V. dahliae*

To reveal the function of the *StPP5* gene, transgenic tobacco protein crude extract was added to the PDA medium for *V. dahliae* culture. The result showed that the growth of *V. dahliae* was significantly inhibited in the medium containing proteins extracted from the leaves of transgenic line P6 compared with wild-type tobacco. The average inhibition rate of line P6 was 55.2%, and wild-type tobacco was 31.1% (Figure 7, Table 1). These results showed that *StPP5* had an inhibitory effect on *V. dahliae* proliferation.

Table 1. Inhibition rate of the protein extract of transgenic and non-transgenic tobacco to *V. dahliae*.

| Treatment | Average Inhibition Zone Diameter (mm) | Inhibition Rate (%) |
|--------------------|---------------------------------------|---------------------|
| Wild-type tobacco | 62.0 | 31.1 |
| Transgenic line P6 | 40.3 | 55.2 * |

*, $p < 0.05$.

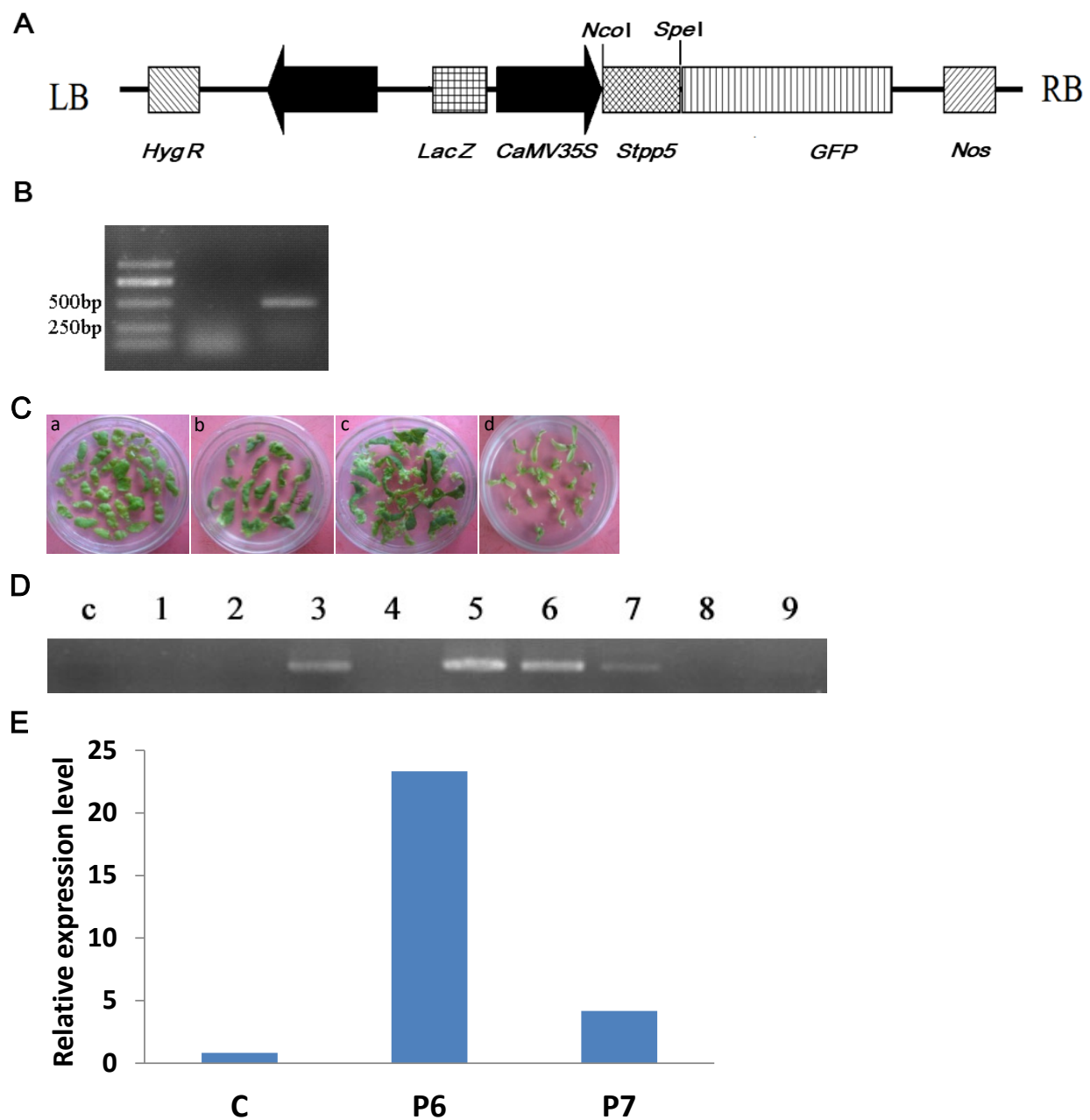


Figure 6. Genetic transformation and regeneration of transgenic *StPP5* tobacco plants: (A) Schematic representation of the binary vector pCMBIA1304-*StPP5*: LB, T-DNA left border; *Hyg^R*, hygromycin resistance; *lacZ*, *lacZ* lapha; *CaMV 35S*, promoter; *GFP*, green fluorescent protein gene; *nos*, *Nos*(nopaline synthase) polyA signal, terminator; RB, T-DNA right border; (B) PCR identification of pCMBIA1304-*StPP5* recombinant plasmid in transformed *A. tumefaciens*: M, Marker; c, non-transformed control; cz, recombinant plasmid pCMBIA1304-*StPP5*; (C) regeneration of transgenic tobacco plants: a, Explants; b, adventitious bud formation; c, the roots selection of 25 mg/L Hyg B; d, transgenic tobacco regeneration; (D) identification of *StPP5* transgenic tobacco lines by PCR; (E) RT-qPCR analysis of *StPP5* expression in transgenic tobacco lines: c, non-transgenic plant; P6 and P7, transgenic line.

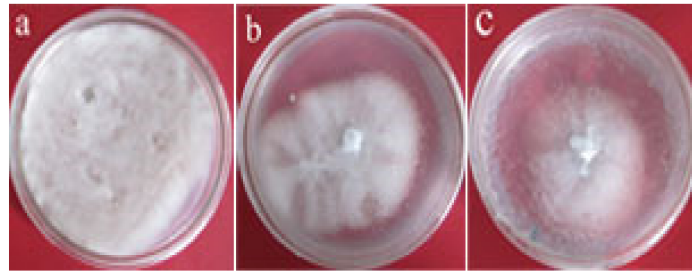


Figure 7. Growth and propagation of *V. dahliae* in the media with different tobacco protein extracts: (a) water control; (b) non-transgenic line; (c) transgenic line P6.

4. Discussion

VW is widespread throughout the world and an important factor in most agricultural economies [21,22]. The plant defense response to VW is a complex process. Based on similarities in the genetic pathway, some VW resistance-related genes, including *Ve1*, *Ve2*, and *PGIP* (polygalacturonase-inhibiting protein), have been tested and found to be expressed after *V. dahliae* infection [7,23]. In the previous study in our laboratory, 118 TDFs were identified from eggplant *S. torvum* before and after inoculation of *V. dahliae* using cDNA-AFLP technology, in which a TDF was closer to serine/threonine-protein phosphatase and named StPP5 [18]. Protein phosphatases that cleave phosphate from phosphorylated serine and threonine in proteins are crucial for the regulation of most cellular processes [24,25]. We isolated an *StPP5* gene from *S. torvum*, which belongs to a kind of PPPs family. The encoded amino acid sequence contains the typical domain structures common to most plant PPPs. The blastp comparison showed that StPP5 is highly homologous to other *Solanaceae*s, including a consistency with tomato of up to 96%. Smart online tools with protein molecules of StPP5 domain analysis revealed that its N terminal domain belonged to the TPR protein family, which is likely the domain interacting with other proteins, and the C terminal has a PP-type protein [26,27].

Serine/threonine-protein phosphatases are involved in cell metabolism, DNA replication, gene expression, signal transduction, cell cycle, cell differentiation, and apoptosis [28,29]. In this study, StPP5 was induced by *V. dahliae* infection. In vitro inhibitory assays showed that the inhibition rates of transgenic tobacco protein extracts to *V. dahliae* reached 55.2%, which was higher than that of wild-type tobacco. Quantitative RT-PCR analysis result indicated that *StPP5* among transgenic tobacco lines was more highly expressed than wild-type tobacco and P6 was 23 times the wild-type control, which was consistent with the result of the inhibitory assay, suggesting that StPP5 might regulate the expression of downstream resistance genes through dephosphorylation, thus regulating the resistance of plants to *V. dahliae*. Similar results were reflected in *StoVe1*: the antifungal assay revealed that transgenic tobacco lines had higher inhibition rates of 35.5%, 29.4%, and 27.3% than the control plants, and the inhibition rates of transgenic potato protein extracts to *V. dahliae* ranged from 39.4 to 45.5% [2,30]. Previous studies discovered that the PP2C-type protein phosphatases gene operated at the junction of drought, heat shock, and oxidative stress [31], and Xb15 encoding a PP2C negatively regulated the XA21-mediated innate immune response in rice [32], consistent with our experimental results about the change of protein phosphatases in stress.

5. Conclusions

In summary, we cloned an *StPP5* gene from *S. torvum* and revealed an inhibitory effect of *StPP5* transgenic tobacco protein extracts to *V. dahliae*, thereby demonstrating the importance of StPP5 in response to VW and possibly providing a potential gene for the breeding of *Solanaceae* resistant to VW.

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