V2 of tomato yellow leaf curl virus can suppress methylation-mediated transcriptional gene silencing in plants

Bi Wang,† Fangfang Li,† Changjun Huang, Xiuling Yang, Yajuan Qian, Yan Xie and Xueping Zhou

State Key Laboratory of Rice Biology, Institute of Biotechnology, Zhejiang University, Hangzhou, PR China

Tomato yellow leaf curl virus (TYLCV) is a DNA virus belonging to the genus Begomovirus. TYLCV replicates using double-stranded DNA intermediates that can become the target of plant transcriptional gene silencing (TGS). Here, we show that the V2 protein of TYLCV can suppress TGS of a transcriptionally silenced green fluorescent protein (GFP) transgene in Nicotiana benthamiana line 16-TGS. Through bisulfite sequencing and chop-PCR, we demonstrated that the TYLCV V2 protein can reverse GFP transgene silencing by reducing the methylation levels in the 35S promoter sequence. Both AtSN1 and MEA-ISR loci in Arabidopsis thaliana were previously reported to be strongly methylated, and we show that the methylation status of both loci was significantly reduced in TYLCV V2 transgenic Arabidopsis plants. We conclude that TYLCV can efficiently suppress TGS when it infects plants, and its V2 protein is responsible for the TGS suppression activity.

Cytosine DNA methylation is a conserved epigenetic silencing mechanism modulating many important biological processes (Bird, 2002; Goll & Bestor, 2005; Zhang et al., 2006) and a defence against biotic stresses such as geminivirus infection (Bisaro, 2006; Raja et al., 2010; Yang et al., 2011). Geminiviruses (family Geminiviridae) are a group of plant-infecting viruses containing circular, single-stranded DNA (ssDNA) genomes packaged into twinned particles (Dry et al., 1993; Fauquet et al., 2008; Zhang et al., 2009; Zhou, 2013). Geminiviruses are transmitted by whiteflies or leafhoppers and can cause significant damage to agronomically important crops (Navot et al., 1991; Hanley-Bowdoin et al., 2004; Rojas et al., 2005). Geminiviruses are classified into four genera (Mastrevirus, Curtovirus, Begomovirus, and Topocuvirus) and replicate in the plant cell nucleus using double-stranded DNA (dsDNA) intermediates as their replication and transcription templates (Pilartz & Jeske, 1992, 2003; Fauquet et al., 2008).

Recent studies have demonstrated that plants employ an RNA-directed methylation strategy to control transcriptional gene silencing (TGS) and use it as a defence mechanism against geminivirus infection (Raja et al., 2008; Rodriguez-Negrete et al., 2009). As a counter defensive measure, geminiviruses express unique proteins to serve as TGS suppressors. For example, the AC2/AL2 proteins encoded by cabbage leaf curl virus and tomato golden mosaic virus, the C2/L2 proteins encoded by beet curly top virus and beet severe curly top virus, and the βC1 protein encoded by tomato yellow leaf curl China betasatellite (TYLCCNB) can decrease DNA methylation and suppress TGS by interfering with the methyl cycle (Buchmann et al., 2009; Yang et al., 2011; Zhang et al., 2011). A recent report indicated that geminivirus replication-associated protein (Rep, also known as C1, AL1 or AC1) could also suppress TGS by reducing the expression of plant DNA methyltransferases (Rodriguez-Negrete et al., 2013).

Tomato yellow leaf curl virus (TYLCV) is a member of the genus Begomovirus and contains a single genome component with six open reading frames (ORFs). Two ORFs (V1 and V2) are located on the viral strand and the other four ORFs (C1, C2, C3 and C4) are on the complementary strand (Zhang et al., 2009). To investigate if TYLCV can suppress TGS, we utilized the Nicotiana benthamiana 16-TGS line. This N. benthamiana line contains a green fluorescent protein (GFP) transgene whose Cauliflower mosaic virus 35S promoter was transcriptionally silenced after infection with a tobacco rattle virus-based vector expressing a portion of the 35S promoter sequence, and the silenced 35S promoter was shown to be highly methylated (Buchmann et al., 2009). Plants at the 4–6 leaf stage were inoculated with a TYLCV infectious clone, pBINPLUS-SH2-1.4A, as previously described (Zhang et al., 2009). The inoculated plants were examined under UV light at

†These authors contributed equally to this paper.

One supplementary table and one supplementary figure are available with the online version of this paper.
2–6 weeks post-inoculation (p.i.). In the TYLCV-infected 16-TGS plants, green fluorescence was observed in the upper systemic leaves, especially phloem tissues (Fig. 1a) coupled with downward leaf curling symptoms and stunting of the plants (data not shown). Northern blot analysis, performed as described previously (Cui et al., 2005), showed that GFP mRNA accumulated in the TYLCV-infected plants (Fig. 1b). Accumulation of GFP was also confirmed in the TYLCV-infected plants by Western blot analysis using a GFP-specific antibody as described previously (Zhang et al., 2012; Fig. 1c). These results indicate that TYLCV can suppress TGS in N. benthamiana line 16-TGS.

To identify the TGS suppressor(s) encoded by TYLCV, a potato virus X (PVX) expression vector was employed to express individual TYLCV ORFs. The TYLCV ORFs were individually amplified from the full-length infectious clone of TYLCV, pBINPLUS-SH2-1.4A, using specific primers listed in Table S1 (available in JGV Online), and then inserted into the PVX vector pgR106 as described (Lu et al., 2003; Yang et al., 2011). The resulting PVX vectors were individually transformed into Agrobacterium strain GV3101 by electroporation.

16-TGS plants were agroinoculated with various PVX vectors expressing different TYLCV ORFs. A PVX vector expressing βC1 (Yang et al., 2011) was used as a positive control and the PVX vector pgR106 was used as a negative control. The development of systemic mosaic symptoms in leaves can be observed after PVX infection, at 7 days p.i.. By 14 days p.i., plants inoculated with the vectors expressing TYLCV-ORF displayed various disease symptoms. For example, PVX-V1 induced mild crumpling, downward curling and mosaic symptoms in leaves. PVX-V2 induced plant stunting, severe curling, vein yellowing and necrotic lesions in the systemic leaves. PVX-C4 induced chlorosis and mild leaf curling symptoms; the others (PVX-C1, PVX-C2 and PVX-C3) induced mild vein yellowing in leaves as previously described (data not shown, Amin et al., 2011). The PVX-βC1-infected plants displayed severe leaf curling, veination and plant stunting as previously described (data not shown, Yang et al., 2011).

At 2–6 weeks p.i., green fluorescence became apparent in leaves, especially phloem tissues of the PVX-V2- and PVX-βC1-infected plants (Fig. 1a). Northern blot analysis revealed that GFP mRNA accumulated in the plants infected with PVX-V2 or PVX-βC1 by 20 days p.i. (Fig. 1b). In addition, a weak accumulation of GFP mRNA was observed in the PVX-C4-infected plants (Fig. 1b). GFP gene expression was, however, not detected in plants infected with PVX, PVX-V1, PVX-C1, PVX-C2 or PVX-C3 (Fig. 1b). Western blot assay confirmed the Northern blot results (Fig. 1c), indicating that the TYLCV V2 protein is a suppressor of TGS of the GFP transgene.

To determine the suppression level of TGS in the PVX-V2-infected N. benthamiana 16-TGS plants, we carried out bisulfite sequencing of the 35S promoter of the GFP transgene. Genomic DNA was extracted from at least three PVX- and PVX-V2-infected 16-TGS plant leaf samples. Bisulfite modification was done using the EZ DNA Methylation Gold™ kit (Zymo Research) to convert unmethylated cytosine to uracil. The 35S promoter sequence was then amplified by PCR using primers specific for sequences with less cytosine (Table S1). The PCR products were cloned into the pGEM-T Easy Vector (Promega), and approximately 20 individual clones were

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**Fig. 1.** In planta assay of TGS suppression. (a) Nicotiana benthamiana 16-TGS plants were inoculated with TYLCV, PVX, PVX-V1, PVX-V2, PVX-C84S/C86S, PVX-C1, PVX-C2, PVX-C3, PVX-C4 or PVX-βC1. Inoculated plants were photographed under UV light at 20 days p.i. (b) Northern blot assay of GFP mRNA accumulation in inoculated plants. Ethidium bromide-stained RNAs were used as RNA loading controls. (c) Western blot assay of GFP accumulation in inoculated plants. Coomassie-stained Rubisco was used as a protein loading control.
sequenced using the M13 reverse primer (Yang et al., 2011). Student’s \( t \)-test was used to compare methylation values from individual clones. The 35S promoter sequence has eight CG, 11 CNG, and 50 CHH sites (Figs 2a and S1a). Our results indicate that the total number of methylated cytosine residues was reduced by about 6% (Fig. S1b). The overall reduction in methylation was not significant. This result might have been due to the tissue-specific expression of the PVX vector (Cruz et al., 1998; Roberts et al., 1997). In order to reduce a potential dilution effect, we extracted DNA samples from virus-enriched vascular tissues instead of whole-leaf tissues. As expected, we obtained much better results of reduction in methylation in either TYLCV- or PVX-V2-infected 16-TGS plants (Fig. 2a–c). To further confirm the effect of V2 on demethylation, a previously described chop-PCR (Qian et al., 2012) was used to examine the methylation status of 35S promoter in TYLCV- and PVX-V2-infected 16-TGS plants. Genomic DNA used for chop-PCR was also extracted from virus-enriched vascular tissues and then digested with the methylation-sensitive endonuclease HindIII, whose cleavage activity is blocked by methylation of cytosine in its target site (G/ANTC). Following digestion, PCR assays were performed to amplify the target sequences. The results showed that the accumulation of 35S promoter in TYLCV- and PVX-V2-infected plants was much less than that in control plants (Fig. 2d). These results indicate that the 35S promoter was demethylated in TYLCV- and PVX-V2-infected plants.

To further explore the mechanism of V2-caused suppression of TGS, we expressed TYLCV V2 in Arabidopsis thaliana through stable transformation. The V2 gene was inserted behind a 35S promoter in the pBA002 vector using the Gateway cloning method (Invitrogen) as described by Zhang et al. (2005). The pBA002-V2 construct was transformed into the Agrobacterium strain GV3101 and then into the Colombia ecotype of A. thaliana (Col-0) through the floral dip transformation procedure as described by Clough & Bent (1998). The resulting T1 seeds were selected on Murashige and Skoog medium (Invitrogen) containing 10 \( \mu \)g ml\(^{-1}\) Basta and then confirmed by PCR using genomic DNA (data not shown). Expression levels of the V2 protein in various transgenic lines were determined with Western blot analysis using an antibody specific for the TYLCV V2 (Fig. 3c). Like the PVX vector-mediated V2 overexpression in N. benthamiana, the vegetative tissues of V2 transgenic Arabidopsis plants did not show any virus-like symptoms. Flowering of the V2 transgenic Arabidopsis was, however, delayed when compared with the wild-type (WT) Col-0 plants (Fig. 3a, b). Since there is increasing evidence that DNA methylation is an important regulatory factor during flower development, this late flowering phenotype suggests that V2 may play a role in the DNA methylation process (Koornneef et al., 1998; Cao & Jacobsen, 2002b).

To determine the effect of V2 on methylation and epigenetic TGS, two independent V2 expression transgenic lines were selected and examined for the methylation status of AtSN1 (a transposon) and MEA-ISR (tandem repeats near the MEA gene) loci through bisulfite sequencing. Both AtSN1 and MEA-ISR loci are known to be silenced in the WT Arabidopsis through methylation (Cao & Jacobsen, 2002a; Yang et al., 2011). The AtSN1 sequence has four CG, seven CNG and 46 CHH sites, and the MEA-ISR sequence has 10 CG, two CNG and 26 CHH sites. DNA was extracted from V2 transgenic and WT plants and treated with the bisulfite reagent as described above. Primers AtSN1-Bis-F and AtSN1-Bis-R, and MEA-ISR-Bis-F and MEA-ISR-Bis-R (Table S1), were used to amplify AtSN1 and MEA-ISR respectively as described by Johnson et al. (2008). The results indicated a significant reduction of DNA methylation at both the AtSN1 and MEA-ISR loci in the V2 transgenic plants compared with that in the WT control plants (Fig. 3d), indicating that expression of V2 can reverse TGS in those plants.

The C84S/C86S amino acid substitution in the V2 of tomato leaf curl virus attenuated viral infection in N. benthamiana (Paddadam et al., 1996) and this mutation of TYLCV V2 abolished its ability to suppress post-transcriptional gene silencing (PTGS) (Zrachya et al., 2007; Glick et al., 2008). We obtained a TYLCV V2 C84S/C86S substitution mutant through PCR using mutagenic overlap primers (Table S1) and the resulting PCR fragment was cloned into the PVX-based expression vector. The 16-TGS plants were then inoculated with the mutant V2-expression PVX vector (PVX-C84S/C86S). By 14 days p.i., plants infected with PVX-C84S/C86S displayed similar symptoms as that shown in the PVX-V2-infected plants (data not shown). By 2 to 6 weeks post-inoculation, the PVX-C84S/C86S-infected plants showed green fluorescence in their systemic leaves, especially in phloem tissues (Fig. 1a), indicating that the TGS in 16-TGS plants was suppressed by the C84S/C86S mutant. As expected, Northern and Western blot assays showed that both GFP mRNA and protein had accumulated in the PVX-C84S/C86S-infected plants (Fig. 1b, c), indicating that the C84S/C86S amino acid substitution in TYLCV V2 did not impede its TGS suppression activity.

Virus-encoded TGS suppressors act through divergent mechanisms. For example, the AC2/AL2 and C2/L2 proteins can interact with adenosine kinase to reduce the levels of cytosine methylation of CNG and CHH. The \( \gamma \)C1 protein encoded by TYLCNB can interact with S-adenosyl homocysteine hydrolase, and reduce all contexts of cytosine methylation. In this paper, we show that the V2 protein encoded by TYLCV can reduce all contexts of cytosine methylation of 35S promoter in 16-TGS plants. However, the reduced methylation observed in AtSN1 and MEA-ISR was only detected at CHH sites in TYLCV V2 transgenic Arabidopsis. There are two explanations of this phenomenon. Firstly, there were many more CHH than CG or CNG sites in AtSN1 and MEA-ISR. Secondly, the experiments were performed with different sequences (genes) in different plant species (N. benthamiana and
Fig. 2. Cytosine methylation levels in the 35S promoter from *Nicotiana benthamiana* 16-TGS plants inoculated with pBINPLUS, TYLCV, PVX or PVX-V2. (a) Diagram of 35S promoter with nucleotide positions. The 338 nt region amplified by PCR for bisulfite sequencing is marked. (b) Cytosine methylation profiles assessed by bisulfite sequencing. The Circles represent cytosine residues in the 35S promoter and are colour coded according to the sequence context (red for CG, blue for CNG, and green for CHH). Each line represents the sequence of an individual clone. (c) Percentage of methylated cytosine in promoter sites. Student’s *t*-test was performed using the methylation values from individual clones. Double asterisks indicate a significant difference (*P* < 0.01) between the two paired samples. (d) Analysis of DNA methylation level of the 35S promoter by chop-PCR. For all the experiments, genomic DNA was extracted from vascular tissues of inoculated plants.
A. thaliana), and cytosine demethylation may occur differently with V2 expression. A previous study showed that highly methylated cytosine occurred exclusively in the 35S promoter of transgenes in gentians but was much less frequent in tobacco or in other promoter-driven transgenes (Mishiba et al., 2005).

TYLCV V2 was also found to suppress PTGS in infected plants via its interaction with a host RNA silencing machinery component, SGS3 (Zrachya et al., 2007; Glick et al., 2008). Interestingly in this study, the C84S/C86S amino acid substitution, which was shown to be essential for PTGS activity and competition of siRNA binding with SGS3 in vitro (Zrachya et al., 2007; Glick et al., 2008), did not affect suppression of TGS by V2. These new findings indicate that the TYLCV V2 protein may contain different domain(s) necessary for its TGS and PTGS suppression activities. Determining the locations of these domains in TYLCV V2 and their biochemical properties requires further investigation.

Taken together, the results presented in this paper demonstrate that the TYLCV V2 protein can suppress TGS when expressed through the PVX vector or expressed stably in transgenic Arabidopsis, and is presumably responsible for the suppression of TGS during TYLCV infection.

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References


