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Virus-induced Silencing of the WRKY1 Transcription Factor that Interacts with the SL1 Structure of *Potato virus X* Leads to Higher Viral RNA Accumulation and Severe Necrotic Symptoms

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Potato virus X (PVX) replication is precisely regulated by regulatory viral sequences and by viral and/or host proteins. In a previous study, we identified a 54-kDa cellular tobacco protein that bound to a region within the first 46 nucleotides (nt) of the 5' non-translated region (NTR) of the viral genome. Optimal binding was dependent upon the presence of an ACCA sequence at nt 10-13. To identify host factors that bind to 5' NTR elements including AC-rich sequences as well as stem-loop 1 (SL1), we used northwestern blotting and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for peptide mass fingerprinting. We screened several host factors that might affect PVX replication and selected a candidate protein, *Nicotiana tabacum* WRKY transcription factor 1 (NtWRKY1). We used a *Tobacco rattle virus* (TRV)-based virus-induced gene silencing (VIGS) system to investigate the role of NtWRKY1 in PVX replication. Silencing of *WRKY1* in *Nicotiana benthamiana* caused lethal apical necrosis and allowed an increase in PVX RNA accumulation. This result could reflect the balancing of PVX accumulation in a systemic *N. benthamiana* host to maintain PVX survival and still produce a suitable appearance of mosaic and mottle symptoms. Our results suggest that PVX may recruit the WRKY transcription factor, which binds to the 5' NTR of viral genomic RNA and acts as a key regulator of viral infection.

Keywords : NbWRKY1, SL1 RNA, RNA-host protein interaction, *Potato virus X*, replication, symptom development

The viral infection cycle involves not only viral proteins such as RNA-dependent RNA polymerase (RdRp), movement protein (MP), and capsid protein (CP) but also host proteins that interact with these viral proteins and/or viral RNA elements. For plant viruses, successful infection is

required for viral genome replication, cell-to-cell movement, and long-distance transport, in which diverse interactions between viral and host factors occur. On the other hand, plant defense against viruses includes the hypersensitive response (HR) and/or RNA silencing. The 5'- and 3'-non-translated-region (NTR) sequences at the end of the viral genome contain RNA elements that are important for RNA accumulation, replication, translation, and regulation (Kim and Hemenway, 1996; Miller et al., 1998; Miller et al., 1999; Pillai-Nair et al., 2003; Srisakanda et al., 1996). The highly ordered stem-loop (SL) structures of the NTR regions, in particular, are implicated in RNA accumulation and replication through interactions with host proteins (Kwon and Kim, 2006).

Potato virus X (PVX) is a flexuous rod-shaped virus in the *Potexvirus* genus (Alphaflexiviridae family). The PVX genome is a positive-sense single-stranded RNA encoding five open reading frames (ORFs). The 5' and 3' ends have a cap structure and a poly(A) tail, respectively (Huisman et al., 1988). The first ORF encodes for the viral replicase, and a region of three overlapping ORFs, called the triple-gene block (TGB), is required for virus cell-to-cell movement (Verchot-Lubicz et al., 2010; Fig. 1A). The final ORF is the viral CP, which is necessary for virion assembly and cell-to-cell movement of the virus (Cruz et al., 1998; Huisman et al., 1988; Kwon et al., 2005; Fig. 1A). The 5' region of the genomic RNA contains an AC-rich single-stranded region and two RNA SL structures, named 5' SL1 and 5' SL2 (Kim and Hemenway, 1996; Miller et al., 1998; Fig. 1B). The 5' SL1 is known as a multifunctional element involved in viral replication, cell-to-cell movement, and virion assembly (Kwon et al., 2005). Several modifications within the 5' SL1 have demonstrated that the wild-type (wt) 5' SL1 and the GAAA sequence within the tetra loop (TL) are important for plus-strand RNA accumulation (Kwon and Kim, 2006; Miller et al., 1999). Previous work has shown that the AC-rich single-stranded region and the 5' SL1 have binding activity toward host protein extracts (Kim et al., 2002; Kwon and Kim, 2006). However, identification and

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Fig. 1. Schematic diagram of the *Potato virus X* (PVX) genome organization and predicted RNA structure adapted from Kim et al. (2002). (A) The five open reading frames are presented as boxes, and subgenomic RNAs are shown as bold arrows. (B) RNA stem-loop (SL) structure of the 5' end of the PVX genome is depicted.

characterization of these host factors have not yet been determined.

Plants have evolved in an environment rich with plant pathogenic viruses and have developed a variety of defense mechanisms to protect themselves from viral infection. Perhaps the best-characterized plant antiviral mechanisms are those mediated by resistance (R) genes. The transcriptional regulation of R gene expression is required for the involvement of transcription factors such as the WRKY transcription factor family (Ulker and Somssich, 2004). Genetic evidence linking a WRKY gene with resistance came from studies of the interaction of tobacco WRKY with Tobacco mosaic virus (TMV). Resistance to TMV is mediated by the tobacco resistance gene N and the down-regulation of tobacco WRKY genes that compromise N-mediated resistance (Liu et al., 2004).

In this study, we searched host proteins involved in PVX RNA accumulation by using the 5' end of the genome as a probe. We used northwestern blotting and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analyses to identify host proteins that bind to the 5' end of the PVX genome. We identified one candidate, the *Nicotiana tabacum* WRKY1 transcription factor (NtWRKY1), by peptide mass fingerprinting (MS-

Fit) and confirmed its binding with the SL1 secondary structure by gel shift assay. Virus-induced gene-silencing (VIGS) analysis was utilized to investigate the biological role of the NtWRKY1 in PVX-infected plants. The results indicate that silencing of NtWRKY1 in *Nicotiana benthamiana* caused plants to exhibit lethal apical necrosis by increasing PVX accumulation, suggesting that NtWRKY1 functions to regulate multiple defense response genes.

Materials and Methods

Preparation of plasmid constructs and RNA transcripts. The plasmid pMON8453 containing a PVX cDNA clone (Hemenway et al., 1990) was used to amplify each DNA fragment including a region from 1-203 nucleotides (nt) and others from 1-43 nt and the SL1 region (nt 32-106). Amplified DNA fragments were cloned into the pGEM[®]-T Easy vector (Promega, Madison, WI, USA) generating the following plasmids: pG-203 containing nt 1-203, pG-43 containing nt 1-43, and pG-SL1 containing nt 32-106 from the 5' end of the PVX genome. Radiolabeled ³²P-RNA probes were obtained by *in vitro* transcription in the presence of 50 Ci of ³²P-CTP (3000 Ci/mmol; GE Healthcare, Piscataway, NJ, USA), 12.5 μM CTP, and 500 μM each of ATP, GTP, and UTP using each linearized DNA template. For the polymerase chain reaction (PCR), strand-specific primers were used as described previously (Kim et al., 2002). Each PCR product was radioactively labeled in the presence of 50 μCi of α-³²P CTP (3000 Ci/mmol; GE Healthcare), 5 mM CTP, and 125 mM each of ATP, GTP, and UTP. Labeled RNA was purified by NucAway Spin Columns (Ambion, Austin, TX, USA).

One- and two-dimensional SDS-PAGE (1-DE and 2-DE). The protein pellets of S100 extracts were solubilized in 250 μl of rehydration buffer containing 8 M urea, 2% CHAPS, 0.5% IPG pH 4-7 buffer (GE healthcare), 18 mM DTT, and bromophenol blue. Isoelectric focusing (IEF) was performed at 3500 V for 20 h at 20°C using IPG strips (pH 4-7 L, 13 cm, GE Healthcare) and an Ettan IPGphor II system (GE Healthcare). Focused strips were equilibrated in 10 ml of 6 M Urea, 30% glycerol, 2% SDS, 125 mM DTT, and 0.992% bromophenol blue in 50 mM Tris-HCL with pH 8.0, twice for 15 min at room temperature (RT) and then treated with 10% tricine acrylamide gel for SDS-PAGE. Proteins were transferred to nitrocellulose membranes and then applied to northwestern blot analysis.

Northwestern blotting and MS-Fit analysis. S100 protein extracts were prepared from *Nicotiana tabacum* cv. Bright Yellow (BY2) suspension cells as described previously (Kim et al., 2002). S100 proteins and PVX CP recombinant

protein were resolved on a 12% SDS-polyacrylamide gel and transferred to a fluoride (PVDF)-P membrane. The membrane was incubated with 6 M guanidine hydrochloride (GuHCl) in northwestern buffer containing 10 mM Tris-HCl (pH 6.8), 25 mM NaCl, 1 mM EDTA, 0.04% BSA, 0.04% Ficoll400, and 0.02% polyvinyl pyrrolidone-40 for 30 min to denature proteins on the membrane, followed by washing with 6, 3, 1.5, 0.75, 0.375, and 0.187 M GuHCl in northwestern buffer to renature proteins. The membranes were blocked with 5% fat-free dry milk and 20 µg/ml yeast tRNA in northwestern buffer for 1 h at RT. After subsequent washing with northwestern buffer three times for 15 min, membranes were air dried and exposed in a BAS imaging plate (IP) for autoradiography (Fuji film, Tokyo, Japan). The bands corresponding to the spots of the two-DE northwestern blot were excised from the gel, digested with trypsin, and analyzed by MALDI-TOF MS. The data from MALDI-TOF MS were analyzed using the Protein Prospector algorithm (<http://prospector.ucsf.edu>) MS-Fit with the NCBI protein database.

Construction and purification of recombinant PVX CP and NtWRKY1. The PVX CP full-length and NtWRKY1 sequences were amplified by PCR from the PVX full-length construct pSPVXp31 (Park and Kim, 2006) and NtWRKY1 cDNA from *N. tabacum* BY2 suspension cells, respectively. PCR products were inserted into the expression vector pMAL-c2X (New England Biolabs, Ipswich, MA, USA). The *Escherichia coli* expression strain BL21 (DE3) was transformed with the plasmid pMAL-c2X containing PVX CP and NtWRKY1. The maltose binding protein (MBP)-PVX CP and MBP-NtWRKY1 fusion proteins were purified according to the manufacturer's instructions (New England Biolabs). The extracted protein concentrations were determined by Bradford analysis.

Electrophoretic gel mobility shift assay (EMSA). Approximately 1 µg of the recombinant proteins were incubated with α -³²P-labelled transcripts corresponding to nt 1-203, 1-43, and 32-106 of PVX RNA (~50,000 cpm) for 10 min on ice with EMSA binding buffer (20 mM Tris-HCl, pH 7.0, 1 mM DTT, 100 mM KCl, 1 mM EDTA, 100 mM NaCl, 25 ng/µl of yeast tRNA, and 4U of RNase inhibitor). After the incubation, the reactions were analyzed by 5% non-denaturing polyacrylamide gel electrophoresis (PAGE) in 0.5X Tris-borate-EDTA buffer. The gels were dried, exposed to a BAS imaging plate (FUJIFILM Fuji Photo Film), and analyzed in a phosphorimager (Fujix BAS 2500, Fuji Film). The band intensities were analyzed with Fuji Multi Gauge v2.2 (Fuji Film) and Excel (Microsoft) software.

Subcellular localization and overexpression. The sub-

cellular localization of NtWRKY1 was determined using the pSITE-2NA-NtWRKY1-GFP fusion protein and nucleus marker RFP:NbH2B as previously described (Chakrabarty et al., 2007). Onion epidermal cell layers were peeled and placed (with insides up) on Murashige and Skoog (MS) agar media. NtWRKY1-GFP and RFP:NbH2B plasmid DNA (0.5 µg) were introduced into the onion cells using a PDS-He biolistic particle gun (Bio-Rad, Hercules, CA, USA). Bombardment was conducted at 10 cm of target distance using 0.7 µm gold microcarriers under the vacuum of 28-inch Hg, helium pressure of 1100 psi (Takeuchi et al., 1992). The bombarded tissues were incubated on the MS plates for 12 h at 25°C in the dark. Samples were observed using a UV light incident fluorescence microscope (Zeiss, Axioskop, Germany). To prepare overexpression constructs of the NtWRKY1 and NtWRKY6, amplified DNA fragments were cloned into pPZP212 vector generating pPZP-NtWRKY1 and pPZP-NtWRKY6. These constructs, pPZP-NtWRKY1 and pPZP-NtWRKY6, were transformed into *Agrobacterium tumefaciens* strain GV2260 and agro-infiltrated together with a Tomato bushy stunt virus (TBSV) p19 (pPZP-p19)-transformed strain of *A. tumefaciens* into the leaves of *N. benthamiana*.

VIGS assay and PVX infection. TRV-induced gene silencing assays using pTRV1 or pTRV2 and its derivatives (pTRV2-PDS and pTRV2-NtWRKY1) were performed as described (Liu et al., 2002). Plants were maintained at 25°C in a growth chamber on a 16-h photoperiod. Ten days post infiltration (dpi) the upper non-infiltrated and carborundum-dusted leaf of the plant was infected with purified PVX by rub inoculation.

Total leaf RNA extraction. Leaves of *N. benthamiana* plants inoculated with TRV-based VIGS vector and purified PVX were harvested at 10 dpi from upper non-inoculated leaves. Total RNA was isolated from 0.1 g of leaf samples with TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Semi-quantitative RT-PCR. Total RNAs extracted from silenced and non-silenced *N. benthamiana* plant leaves were treated with TURBO DNA-free™ (Ambion) to remove contaminating genomic DNA. Next, 1 µg of total RNA was reverse-transcribed using M-MuLV reverse transcriptase (Promega) and either an NtWRKY1 specific 3' primer (5'-TCAGTTAAGGAAAGAGCTG-3') or oligo (dT) 3' primer. Semi-quantitative RT-PCR was performed as previously described (Kim et al., 2010; Liu et al., 2002). PCR was performed for 15, 20, 27, 30, and 35 cycles. The intensities of the PCR bands were analyzed with GEL DOC 2000 and QUANTITY ONE (Bio-Rad). As for detection of PVX, the

Fig. 2. Northwestern blot analysis used to identify host proteins that bind to the first 203 nucleotides (nt) of viral RNA. (A) One-dimensional electrophoresis (DE) northwestern blot analysis of S100 and recombinant PVX capsid protein (CP) with ^{32}P -labeled 5' 203 nt RNA. Detected host proteins are designated by arrowheads, and PVX CP are denoted by asterisks. (B) Two-DE northwestern blot analysis with S100 and recombinant PVX CP. Radiolabeled ^{32}P 5' 203 nt RNA was used as a probe. Numbers two and three were identified as NtWRKY1 and PVX CP, respectively, by MADI-TOF MS and MS-Fit.

total RNA extracted from upper non-inoculated leaves was used to detect the presence of the PVX genome by RT-PCR using PVX CP gene primers (5'-ATGTCAGCACCAGCTA-GCAC-3' and 5'-TTATGGTGGTGGGAGAGTGAC-3').

Results

Host proteins that interact with the first 5' 203 nt of the PVX RNA were identified by northwestern blot analysis.

It has been reported that RNA-protein complexes between nt 1–203 in the PVX genome (5' 203 nt) and host proteins have been formed in EMSA (Kwon et al., 2005). Interaction between the 5' end of the PVX genome and host proteins was expected at various steps of the viral infection cycle due to the multiple roles of the 5' end. Northwestern blot analysis was used to identify host proteins from S100 extracts of *N. tabacum* BY-2 suspension cells that interact with the 5' 203 nt of viral RNA. First, 1-DE northwestern blot analysis confirmed the binding ability of the 5' 203 nt RNA to host proteins. The purified CP of PVX was used as a positive control because binding ability of the CP to the 5' SL1 in the 5' 203 nt has previously been reported (Kwon et al., 2005). Multiple band patterns were found, some with smaller molecular weights than CP and one band with a higher molecular weight, around 50 kDa (Fig. 2A). The 1-DE northwestern blot analysis is insufficient to effectively resolve complex protein mixtures. To determine how many distinct host proteins that interact with the 5' 203 nt viral RNA were present in the S100 extracts, we further fractionated the S100 extracts by 2-DE SDS-PAGE. The 5' 203 nt RNA probe detected diverse proteins including the positive control, PVX CP (spot number 3), on the 2-DE northwestern blot (Fig. 2B). We excised a band from the gel corresponding to spot number 2 of the two-DE northwestern blot and analyzed it by MALDI-TOF MS. The data from MALDI-TOF MS were analyzed using MS-Fit with fixed default parameters and 50–60 kDa molecular mass, since a 54-kDa cellular protein (p54) was identified in our previous study (Kim et al., 2002). The identified proteins included a transcription factor, cyclins, a protein kinase, and

Fig. 3. Electrophoretic mobility shift assay (EMSA) of the maltose binding protein (MBP)-NtWRKY1 expressed in *Escherichia coli* with 5' 203 nt, 5' 43 nt, and SL1 RNA probes. (A) MBP and MBP-NtWRKY1 were purified by affinity chromatography and visualized by Coomassie-blue staining after electrophoresis through a 12% SDS-PAGE gel. The molecular weight marker is indicated as M. (B, C, and D) The MBP or MBP-NtWRKY1 was assayed by EMSA for binding to the α - ^{32}P -CTP-labeled 5' 203 nt (B), 5' 43 nt, (C) and SL1 (D) RNA probes. No protein, MBP-NtWRKY1, and MBP were added (lanes 1, 2, and 3, respectively). RNA and RNA-protein complexes were separated on a 4% non-denaturing polyacrylamide gel. Closed and open arrowheads represent retarded movement by the formation of PVX 5' RNA-MBP-NtWRKY1 and PVX 5' RNA-MBP complexes, respectively.

a dehydrogenase. The protein with the highest score, *N. tabacum* WRKY1 transcription factor (NtWRKY1), was selected and used for additional characterization. The accession number of the full-length cDNA for this protein is AB022693.

NtWRKY1 binds to the SL1 stem-loop structure of the 5' region of the PVX genome. The 5' region of the PVX genome contains an AC-rich single-stranded region and RNA stem-loop structures that are required for viral plus-strand accumulation, virus assembly, and cell-to-cell movement (Kim and Hemenway, 1996; Kwon et al., 2005; Lough et al., 2006; Miller et al., 1998; Miller et al., 1999). To confirm the *in vitro* binding activity of NtWRKY1 with the 5' end regions of the PVX genome, EMSA was conducted with non-secreted MBP-NtWRKY1 fusion protein (Fig. 3A). Three RNA probes, 1-203 nt, 1-43 nt, and the SL1 RNA, were prepared by *in vitro* transcription with α - 32 P-CTP. Distinct RNA-protein complexes exhibiting slower electrophoretic mobility were displayed when the 5' 203 nt and 5' SL1 RNA probes were incubated with the MBP-NtWRKY1 fusion protein, whereas no binding was observed between MBP and either RNA probe (Fig. 3B and C). The 5' 43 nt probe containing the AC-rich single-stranded region (5' 43 nt) also bound to the MBP-NtWRKY1 fusion protein, since there was binding affinity for the MBP (Fig. 3C). Because the 5' 43 nt RNA probe possessed non-specific binding affinity to MBP, the binding of the 5' 43 nt RNA with MBP-NtWRKY1 fusion protein was not specific. These results indicated that the 5' SL1 stem loop structure in the 5' region of the PVX genome could bind NtWRKY1 *in vitro*.

***In vivo* localization and overexpression of NtWRKY1.** The tobacco WRKY1 transcription factor has been shown to be localized in the nucleus and to function as a trigger of HR-like cell death when the salicylic acid-induced protein kinase (SIPK) is co-expressed in a dose-dependent manner (Menke et al., 2005). To confirm these findings in our system, the NtWRKY1 coding sequence was fused to the amino termini of green fluorescent protein (GFP) in the pSITE-2NA and introduced into the pZP vector, which allows expression of NtWRKY1 within the plant without autofluorescence (Chakrabarty et al., 2007). As we expected, NtWRKY1:GFP was mainly present in the nucleus when transiently expressed with the red fluorescent protein (RFP)-fused nucleus marker, RFP:NbH2B harboring *N. benthamiana* histone 2B gene, in onion epidermal cells (Fig. 4).

In contrast to previous work (Menke et al., 2005), our results show that transient overexpression of NtWRKY1 without the expression of SIPK caused a hypersensitive

Fig. 4. Subcellular localization of NtWRKY1-GFP. NtWRKY1-GFP and RFP:NbH2B (nucleus marker) were bombarded into onion epidermal cells with DNA-coated gold particles, and GFP and RFP expression was then visualized after 16 h.

Fig. 5. Overexpression of NtWRKY1 by the silencing suppressor p19. Symptom appearances on NtWRKY6 (A and B) and NtWRKY1-overexpression (C and D) plants are shown.

response (HR)-like cell death when NtWRKY1 was co-expressed with the RNA silencing suppressor TBSV p19 in the *N. benthamiana* (Fig. 5C and D), whereas overexpression of NtWRKY6 as control did not induce cell death (Fig. 5A and B). These results suggest that NtWRKY1 activates defense genes related to HR-like cell death in the nucleus and co-expression of NtWRKY1 with either SIPK or viral RNA silencing suppressor p19 leads to more rapid cell death than the expression of NtWRKY1 alone.

Fig. 6. Expression levels of *NtWRKY1* and elongation factor 1- α (*EF1- α*) genes after PVX infection. (A) *NtWRKY1* mRNA levels were accumulated rapidly within 0.5 hours post-infection (hpi) and gradually decreased until 1.5 hpi. *EF1- α* was used as an endogenous gene control.

Changes in mRNA levels of *NtWRKY1* during PVX infection. In another plant virus study, transcripts of the WRKY-type transcription factor *TIZZ* (TMV-induced leucine zipper zinc-finger) were induced in TMV-infected *nahG* transgenic tobacco plants (Yoda et al., 2002). To test whether PVX infection affects *NtWRKY1* mRNA levels, RT-PCR was performed with total RNAs extracted from PVX-infected *N. benthamiana* at different time points. The results demonstrated that *NtWRKY1* mRNA levels accumulated rapidly within 0.5 hours post-infection (hpi) (Fig. 6A). The level of *NtWRKY1* mRNA reached a maximum at about 0.5 hpi and gradually decreased until 1.5 hpi (Fig. 6A), whereas very little or no difference was detected for mRNA levels of *EF1- α* (Fig. 6B). This result indicates that *NtWRKY1* was induced early following PVX infection.

Effects of silencing the *WRKY1* gene on PVX infection. The TRV-based VIGS vector has been used to silence genes of interest. Hence, an ortholog of the *NtWRKY1* gene (*OtWRKY1*) and a gene for endogenous phytoene desaturase (*PDS*) were silenced in order to examine the function of the *OtWRKY1* transcription factor in *N. benthamiana* (Liu et al., 2002). The *PDS* gene was used for assessment of the

Fig. 7. Silencing of the *PDS* and *NtWRKY1* genes. (A and B) Recombinant TRV constructs carrying either the *PDS* or the *NtWRKY1* sequence were infiltrated into *Nicotiana benthamiana* plants to silence endogenous *PDS* and an ortholog of *NtWRKY1*. Whereas *PDS*-silenced plants had inhibited carotenoid biosynthesis, resulting in a photobleached phenotype (A), no symptom differences were detected in the ortholog of *NtWRKY1*-silenced plants (B). The whole plant is presented on the left, and an enlarged single leaf is presented on the right. (C) RT-PCR analysis was performed on *N. benthamiana* silenced for *PDS* and the ortholog of *NtWRKY1* to confirm silencing.

gene-silencing efficiency of our TRV VIGS system (Kumagai et al., 1995). The highest silencing efficiency of the *PDS* gene was observed in the upper non-infiltrated leaves at 10 dpi indicating that the TRV-VIGS system could be used to induce silencing of *OtWRKY1* (Fig. 7A). After 10 dpi, no visible phenotypic difference was detected in *OtWRKY1*-silenced *N. benthamiana* when compared to that of the TRV-VIGS vector alone (Fig. 7B). Semi-quantitative RT-PCR analysis suggested that mRNA levels of *PDS* and *OtWRKY1* were reduced to ~10–20% of the TRV-vector infiltrated control plant (Fig. 7C).

To verify the role of the *OtWRKY1* transcription factor in PVX infection, *WRKY1*-silenced and TRV-VIGS vector-infiltrated control plants were infected with PVX and monitored for 10 dpi. In TRV-VIGS vector-infiltrated control plants, PVX induced severe mosaicism with leaf curling symptoms, whereas PVX infection caused lethal apical

Fig. 8. Effect of *NtWRKY1* silencing in *N. benthamiana* on symptom development and viral RNA accumulation. (A and B) Symptom development was observed at 10 dpi after PVX infection. Symptoms caused by PVX on *Tobacco rattle virus* (TRV) alone infected (A) and *NtWRKY1* silenced (B) plants. (C) Viral RNA accumulation analyzed by semi-quantitative RT-PCR and 20 cycles of reactions were represented. Total RNAs were extracted from TRV alone infected (lane 1) and *NtWRKY1* silenced (lane 2) plants. The molecular weight markers are indicated as M.

necrosis (Fig. 8A and B). The levels of PVX RNA accumulation in the upper non-infiltrated leaf were analyzed by semi-quantitative RT-PCR analysis. The result indicated that silencing of *OtWRKY1* increased the levels of PVX RNA accumulation compared to the non-silenced plant (Fig. 8C). The results suggest that *OtWRKY1* might be implicated in balanced accumulation of the PVX genome and maintaining mosaic symptoms in *N. benthamiana*.

Discussion

We used 2-DE northwestern blot analysis to identify host proteins that bound to the first 203 nt of the PVX genome. It has been reported that the 5' end of the PVX genomic RNA is important for accumulation of genomic RNAs and sgRNAs, cell-to-cell movement, and virion assembly (Kim and Hemenway, 1996; Kwon et al., 2005; Lough et al., 2006; Miller et al., 1998; Miller et al., 1999). The fact that host proteins bound to the 5' end of PVX indicates that viral RNA undergoes multiple interactions with host factors and that these interactions are essential for the PVX infection cycle. Among those host proteins, the *NtWRKY1* transcription factor was selected for further investigation. EMSA using the recombinant MBP-*NtWRKY1* fusion protein demonstrated that the 5' SL1 element, in the 5' end of the PVX genome, is the direct binding partner for *NtWRKY1*.

WRKY transcription factors are involved in the regulation of plant defense gene expression against diverse biotic and abiotic stresses (Pandey and Somssich, 2009). The resistance response of plants is required for large-scale transcriptional reprogramming by various transcription factor families such as the *WRKY* genes (Eulgem, 2005; Naoumkina et al., 2008; Ryu et al., 2006). Nuclear localization was predicted for WRKY proteins and has been demonstrated

for the WRKY transcription factors of parsley and tobacco (Eulgem et al., 1999; Hara et al., 2000). We also showed that the predominant nuclear localization of *NtWRKY1* was confirmed in transient expression assays by particle bombardment in onion epidermal cells. Whereas DNA viruses and retroviruses are accessible to proteins that are resident in the nucleus, the site of viral-genome replication of RNA viruses is usually the cytoplasm. Therefore, the rationale for employment of transcription factors in positive-stranded RNA viruses such as PVX is not immediately understood. Viruses have evolved a variety of mechanisms to overcome host defenses. This raises the possibility that transcription factors are required for limiting the host resistance response and reprogramming metabolic functions of the host in order to obtain a more favorable environment for viral multiplication. Furthermore, not all of the *NtWRKY1* protein resides in the nucleus of the onion cell, which means that this protein can traffic from the nucleus to the cytoplasm. In our study, expression of *NtWRKY1* was activated rapidly, within 30 min of PVX infection, and then gradually decreased, suggesting that *NtWRKY1* residence in the nucleus is time- and/or condition-dependent. In *Arabidopsis thaliana*, nearly 1 out of 4 of the 72 *AtWRKY* proteins were transcriptionally upregulated within 2 h of pathogen infection or salicylic acid (SA) treatment, although almost 70% of the *AtWRKY* genes were differentially regulated (Dong et al., 2003; Kalde et al., 2003).

Previous studies have shown that overexpression of SIPK-triggered cell death and transient expression of tobacco WRKY1 in the presence of SIPK induced more rapid cell death than the expression of SIPK alone (Menke et al., 2005; Zhang and Liu, 2001). This suggested that WRKY1 may be a component of the signaling cascade downstream of SIPK involved in HR-like cell death (Menke et al., 2005). Cell death in the *N. benthamiana* was observed

only after 1 week with transiently expressed NtWRKY1 alone. However, overexpression of NtWRKY1 in combination with the silencing suppressor p19 accelerated cell death of the plant suggesting that NtWRKY1 may activate and regulate defense response genes related to HR-like cell death. We suspect that plants precisely regulate the expression of NtWRKY1 to prevent themselves from the prolonged expression of NtWRKY1. The function of NtWRKY1 was further investigated using the TRV-based VIGS system. Our silencing results show that PVX infection induced very severe lethal apical necrotic symptoms in NtWRKY1-silenced plants, in which the levels of PVX RNA accumulation were increased compared to control plants. Together with overexpression results, this suggests that NtWRKY1 plays an important role in the control of PVX genome accumulation and maintaining mosaic symptoms in *N. benthamiana*.

In summary, our findings demonstrate the multifunctional nature of the 5' end of the PVX genome, specifically the SL1, which can interact with host proteins and maintain the correct balance of viral accumulation during PVX infection by interacting with WRKY1. This may be one of multiple strategies of plant viruses for survival in the systemic host, *N. benthamiana*.

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