Protein Extract of Tobacco Expressing *StoVe1* Gene Inhibits *Verticillium dahliae* Proliferation

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Abstract

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Verticillium dahliae is a principal pathogen causing verticillium wilt in *Solanaceae* crops. *StoVe1* is a gene resisting to verticillium wilt isolated from *Solanum torvum*. In order to generate resistant tobacco plants, *StoVe1* was inserted in the orientation behind CaMV 35S promoter of vector pGS and this construct was introduced into tobacco by *Agrobacterium*-mediated transformation. A total of 12 kanamycin-resistant plants were generated and 7 independent transgenic lines were identified by PCR analysis. Quantitative RT-PCR analysis showed that the levels of *StoVe1* transcript in transgenic lines were up to 2–6 fold higher than in the control. Anti-fungal assay indicated that the protein extract of transgenic tobacco lines showed strong inhibition activity to *V. dahliae*, 2 fold or higher compared to control plants. This result reveals that *StoVe1*, as a *V. dahliae* resistance gene, has an application potential in plant breeding for verticillium wilt resistance.

Keywords: disease resistance; regeneration; *StoVe1*; transformation; tobacco

Verticillium wilt (VW) is a common fungal disease of over 300 species of eudicot plants caused by one of the two species of *Verticillium* fungus, *Verticillium dahliae* Kleb and *Verticillium alboatrum* Reinke et Berth. Pathogens causing VW depend on climatic conditions: for instance *V. dahliae* causes VW in warm production regions and *V. alboatrum* in cooler areas (Rowe & Powelson 2002). Once the pathogen enters the host, it makes its way to the vascular system, specifically the xylem, and can quickly colonize the plant, causing symptoms such as stunting, chlorosis or yellowing of the leaves, necrosis or tissue death, and defoliation. Due to the fact that VW can spread in many ways and most vegetable species have some susceptibility, it reduces the quality and quantity of many crops including cotton, eggplant, potato, tomato and sunflower (Chai *et al*. 2003). There are many ways to control VW, such as planting disease free plants in uncontaminated soil, planting resistant varieties, and avoiding planting susceptible crops in areas that have been used repeatedly for *Solanaceous* crops. Soil fumigation can also be used, but it is generally too expensive over large areas of culture (Agrios 2005). Among all the measures against VW, host plant resistance offers a viable alternative long-term control method.

The *Ve* locus, which was identified from tomato several decades ago (Schaible *et al*. 1951), includes two genes (*Ve1* and *Ve2*) (Kawchuk *et al*. 2001) which both contain a lengthy leucine-rich repeat (LRR) domain, a common plant disease

resistance gene feature. The *Ve* gene products are predicted to be cell surface receptor proteins based on conserved amino acid sequence motifs and the receptor protein plays a role in the recognition of pathogen-derived molecules (Kawchuk *et al*. 2001). When plants are challenged by pathogens, complex integrated sets of defence responses, both constitutive and induced, are triggered and mediated by high affinity plant cell surface receptors (Dong 1998; Kunkel & Brooks 2002; Nishimura & Dangl 2010; Yang *et al*. 2010; Antonios *et al.* 2012). Then, the plants rapidly express nucleic acid binding leucine-rich repeat (NB-LRR) gene-based recognition patterns to establish a series of effector-triggered immunity reactions (Jones & Dangl 2006; Lacombe *et al*. 2010).

Ve gene, as a VW resistance gene, has been isolated from different plants such as tomato (Kawchuk *et al*. 2001), *Solanum lycopersicoides* (Chai *et al*. 2003), potato (Simko *et al*. 2004a, b), *Solanum torvum* (Fei *et al*. 2004; Shi *et al*. 2006), mint (Vining & Davis 2009) and *Gossypium barbadense* (Zhang *et al*. 2011) etc. In a study where the *Ve* gene was cloned from tomato and transformed into potato, results showed that the *Ve* gene conferred the transgenic potato resistance to *V. alboatrum* (Kawchuk *et al*. 2001). In another study it was found that transgenic *Arabidopsis thaliana* with *GbVe* cloned from *Gossypium barbadense* had greater levels of resistance to *V. dahliae* (Zhang *et al*. 2011).

StoVe1 (accession No. DQ020574) is a *Ve* homologue cloned from *S. torvum* (Shi *et al*. 2006), which has 91% identity in amino acid sequence with its homolog *StVe* (Fei *et al*. 2004). Recently, it was introduced into the potato cultivar Désirée and transgenic plants demonstrated resistance to *V. dahliae* (Liu *et al*. 2012). Tobacco is a *Solanaceae* species and a model plant in biological studies. No report on the genetic transformation of *StoVe1* gene in tobacco has been found to date. In this paper, we present the result of the production of transgenic tobacco plants with *StoVe1* by *Agrobacterium*-mediated transformation, which revealed that the protein extract of tobacco expressing *StoVe1* gene inhibited *V. dahliae* proliferation.

MATERIAL AND METHODS

Plant material and growth conditions. Tobacco (*Nicotiana tabacum* L. cv. NC89) used in the experiment was preserved in our laboratory. The tobacco seeds were disinfected using 70% alcohol and then 10% NaClO, and subsequently inoculated and germinated on MS medium (Murashige & Skoog 1962) containing 3% sucrose and solidified with 0.8% agar at pH 5.8 under a 16 h light : 8 h dark regime. Leaves from 4-week-old plants were taken and used as explants for a transformation experiment.

Tobacco transformation and regeneration. The ORF sequence of *StoVe1* gene was inserted in the orientation behind CaMV 35S promoter of the vector pGS (provided by Dr. Chonglin Huang, Beijing Academy of Agriculture and Forestry Sciences, Beijing), designated as pGS-*StoVe1* (Figure 1). After enzyme digestion identification and sequencing, the construct pGS-*StoVe1* was introduced into *Agrobacterium tumefaciens* strain GV3101 using a freeze-thaw method (Hofgen & Willmitzer 1988), followed by PCR detection with middle fragment (956 bp) of *StoVe1* primers Meds (5'-CCTTTCACG-TAATGGTCTAAC TGG-3') and Meda (5'-GTTC-CCAAGATTCAGCACTCCAAG-3'). The bacteria were cultured in LB medium (Sambrook *et al*. 2000) supplemented with 100 mg/l kanamycin (Kan) and 50 mg/l rifampicin at 28°C until the optical density of OD_{600} = 0.6.

Tobacco leaf-disc explants were pre-cultured on T1 medium [MS + 1.0 mg/l 6-benzylaminopurine (6-BA) + 0.1 mg/l α-naphthalene acetic acid (NAA)] in darkness at 25°C for 2 days, immersed in bacterial suspension for 8 min, dried by sterile filter paper, and subsequently co-cultured on T1 medium under the same conditions as mentioned above for 2 days. Next, the inoculated leaf-disc explants were placed on T2 medium [MS + 1.0 mg/l 6-BA + 0.1 mg/l NAA + 200 mg/l Cefotaxime (Cef) + 50 mg/l Kan] and incubated under 12/12 h photoperiod at 25°C. Subculture was done on fresh T2 medium on a weekly basis. Approximately 4 weeks later, shoots (1 cm long) were excised and rooted on MS medium supplemented with 50 mg/l Kan. Surviving plantlets were propagated on MS medium.

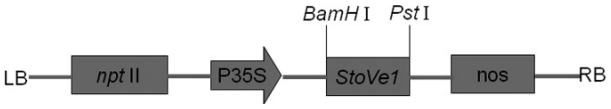

Figure 1. Structure of the expression cassette of pGS-*StoVe1* vector and its restriction sites
LB: T-DNA left border; *npt* II: neomycin phosphotransferase II; P35S: promoter from cauliflower mosaic virus; nos: nos terminator; RB: T-DNA right border

Transgenic plant identification. Genomic DNA was extracted from young leaves of transgenic and wild-type plants using CTAB method (Hu *et al.* 2003). PCR was performed using primers which contain the partial sequence of pGS vector Yans (5'-AAGGACAGTAGAAA AGGAAGGTGGC -3') and Yana (5'-CACTTGTCTTGTGGTTCCATTTT-GC-3') under these thermal conditions: 94°C for 4 min, followed by 33 cycles of 30 s at 94°C, 30 s at 56°C, 40 s at 72°C, and final 10 min incubation at 72°C. The presence of *StoVe1* gene was identified by the PCR product expected to be a 660 bp fragment.

Analysis of gene expression. Expression analysis of *StoVe1* was carried out by quantitative RT-PCR (qRT-PCR) with primers *StoVe1*-RTS (5'-AGGTC-CTCTCACTTTCTTCCAAC-3') and *StoVe1*-RTA (5'-CAACTGAGGAAATGCGAAAGAGG-3') designed according to the conservative fragment of *StoVe1*. Total RNA was extracted from the root of transgenic and wild-type tobacco plants using TRIzol Reagent (Tiangen, Beijing, China) following the manufacturer's instructions. cDNA was synthesized using random primers and Reverse Transcription System kit (Takara, Dalian, China). The tobacco gene glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) was used as an internal control. PCR reaction of *gapdh* used specific primers *gapdh*-S (5'-CAAGGACTGGAGAGGTGG-3') and *gapdh*-A (5'-TTCACTCGTTGTCGT ACC-3'). The qRT-PCR was accomplished on Mastercycler ep realplex machine (Eppendorf, Hamburg, Germany) with the following SYBR Premix Ex Taq (Takara, Dalian, China) reaction system and steps: 94°C for 1 min, (95°C 15 s, 54°C 30 s) × 40. Correspondence expression content ($2^{-\Delta\Delta CT}$) was calculated following the formula: $\Delta\Delta CT = (C_{T.Target} - C_{T.GAPDH}) \times X - (C_{T.Target} - C_{T.GAPDH}) \times 0$. X stands for different lines and 0 stands for one time expression of *StoVe1* after calibration by *GAPDH*. Each treatment was repeated three times.

Antifungal assay. One gram of leaf tissue was ground with phosphate buffer solution (PBS). After centrifugation at 15 000 g for 10 min, the supernatant liquid containing proteins was collected as crude protein solution.

Antifungal assay *in vitro* was carried out according to the method of Hu *et al.* (1999). *V. dahliae* was inoculated in the central hole of the PDA medium plate, and subsequently cultivated at 25°C in darkness for two days. When the mycelium diameter was 2 cm, 32 μg of protein extract was added to the other two holes. The plate was put at 4°C overnight until the protein solution permeated into the nutrient medium. The radius of inhibition zone was measured after 10 days of culture. The experiment consisted of 4 treatments which were wild-type tobacco, T_{0-7}, T_{0-11} and T_{0-14}. Each treatment was repeated three times. Inhibition rate was calculated using the following formula: inhibition rate = (the inhibition zone radius of non-treatment – the inhibition zone radius of treatment)/the inhibition zone radius of non-treatment × 100 (%).

RESULTS

Regeneration of transgenic plants

The leaf-disc explants infected with *A. tumefaciens* were cultured on T2 medium for callus and shoot induction (Figure 2a). Callus appeared two weeks after inoculation (Figure 2b). Then the explants with callus were transferred to fresh T2 medium for shoot formation. Shoots formed after about four to five weeks of culture (Figure 2c). Finally, they were selected on Kan-selective medium for rooting (Figure 2d). A total of 12 independent Kan-positive lines were obtained.

Figure 2. Regeneration of *StoVe1* transgenic tobacco plants
a: explants; b: callus formation; c: shoot formation; d: selection of transgenic plantlets in MS with 50 mg/l Kan

Identification of transgenic plants

Transgenic lines were identified by PCR analysis using the specific primers of pGS-*StoVe1*. A 660 bp band was amplified in seven Kan resistant lines. No band was observed in the wild-type plant (Figure 3).

Analysis of *StoVe1* gene expression

For the reason that plants were infected with VW mainly through roots, the expression of *StoVe1* gene was analysed in roots of three transgenic lines which were randomly selected. As shown in Figure 4, the respective contents of transcript in line $T_{0\text{-}7}$, $T_{0\text{-}11}$ and $T_{0\text{-}14}$ were 2 fold, 6 fold and 3 fold higher as compared to the wild type control. Hence all of the transgenic lines increased the transcript content compared to the wild-type control, among which line $T_{0\text{-}11}$ showed the highest level of expression. It was also observed that there were considerable differences in expression among the transgenic lines which might result from the position and copy number of integrated gene in the genome of transgenic plants (Emani *et al.* 2003; Vidal *et al.* 2006; Shah *et al.* 2010).

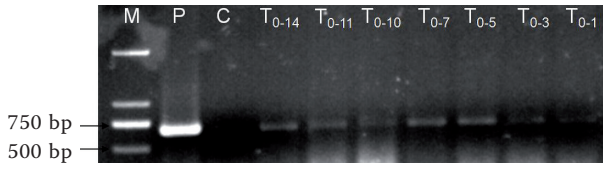

Figure 3. Identification of *StoVe1* transgenic tobaccos by PCR analysis
M: DNA marker; C: wild-type tobacco; P: pGS-*StoVe1*; $T_{0\text{-}1}$, $T_{0\text{-}3}$, $T_{0\text{-}5}$, $T_{0\text{-}7}$, $T_{0\text{-}10}$, $T_{0\text{-}11}$, $T_{0\text{-}14}$: transgenic plants

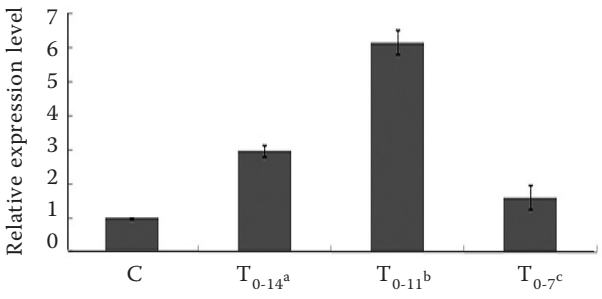

Figure 4. Expression of *StoVe1* gene in selected transgenic tobacco lines.
C: wild-type tobacco; $T_{0\text{-}7}$, $T_{0\text{-}11}$, $T_{0\text{-}14}$: transgenic lines; a, b, c: *P* value of transgenic lines, a < 0.05, b < 0.01, c < 0.05

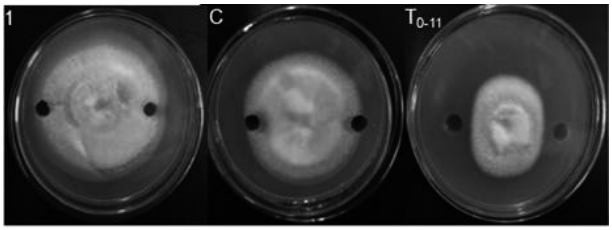

Figure 5. Colonies of *V. dahliae* on the media containing the protein extracts from transgenic and wild-type tobaccos
1: PBS control; C: wild-type tobacco; $T_{0\text{-}11}$: transgenic tobacco $T_{0\text{-}11}$

Inhibitory effect of transgenic tobacco protein extract on *V. dahliae*

V. dahliae was inoculated on media containing protein extracts of transgenic lines or wild-type plants. Observations demonstrated that the radius of the colony on the medium containing the protein extract of transgenic plant (Figure 5) was much more reduced compared to that of the control ($P < 0.05$), which revealed that the growth of *V. dahliae* was significantly inhibited by the protein extract of transgenic plants. The analysis indicated that the inhibition rates were visibly higher in the three transgenic lines, about twice or more than in the wild-type control (Table 1).

DISCUSSION

Tobacco is an important economic crop which can be damaged by numerous diseases such as brown spot, verticillium wilt, black shank, bacterial wilt, mosaic virus disease etc. Although *V. dahliae*-

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

Treatment	Radius of inhibition zone (mm)	Inhibition rate (%)
WT	28.3	14.2
$T_{0\text{-}11}$	21.3	35.5^{a}
$T_{0\text{-}14}$	23.3	29.4^{b}
$T_{0\text{-}7}$	24.0	27.3^{c}

WT – wild-type tobacco; $T_{0\text{-}11}$, $T_{0\text{-}14}$, $T_{0\text{-}7}$ – transgenic tobacco; a, b, c – *P* value of transgenic lines, a < 0.05, b < 0.05, c < 0.01

induced verticillium wilt of tobacco constituted an economic problem in some countries such as in New Zealand in 1969 (Mahanty 1970), there is nonetheless limited literature about verticillium wilt of tobacco. Recently, a sea-island cotton (*Gossypium barbadense* L.) thaumatin-like protein gene (*GbTLP1*) has been overexpressed in tobacco. The transgenic tobacco with constitutively higher expression of the *GbTLP1* showed enhanced resistance against *V. dahliae* (Munis *et al.* 2010). In the meantime, as VW resistance genes, *Ve* genes isolated from different plants have been transferred into some crops such as potato (Kawchuk *et al.* 2001; Liu *et at.* 2012) and *Arabidopsis thaliana* (Zhang *et al.* 2011). In this study, we successfully generated tobacco plants expressing *StoVe1* gene and we demonstrated that *StoVe1* independently confers resistance to *V. dahliae*, which has not been reported to date as to our knowledge.

In-vitro inhibitory assays showed that the inhibition rates of transgenic tobacco protein extracts to *V. dahliae* ranged from 27.3 to 35.5%, which was noticeably higher than that of wild-type tobacco whose inhibition rate was 14.2%, but lower than in transgenic potato whose inhibition rate ranges from 39.4 to 45.5% (Liu *et al.* 2012), suggesting that the expression of *StoVe1* varies across different recipients. In this study, we observed differences in inhibition rate among the transgenic lines. To verify whether the difference is a consequence of the expression of the transgene, quantitative RT-PCR analysis was performed. The result indicated that *StoVe1* gene was differentially expressed among transgenic tobacco lines and this was consistent with the result of the inhibitory assay. In genetic transformation, the difference in gene expression between transgenic lines has frequently been observed, which mainly results from the position and copy number of integrated gene in the genome of transgenic plants (Emani *et al.* 2003; Vidal *et al.* 2006; Shah *et al.* 2010).

Until now, the VW resistance mechanism of *Ve* genes across different plants has been obscure. It is clear that both *V. dahliae* and *V. alboatrum* are causes of VW. In susceptible potato plants, the expression of individual *Ve* loci (*Ve1* and *Ve2*) conferred resistance to *V. alboatrum* (Schaible *et al.* 1951; Kawchuk *et al.* 2001). Both transgenic *Arabidopsis thaliana* with *GbVe* and transgenic potato cultivar Désirée with *StoVe1* (Liu *et al.* 2012) had greater levels of resistance to *V. dahliae* (Zhang *et al.* 2011). All above examples indicate that there is a conservation of VW resistance signalling mechanisms across different plant species, and that the expression of orthologous *Ve* genes can confer resistance not only to *V. alboatrum* but also to *V. dahliae*. This is further confirmed by our experiment in which the protein extract of transgenic tobacco expressing *StoVe1* gene cloned from *S. torvum* inhibited *V. dahliae* proliferation. Orthologous *Ve* resistance genes contain a lengthy leucine-rich repeat (LRR) domain and their products are predicted to be cell surface receptor proteins (Kawchuk *et al.* 2001). All of the aforementioned features of *Ve* genes suggest that they are different from other VW resistance genes, such as endochitinase which is capable of degrading chitin of the cell wall of most fungi to resist *V. dahliae* (Tohidfar *et al.* 2012).

In this paper, it was revealed that the protein extract of tobacco expressing *StoVe1* gene inhibited *V. dahliae* proliferation, which has a great potential in plant breeding for VW resistance. In the meantime, further investigation is needed to analyse the stability transformation in the next sexual generations and understand which, if any, amino acids in the LRR domain and conserved amino acid motifs of Ve are key determinants of wilt resistance.

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