Variability in the level of RNA silencing suppression caused by triple gene block protein 1 (TGBp1) from various potexviruses during infection

Hiroko Senshu,1 Johji Ozeki,1 Ken Komatsu,1 Masayoshi Hashimoto,1 Kouji Hatada,1 Michiko Aoyama,1 Satoshi Kagiwada,2 Yasuyuki Yamaji1 and Shigetou Namba1

1Laboratory of Plant Pathology, Department of Agricultural and Environmental Biology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
2Department of Clinical Plant Science, Faculty of Bioscience and Applied Chemistry, Hosei University, Tokyo, Japan

RNA silencing is an important defence mechanism against virus infection, and many plant viruses encode RNA silencing suppressors as a counter defence. In this study, we analysed the RNA silencing suppression ability of multiple virus species of the genus Potexvirus. Nicotiana benthamiana plants exhibiting RNA silencing of a green fluorescent protein (GFP) transgene showed reversal of GFP fluorescence when systemically infected with potexviruses. However, the degree of GFP fluorescence varied among potexviruses. Agrobacterium-mediated transient expression assay in N. benthamiana leaves demonstrated that the triple gene block protein 1 (TGBp1) encoded by these potexviruses has drastically different levels of silencing suppressor activity, and these differences were directly related to variations in the silencing suppression ability during virus infection. These results suggest that suppressor activities differ even among homologous proteins encoded by viruses of the same genus, and that TGBp1 contributes to the variation in the level of RNA silencing suppression by potexviruses. Moreover, we investigated the effect of TGBp1 encoded by Plantago asiatica mosaic virus (PlAMV), which exhibited a strong suppressor activity, on the accumulation of microRNA, virus genomic RNA and virus-derived small interfering RNAs.

INTRODUCTION

RNA silencing is an RNA-guided gene regulatory mechanism that operates in a wide variety of eukaryotic organisms. RNA silencing begins with processing of an RNA trigger into small RNAs (~21–30 nt) by the RNase III-type enzyme Dicer (Bernstein et al., 2001). In plants, small RNAs are divided into two classes: small interfering RNAs (siRNAs) and microRNAs (miRNAs) (Mallory & Vaucheret, 2006; Brodersen & Voinnet, 2006). siRNAs are generated from double-stranded RNAs (dsRNAs) which are usually derived from transgenes, viruses or endogenous non-coding RNA genes. On the other hand, miRNAs are generated from genome-encoded precursor RNAs with imperfect stem–loop structures. These small RNAs are then incorporated into multicomponent RNA-induced silencing complexes (RISC), which contain an Argonaute (AGO) family protein and lead to homologous RNA cleavage or translational repression, and (or) DNA/chromatin methylation (Hammond, 2005; Xie et al., 2004; Chapman & Carrington, 2007).

One of the important roles of RNA silencing in plants is antiviral defence (Ratcliff et al., 1997; Vance & Vaucheret, 2001; Baulcombe, 2004; Ding & Voinnet, 2007). During virus infection, dsRNAs derived from viral replication intermediates or highly structured viral genomic RNAs trigger RNA silencing directed against the viruses. Consequently, initially symptomatic plants recover from infection and become resistant to secondary infection with homologous viruses. To counteract RNA silencing, viruses have evolved RNA silencing suppressors. More than 40 RNA silencing suppressors have been identified in plant, animal, insect and fungal viruses (Li & Ding, 2006). These suppressors do not have obvious sequence similarity to one another, and they block host RNA silencing by targeting different steps in the silencing pathway. For example, tombusviral p19 binds siRNAs to interfere with their incorporation into the RISC (Silhavy et al., 2002; Lakatos et al., 2004). Similarly, many viral suppressors are known to bind dsRNAs (Merai et al., 2006; Lakatos et al., 2006). Furthermore, some viral suppressors interact with the host protein components of the silencing machinery. Both the
2b protein encoded by cucumber mosaic virus (CMV) and the potexvirus P0 protein target AGO1 (Zhang et al., 2006; Baumberger et al., 2007; Bortolamiol et al., 2007), and p38 of turnip crinkle virus affects Dicer-like 2 and 4 (Deleris et al., 2006). Besides interfering with host antiviral RNA silencing pathways, some viral suppressors also affect the miRNA pathway that is mainly involved in the developmental regulation of host plants, suggesting that both pathways share common components (Chapman et al., 2004; Dunoyer et al., 2004). Most of these viral suppressors were originally identified as pathogenicity determinants (Voinnet, 2005; Li & Ding, 2006). Therefore, the identification and functional analysis of viral silencing suppressors provide important clues to understanding the mechanisms of successful virus infection, host range determination and virus virulence.

Members of the genus Potexvirus have a monopartite, positive-strand RNA genome containing five open reading frames (ORFs) (Verchot-Lubicz et al., 2007). ORF1 encodes a viral replicase and ORF5 encodes a coat protein (CP). Three overlapping ORFs in the central region of the genome, ORFs 2–4, encode movement proteins named triple gene block (TGB) protein 1, TGBp2 and TGBp3 (Verchot-Lubicz, 2005). Functional analysis of the encoded proteins of potato virus X (PVX), the type species of the genus, showed that TGBp1 also functions as an RNA silencing suppressor (Voinnet et al., 2000). To date, PVX TGBp1 has been the only suppressor of RNA silencing identified in the genus. Voinnet et al. (1999) reported that closely related members of the genus Potexvirus showed strong variations in the ability to suppress RNA silencing in Nicotiana benthamiana. To date, however, neither the viral factors nor the mechanisms underlying these variations have been elucidated.

Plantago asiatica mosaic virus (PlAMV) is a member of the genus Potexvirus which has a relatively broad host range including the monocotyledon lily, dicotyledon Plantago asiatica L. and the woody plant Nandina domestica (Komatsu et al., 2008; Kostin & Volkov, 1976; Solovyev et al., 1994; Hughes et al., 2005). We previously determined the complete genomic sequence of PlAMV lily isolates (Komatsu et al., 2008). In this study, we analysed the RNA silencing suppression ability of PlAMV, PVX, asparagus virus 3 (AV3), white clover mosaic virus (WCIMV) and tulip virus X (TVX). These potexviruses showed variations in their ability to suppress RNA silencing. Moreover, we suggest that these variations result from the suppressor activity of TGBp1. Our findings demonstrate that homologous proteins encoded by different virus species of the same genus suppress RNA silencing at different activity levels, although they may employ a similar mechanism.

**METHODS**

**Plasmid constructs.** The ORF2s encoding TGBp1 of PVX (Baulcombe et al., 1995), PlAMV-Li1 (Ozeki et al., 2006), AV3 (Hashimoto et al., 2008), WCIMV-RC (Nakabayashi et al., 2002) and TVX-J (Yamaji et al., 2001), as well as CMV-Y (Suzuki et al., 1991) and tobacco etch virus strain AT (TEV-AT, ATCC PV-633; Pirrone & Thornbury, 1983) ORFs encoding 2b and HC-Pro, respectively, were PCR-amplified from the cDNA clones with 5’- and 3’-specific primers containing suitable restriction sites. The PCR products were cloned into the binary vector pCAMBIA1301 using Ncol and PmeCI sites. Plasmid pBin19 containing the sequence of the tomato bushy stunt virus (TBSV) p19 in Agrobacterium tumefaciens strain C58C1 was kindly provided by Dr D. C. Baulcombe (Sainsbury Laboratory, Norwich, UK). To generate TGBp1 constructs with a 7b epitope tag, which corresponds to the first 11 residues of the bacteriophage T7 gene 10 capsid protein, TGBp1 ORFs were PCR-amplified using primers that introduce the 7b epitope tag at the C terminus of the ORFs, separated by a CslI site, and contain suitable restriction sites. The PCR products were cloned into pCAMBIA1301 using the same sites.

For construction of pBl–green fluorescent protein (GFP), full-length GFP cDNA was amplified by RT-PCR using 5’- and 3’-specific primers containing suitable restriction sites from total RNA extracted from GFP-expressing transgenic N. benthamiana line 16c plants (Brignetti et al., 1998; kindly provided by Dr D. C. Baulcombe) and cloned into the binary vector pBI121 using BanHI andSacI sites. To produce pBl–dsGF, the ‘GF’ cDNA fragment was PCR-amplified with primers: Xbbm-16c1F (5’-TTTTCTAGAGAATAGAATGAG-3’) and Xbbm-16c1R (5’-TTTTCTAGATGAGAATAGAATGAG-3’). The BamHI/SacI-digested sense GF fragment and the Xbal/SnaBI-digested antisense GF fragment were inserted in inverted-reverse orientation around an intron derived from the phaseolin gene in pE7133-GUS (Mitsurua et al., 1996). The resulting plasmid was digested with EcoRV (blunt-end) and EcoRI to generate inverted-repeat GF fragments. The inverted-repeat GF fragment was cloned into pBI121 digested with HindIII (followed by filling in with the Klenow fragment) and EcoRI.

For construction of PlAMV–ΔTGBp2, two cDNA fragments were amplified from the PlAMV-Li1 cDNA clone, one using primer 2606F (5’-CAAGTCTCCACATAGTGG-3’) and LidelTG2R (5’-CAGCTTGGGGTCCCGGAGG-3’) and LidelTG2R (5’-CAGCTTGGGGTCCCGGAGG-3’), the mutated start codon of TGBp2 ORF is underlined), and the other using LidelTG2F (5’-ACCGGAGCCCCAAATCGACTGCGGAGG-3’) and LidelTG2R (5’-ACCGGAGCCCCAAATCGACTGCGGAGG-3’), which is the reverse complement of LidelTG2R) and 6095R (5’-CAGAATTCGATGCGGAGG-3’) and p38 (nt 3591) sites. For construction of PlAMV–ΔTGBp1ATGBp2, these procedures were repeated using pAMV–ΔTGBp2 as a PCR template instead of pLi1 and different primers: LidelTG1R (5’-CGTCGATGCGGAGG-3’) and p38 (nt 3591) sites. For construction of PlAMV–ΔTGBp1ATGBp2, these procedures were repeated using pAMV–ΔTGBp2 as a PCR template instead of pLi1 and different primers: LidelTG1R (5’-CGTCGATGCGGAGG-3’) and p38 (nt 3591) sites.

**Viruses and plant inoculation.** Inoculation of wild-type N. benthamiana with PVX or PlAMV-Li1 was done by Agrobacterium-mediated inoculation (Takahashi et al., 2006; Ozeki et al., 2006). Bacterial cells were harvested and resuspended in infiltration buffer to a final OD600 = 1.0 for infiltration. For AV3 and WCIMV-RC inoculation, carborundum-dusted leaves of N. benthamiana were rubbed with an extract of each virus-infected N. benthamiana plant prepared by grinding infected leaf tissue in 0.1 M phosphate buffer (pH 7.0) (Hashimoto et al., 2008; Nakabayashi et al., 2002).

**Plant materials and agroinfiltration.** Plants were maintained in growth chambers at 20–25°C throughout the assays. A. tumefaciens inoculation was performed as described previously (Takahashi et al., 2006). Bacterial cells were harvested and resuspended in infiltration buffer to a final OD600 = 1.0 for general infiltration. In co-infiltration
experiments, equal volumes of each suspension were mixed before infiltration. GFP fluorescence was observed under long-wavelength UV-light (B-100 Black-Ray long wave UV lamp; UV Products) and photographed using a Canon EOS Kiss digital camera with a yellow filter.

**Immunoblot analysis.** Immunoblot analysis was performed as described previously (Yamaji et al., 2006). Total protein extracted from infiltrated leaf tissue with 8 M urea was separated on a SDS-polyacrylamide (15 %) gel, blotted onto a PVDF membrane (Millipore), and detected by an anti-T7 antibody (Chemicon). Equal volumes of total protein were separated on a SDS-polyacrylamide (15 %) gel and stained with Coomassie brilliant blue as loading controls.

**RNA isolation and analysis.** Infiltrated leaf tissue was ground to a fine powder in liquid nitrogen, and total RNA was extracted with Isogen solution (Nippon Gene) according to the manufacturer’s protocol. After fractionation of total RNA with 8 M LiCl, the pellet fraction containing high-molecular-mass RNA (HMM-RNA) was resuspended in water. The supernatant fraction containing low-molecular-mass RNA (LMM-RNA) was ethanol-precipitated and then resuspended in water. For Northern blot analysis of GFP mRNA, 6 μg HMM-RNA was separated on a 1 % agarose gel containing 5 % formaldehyde and transferred to a positively charged nylon membrane (Roche Diagnostics). For analysis of siRNAs or miR159, 8 μg LMM-RNA was separated on an 8 M urea/16 % polyacrylamide gel and transferred to a membrane. The membranes were hybridized with digoxigenin (DIG)-labelled complementary RNA probes corresponding to a fragment of the GFP sequence (nt 448–717), or with DIG-labelled DNA nucleotides complementary to the miR159 sequence. The hybridized membranes were then immunodetected with anti-DIG Fab fragments coupled to alkaline phosphatase (Roche Diagnostics) and visualized with a chemiluminescent substrate (CDP-Star; New England BioLabs) using the luminescent image analyser LAS-1000 plus (Fujifilm). Accumulation levels of each lane were calibrated using ImageJ version 1.41 software.

**RESULTS**

**Members of the genus Potexvirus show various degrees of RNA silencing suppression**

In this study, we used PVX, the type species of the genus Potexvirus, together with four potexviruses isolated in Japan: PIAMV, AV3, WClMV and TVX. First, we examined the silencing suppression activity during virus infection. Although PVX, PIAMV, AV3 and WClMV infected *N. benthamiana* systemically and caused symptoms by 7–10 days post-inoculation, TVX did not cause symptoms, and RT-PCR analysis failed to detect this virus in the upper leaves (data not shown). Therefore, we used PVX, PIAMV, AV3 and WClMV for this assay. The test for silencing suppression was based on previously described experiments using GFP-expressing transgenic *N. benthamiana* line 16c plant seedlings [Fig. 1a(i); Brigneti et al., 1998]. Systemic silencing was induced by infiltration with an *A. tumefaciens* strain carrying the homologous GFP sequence under the control of the 35S promoter. At 17 days post-infiltration (p.i.), when the GFP transgene was completely silenced and the plants appeared uniformly red under UV illumination due to chlorophyll fluorescence [Fig. 1a(ii)], these plants were inoculated with PVX, PIAMV, AV3 and WClMV. At 14 days post-inoculation (i.e. 31 days p.i.), the extent of green fluorescence was assessed under UV illumination. In all PIAMV-infected plants (10 out of 10), strong green fluorescence was observed in the mesophyll tissues of new emerging leaves [Fig. 1a(iii)]. In 8 out of 10 PVX-infected plants, faint green fluorescence appeared in the petioles of the upper leaves. However, the fluorescence did not spread into the surrounding tissue [Fig. 1a(iv)]. Most of the AV3-infected plants (8 out of 10) showed no reversal of green fluorescence, although fluorescence was observed only in the growing tips of the other two plants [Fig. 1a(v); note that the bright fluorescence in the AV3-infected plants comes from necrotic autofluorescence of AV3 symptoms]. In WClMV-infected plants (10 out of 10), green fluorescence was observed along the veins of new emerging

---

**Fig. 1.** RNA silencing suppression caused by potexviruses in a GFP-silenced *N. benthamiana* line 16c. (a) *Agrobacterium* strain carrying a homologous GFP sequence was infiltrated into *N. benthamiana* line 16c leaves. After the GFP transgene was systemically silenced at 17 days p.i., each potexvirus was inoculated. Photographs were taken at 14 days p.i. (i) Non-silenced plant, (ii) GFP-silenced plant. (iii–vi) GFP-silenced plant infected with PIAMV (iii), PVX (iv), AV3 (v) or WClMV (vi). (b) Northern blot analysis of GFP mRNA extracted from upper leaves of plants shown in (a). Ethidium bromide-stained rRNA is shown as a loading control.
leaves, although the fluorescence was weaker than in the PIAMV-infected plants [Fig. 1a(vi)]. Northern blot analysis of GFP mRNA extracted from upper leaves of each virus-inoculated plant confirmed the fluorescence observations (Fig. 1b). These results suggest that potexviruses vary significantly in their ability to suppress RNA silencing.

**TGBp1s have various levels of silencing suppressor activity**

It was reported that TGBp1 encoded by PVX is an RNA silencing suppressor (Voinnet et al., 2000). To investigate whether TGBp1s of other potexviruses function as an RNA silencing suppressor, we performed an Agrobacterium-mediated transient expression assay (Johansen & Carrington, 2001). In this assay, suppressor activity on sense transgene-induced RNA silencing (S-RNAi) was tested. TGBp1s of PVX, PIAMV, AV3, WClMV and TVX were cloned into a binary vector, and the resulting plasmids were introduced into Agrobacterium. Each of the bacterial strains was mixed with a strain containing the GFP transgene (pBI–GFP) and then co-infiltrated into leaves of wild-type *N. benthamiana*.

In control experiments, when the β-glucuronidase (GUS) gene (contained in an empty binary vector) and the GFP gene were co-expressed in leaves by agroinfiltration, GFP fluorescence was observed in infiltrated patches at 2–3 days p.i., followed by the decrease and disappearance of fluorescence due to RNA silencing at 4 days p.i. [Fig. 2a(i–v), right upper patches; Johansen & Carrington, 2001]. In contrast, patches expressing the well-characterized RNA silencing suppressor p19 (Voinnet et al., 1999) together with GFP showed bright green fluorescence at 2–3 days p.i., and the high level of fluorescence persisted for at least 12 days [Fig. 2a(i–v), right lower patches]. At 4 days p.i., patches expressing PVX TGBp1 showed obvious GFP fluorescence [Fig. 2a(i), left patch], indicating that PVX TGBp1 has a silencing suppressor activity, as described previously (Bayne et al., 2005). Strikingly, when TGBp1 of PIAMV, WClMV, and TVX was expressed, much stronger GFP fluorescence was observed [Fig. 2a(ii, iv, v), left patches]. Patches expressing AV3 TGBp1 showed slightly stronger GFP fluorescence than those co-expressing GUS and GFP, but much weaker than those expressing PVX TGBp1 [Fig. 2a(iii), left patch].

Northern blot analysis of GFP mRNA extracted from infiltrated patches was performed. High-level accumulation of GFP mRNA was detected in the patches expressing p19,

---

**Fig. 2.** Suppressor activity of potexvirus TGBp1s on S-RNAi. (a) Wild-type *N. benthamiana* leaves were infiltrated with Agrobacterium mixtures containing a vector expressing GFP and either GUS (right upper patches of each panel), p19 (right lower patches), or TGBp1 of PVX (i), PIAMV (ii), AV3 (iii), WClMV (iv), TVX (v) expression vector (left patches). GFP fluorescence was visualized under UV light at 4 days p.i. (b) Northern blot analysis of GFP mRNA (upper panel) and siRNAs (lower panel) extracted from infiltrated patches shown in (a). RNA extracted from uninfiltreted leaves was used as a negative control (–). Ethidium bromide-stained rRNA or low-molecular-mass RNA (LMM-RNA) is shown below each panel as a loading control. The numbers below each lane in the panels refer to accumulation levels relative to GUS, after normalization against the loading control. Molecular size markers are indicated on the right. (c) Expression levels of potexvirus TGBp1. Total protein was extracted from T7 epitope-tagged TGBp1 expressing patches at 2 days p.i. Protein extracted from uninfiltrated leaves was used as a negative control (–). Coomassie brilliant blue-stained total protein was used as a loading control. Potexviruses encoding each TGBp1 are shown at the top of the panels in (b) and (c).
whereas the GFP mRNA level was quite low in the patches expressing GUS (Fig. 2b, upper panel, lanes 2, 3). GFP mRNA accumulation levels in the patches expressing PVX TGBp1 were somewhat higher than in those expressing GUS (Fig. 2b upper panel, lane 4), while in the patches expressing either PIAMV, WCIMV or TVX TGBp1, GFP mRNA levels were much higher than in those expressing GUS and comparable to those in patches expressing p19 (Fig. 2b upper panel, lanes 5, 7, 8). Slightly higher level of GFP mRNA was detected in the patch expressing AV3 TGBp1 compared with the negative control (Fig. 2b upper panel, lanes 6). These results indicate that potexvirus TGBp1s have different levels of silencing suppressor activity. It was also noted that these differences in TGBp1 silencing suppressor activity occurred in parallel with the variation in ability to suppress silencing during virus infection with respect to PVX, PIAMV, AV3 and WCIMV (Fig. 1).

GFP-specific siRNA was also analysed to determine whether the potexvirus TGBp1s affect its accumulation. Inversely correlated with the levels of GFP mRNA, the accumulation levels of GFP-specific siRNAs were significantly lower in patches expressing either p19, PIAMV TGBp1, WCIMV TGBp1 or TVX TGBp1 than in those expressing either GUS, PVX TGBp1 or AV3 TGBp1 (Fig. 2b, lower panel).

This marked difference in suppressor activity of TGBp1 might be related to its expression level. To test this, total protein was extracted from the infiltrated patches and analysed by immunoblot analysis. Each potexvirus TGBp1 construct with a T7 epitope tag was expressed in leaves by agroinfiltration. We confirmed that T7 epitope-tagged TGBp1s showed similar silencing suppressor activity to that of non-tagged proteins (data not shown). Although PIAMV and TVX TGBp1 exhibited much stronger suppressor activity than PVX TGBp1 [Fig. 2a(i, ii, v), and Fig. 2b lanes 4, 5, 8], PVX, PIAMV, and TVX TGBp1 protein accumulated at similar levels (Fig. 2c, lanes 2, 3, 6). WCIMV TGBp1 accumulated at relatively low levels (Fig. 2c lane 5), despite its strong suppressor activity [Fig. 2a(iv), Fig. 2b lane 7]. Regarding AV3 TGBp1, which did not obviously show suppressor activity [Fig. 2a(iii), Fig. 2b lane 6], the protein could rarely be detected (Fig. 2c, lane 4). Collectively, the levels of silencing suppressor activity of potexvirus TGBp1s were not necessarily correlated with their accumulation levels.

**TGBp1 is the only suppressor of RNA silencing in the PIAMV genome**

Since PIAMV showed the strongest suppression ability against RNA silencing during viral infection among all the potexviruses screened (Fig. 1), it seemed possible that other proteins encoded by this virus might contain suppression activity. To this end, we performed the same Agrobacterium-mediated transient expression assay on PIAMV-encoded proteins other than TGBp1. Each protein encoded by PIAMV was expressed by agroinfiltration in leaves of *N. benthamiana* with GFP. At 4 days p.i., only the patches expressing TGBp1 showed significant GFP enhancement, whereas GFP fluorescence nearly disappeared in patches expressing the other four proteins (Fig. 3a). These observations were confirmed by Northern blot analysis of GFP mRNA (Fig. 3b). The results suggest that TGBp1 alone is responsible for the RNA silencing suppression by PIAMV.

**Comparison of TGBp1 with RNA silencing suppressors of viruses in other genera**

To date, PVX TGBp1 has been the only RNA silencing suppressor identified in the genus *Potexvirus*, although its activity was shown to be relatively weak (Chiba et al., 2006). In this study, some potexvirus TGBp1s, including PIAMV TGBp1, showed stronger silencing suppressor activity than PVX TGBp1. Therefore, we compared the suppressor activities of PVX TGBp1 and PIAMV TGBp1 with those of known silencing suppressors from diverse genera. The results suggested that TGBp1 is the only suppressor of RNA silencing in the PIAMV genome.
plant viruses. GFP was co-expressed with either PIAMV TGBp1, PVX TGBp1 (p25), the 2b protein of CMV, HC-Pro of tobacco etch virus (TEV), or p19 of tomato bushy stunt virus (TBSV) by agroinfiltration in leaves of *N. benthamiana*. At 4 days p.i., highly intense GFP fluorescence was observed in patches expressing HC-Pro or p19 [Fig. 4a(v, vi)]. In patches expressing 2b or PIAMV TGBp1, the intensity of GFP fluorescence was slightly lower than in those expressing HC-Pro or p19, but remarkably higher than in those expressing PVX TGBp1 [Fig. 4a(ii, iv)]. GFP mRNA accumulation detected by Northern blot analysis corresponded well to the intensity of GFP fluorescence (Fig. 4b upper panel). GFP mRNA accumulated at high levels in patches expressing p19, HC-Pro, 2b and PIAMV TGBp1, although the levels of GFP mRNA in patches expressing PIAMV TGBp1 and 2b were slightly lower than those in patches expressing p19 and HC-Pro (Fig. 4b upper panel, lanes 3, 5, 6, 7). In patches expressing PVX TGBp1, the GFP mRNA level was higher than in the negative control, but critically lower than in those expressing the other suppressors (Fig. 4b upper panel, lanes 2 and 4). GFP fluorescence disappeared around 7 days p.i. in patches expressing 2b and PIAMV TGBp1, while HC-Pro and p19 showed persistent fluorescence until at least 10 days p.i. (data not shown). The accumulation of GFP-specific siRNAs roughly coincided with suppression levels of GFP fluorescence and GFP mRNA (Fig. 4b lower panel). Remarkably, GFP-specific siRNAs were almost completely eliminated in patches expressing PIAMV TGBp1 (Fig. 4b lower panel, lane 3).

**Effects of PIAMV TGBp1 on different RNA silencing pathways**

PIAMV TGBp1 showed significantly higher suppressor activity than PVX TGBp1. This raises the possibility that PIAMV TGBp1 acts at different steps in the silencing pathway from PVX TGBp1, which is the only characterized suppressor of potexviruses to date. Therefore, we next dissected the mechanism of PIAMV TGBp1-mediated suppression of silencing. We first tested whether PIAMV TGBp1 suppresses the RNA silencing induced by an inverted repeat transgene construct (IR-RNAi). At 4 days p.i., patches of *N. benthamiana* line 16c leaves agroinfiltrated with *Agrobacterium* mixtures containing a vector expressing an inverted-repeat sequence of GFP and either the GUS (left upper patches of each panel), p19 (left lower patches) or TGBp1 expression vector (right patches). PVX TGBp1 (a) and PIAMV TGBp1 (b) were used. GFP fluorescence was visualized under UV light at 4 days p.i.

![Fig. 4](http://vir.sgmjournals.org) Comparison of the suppressor activity of TGBp1s and other viral suppressors on S-RNAi. (a) Suppressor activity of PIAMV TGBp1 (i), PVX TGBp1 (ii), CMV 2b (iv), TEV HC-Pro (v) and TBSV p19 (vi) were assessed by the same *Agrobacterium*-mediated transient expression assay as described in Fig. 2. The GUS expression vector was used as a control (i). Photographs were taken at 4 days p.i. (b) Northern blot analysis of GFP mRNA (upper panel) and siRNAs (lower panel) extracted from infiltrated patches shown in (a). RNA extracted from uninfected leaves was used as a negative control (–). Ethidium bromide-stained rRNA or low-molecular-mass RNA (LMM-RNA) is shown below each panel as a loading control. Accumulation levels were calculated as described in Fig. 2(b) and shown below each lane. Molecular size markers are indicated on the right.

![Fig. 5](http://vir.sgmjournals.org) Effect of potexvirus TGBp1s on IR-RNAi. *N. benthamiana* line 16c leaves were infiltrated with *Agrobacterium* mixtures containing a vector expressing an inverted-repeat sequence of GFP and either the GUS (left upper patches of each panel), p19 (left lower patches) or TGBp1 expression vector (right patches). PVX TGBp1 (a) and PIAMV TGBp1 (b) were used. GFP fluorescence was visualized under UV light at 4 days p.i.
contrast, enhancement of GFP fluorescence was observed in patches co-expressing pBI-dsGF and p19 (Fig. 5, left lower patches). However, in patches co-expressing pBI-dsGF and either PVX or PlAMV TGBp1, enhancement of fluorescence was not observed, and the fluorescence was decreased similarly to negative control patches (Fig. 5, right patches).

Transient expression assays using reporter genes are a rapid and useful method to analyse RNA silencing suppressor activities; however, this differs from the antiviral RNA silencing induced by viruses during virus infection (Voinnet, 2005; Ding & Voinnet, 2007). Thus, we next investigated the effect of TGBp1 on the accumulation of virus genomic RNA and virus-derived siRNAs. For this purpose, we introduced a mutation into TGBp1 of PlAMV (PlAMV-DTGBp1). Since TGBp1 is also involved in virus cell-to-cell movement and a defect of TGBp1 leads to a reduction of virus accumulation in inoculated regions (Verchot-Lubicz, 2005), TGBp2, which is another movement protein, was also deleted in both PlAMV and PlAMV-DTGBp1 to solely evaluate the effect of silencing suppressor activity of TGBp1 on virus accumulation. These PlAMV mutants (designated PlAMV-DTGBp2 and PlAMV-DTGBp1DTGBp2) were inoculated into leaves of N. benthamiana by agroinfiltration. Northern blot analysis of total RNA extracted from the inoculated leaves showed that the accumulation level of PlAMV-DTGBp1DTGBp2 was comparable to that of PlAMV-DTGBp2 (Fig. 6a, upper panel). In addition, there were no significant differences in the levels of these two mutant virus-derived siRNAs (Fig. 6a, lower panel). These results suggest that PlAMV TGBp1 does not significantly affect the accumulation of PlAMV RNA or PlAMV-derived siRNAs.

Since several silencing suppressors have been shown to affect the miRNA pathway (Chapman et al., 2004; Dunoyer et al., 2004), we investigated the effect of PlAMV TGBp1 on miRNA accumulation. We assayed levels of miR159 (Axtell & Bartel, 2005) and miR159* in patches expressing either PlAMV TGBp1 or GUS in N. benthamiana. Expression of PlAMV TGBp1 did not alter accumulation levels of miR159 or miRNA159* (Fig. 6b), indicating that PlAMV TGBp1 does not inhibit the miRNA pathway.

**DISCUSSION**

The ability of potexviruses to suppress RNA silencing varies significantly (Voinnet et al., 1999). However, since the experiments were performed via whole virus infection, neither the suppressor gene nor the gene responsible for the variation in suppression ability was identified. In our study, we also demonstrated that potexviruses showed variations in RNA silencing suppression ability in infected N. benthamiana (Fig. 1). Consistent with the previous report on PVX (Voinnet et al., 2000), TGBp1 itself was the suppressor of RNA silencing in the PlAMV genome (Fig. 3), suggesting that TGBp1 is the commonly conserved silencing suppressor among potexviruses. In fact, our results revealed that TGBp1s derived from PVX, PlAMV,
Silencing suppressor activities of potexvirus TGBp1s

AV3, TVX and WClMV have different levels of RNA silencing suppressor activity (Fig. 2a, b). These results suggest that the variations in the RNA silencing suppression ability of potexviruses during infection were caused by the different suppressor activities of TGBp1s. To date, some homologous proteins encoded by viruses in other genera, such as p19 of tombusviruses and HC-Pro of potyviruses, have been reported to function similarly as an RNA silencing suppressor (Ye et al., 2003; Vargason et al., 2003; Johansen & Carrington, 2001; Mallory et al., 2002). However, the differences in the activities of these suppressors were rarely evaluated. Considering that each potexvirus TGBp1 showed different levels of silencing suppressor activity, there is a need for further comparative analysis of each viral suppressor within a given genus, including p19 and HC-Pro.

In this study, we demonstrated that multiple potexviruses showed strong variations in the ability to suppress RNA silencing and these variations resulted from the suppressor activity of TGBp1. However, the variations of suppressor activity were not necessarily correlated with viral infectivity in N. benthamiana. For example, TVX could not infect N. benthamiana, though TVX TGBp1 showed strong suppressor activity [Figs 1, 2a(v), b lane 8]. In contrast, AV3 consistently infected N. benthamiana, despite its weak suppressor activity of TGBp1 [Figs 1, 2a(iii), b lane 6]. These results suggest that RNA silencing suppressor activity of TGBp1s does not simply affect the viral infection in N. benthamiana. Considering that many silencing suppressors of plant viruses have been identified as ‘pathogenicity determinants’ or ‘symptom determinants’ (Li & Ding, 2006), the silencing suppressor activity of TGBp1s may have roles that enhance the expression of other factors required for infection. However, it is also possible that the silencing suppressor activity of TGBp1s may be required for viral infection in plant species other than N. benthamiana.

Although the accumulation of TGBp1 from PVX, PIAMV and TVX in the transient expression assay was comparable, the silencing suppressor activity of PIAMV and TVX TGBp1 was much stronger than that of PVX TGBp1 (Fig. 2). Moreover, WClMV TGBp1 showed strong suppressor activity, despite its low level of accumulation. Thus, WClMV TGBp1 may be a forceful suppressor. Collectively, these results suggest that the levels of suppressor activity were not necessarily correlated with protein accumulation levels. In contrast, AV3 TGBp1 did not display substantial suppressor activity in this study. This might be due to the reduced stability of the TGBp1s of AV3 (Fig. 2c). Another possibility is that AV3 TGBp1 was indeed stable, but that its suppressor activity was so weak, or absent altogether, that TGBp1 miRNAs were degraded by RNA silencing.

PVX TGBp1 (p25) is one of the well-characterized silencing suppressors. Previous studies using TGBp1-deficient PVX mutants or PVX TGBp1-expressing transgenic Arabidopsis thaliana plants have demonstrated that PVX TGBp1 inhibited the accumulation of transgene-derived siRNAs, but that it neither affected the miRNA pathway nor the trans-acting siRNA pathway (Bayne et al., 2005; Hamilton et al., 2002; Dunoyer et al., 2004; Moissiard et al., 2007). In this work, we showed that PIAMV TGBp1 also completely inhibited the accumulation of transgene-derived siRNAs (Figs 2b, 4b) and had no effect on miRNA accumulation (Fig. 6b). In contrast, other viral suppressors such as 2b, HC-Pro and p19 alter the accumulation of miRNA (Dunoyer et al., 2004; Mallory et al., 2002; Moissiard et al., 2007). Therefore, it is likely that potexviruses employ a different RNA silencing suppression mechanism from that of the well-known viral suppressors. In addition, all the potexvirus TGBp1s examined in this study did not effectively suppress IR-RNAi (Fig. 5, data not shown). This result contradicts previous work on PVX TGBp1, which could suppress RNA silencing induced by inverted repeats (Bayne et al., 2005; Moissiard et al., 2007). Because dsRNAs constructs induce silencing more effectively than sense transgene constructs (Johansen & Carrington, 2001), GFP expression levels could possibly be below the detection limit in our experiment. To date, much effort has been devoted to elucidating the molecular mechanism of RNA silencing suppression by PVX TGBp1. Nevertheless, the component of the host-silencing pathway with which PVX TGBp1 directly interacts has yet to be identified. PIAMV TGBp1, which showed higher suppressor activity than that of PVX in N. benthamiana, may provide a clue to the mechanism of RNA silencing suppression by potexvirus TGBp1.

Both PIAMV and PVX TGBp1 had no effect on viral accumulation in the inoculated cells (Fig. 6a; Angell & Baulcombe, 1999). Thus, potexvirus TGBp1 seems to be dispensable for suppression of antiviral RNA silencing during the initial stages of infection. Consistent with this, previous studies have shown that PVX TGBp1 prevents spread of the silencing signals, and that TGBp1 suppression of RNA silencing is required for the cell-to-cell movement of PVX (Voinnet et al., 2000; Bayne et al., 2005).

Amino acid sequence analysis revealed that there is a high sequence identity between the TGBp1s of PIAMV and TVX (69%). These viruses showed similar levels of suppressor activity (Fig. 2). These findings imply the presence of important amino acids that contribute to suppressor activity in TGBp1. Seven amino acid residues were conserved in potexvirus TGBp1s which showed significant suppressor activity: PVX, PIAMV, TVX and WClMV (Fig. 7, boxed). However, these residues were dispersed and no obvious functional domain involved in RNA silencing suppression was found in TGBp1. This is consistent with previous data from mutagenic analysis of PVX TGBp1 (Bayne et al., 2005). Therefore, the overall protein structure of TGBp1, rather than a particular domain, may play an important role in silencing suppression.

TGB proteins, involved in virus movement, are conserved in the genera Potexvirus, Carlavirus, Allexivirus, Foveavirus,
Hordeivirus, Benyvirus, Pomovirus and Pecluvirus (Morozov & Solovyev, 2003; Verchot-Lubicz, 2005). The results presented here suggest that TGBp1 generally functions as an RNA silencing suppressor in members of the genus Potexvirus. Interestingly, TGBp1 encoded by barley stripe mosaic virus (BSMV, genus Hordeivirus) or peanut clump virus (PCV, genus Pecluvirus) does not function as an RNA silencing suppressor, although it contains NTPase/helicase conservative motifs that cover the entire sequence of potexvirus TGBp1 (Fig. 7; Morozov & Solovyev, 2003). Instead, in these viruses, proteins other than TGBp1, i.e. cb of BSMV and p15 of PCV, have been identified as silencing suppressors (Yelina et al., 2002; Dunoyer et al., 2002). Thus, homologous proteins encoded by members of different genera are not necessarily silencing suppressors. Since phylogenetic analysis of the NTPase/helicase sequence allows clustering of TGBp1 into two major groups corresponding to filamentous viruses (genera Potexvirus, Carlavirus, Allexivirus, Foveavirus and Allexivirus) and rod-shaped viruses (genera Hordeivirus, Benyvirus, Pomovirus and Pecluvirus), it is possible that only TGBp1 of the filamentous viruses can suppress RNA silencing. Further studies of the silencing suppressor activity of TGBp1 in these genera are required.

In this study, some potexvirus TGBp1s showed weak suppressor activity in N. benthamiana, but these TGBp1s may function as an effective silencing suppressor in other plant species. Furthermore, considering that potexviruses with various levels of silencing suppressor activity could infect N. benthamiana efficiently, virus host range determination depends not only on silencing suppressor activity, but also on other factors. Still, our results imply that viruses even in the same genus have evolved diverse mechanisms to counteract host antiviral RNA silencing. Comprehensive analyses of RNA silencing suppressors from closely related viruses in multiple plant species may shed new light on the effect of RNA silencing on host specificity and viral virulence.

ACKNOWLEDGEMENTS

We thank Dr D. C. Baulcombe (Sainsbury Laboratory, Norwich, UK) for providing cDNA clone of PVX, plasmid pBin19, and Nicotiana benthamiana line 16c. This work was supported by a Grant-in-Aid from the Japan Society for the Promotion of Science.

REFERENCES


Silencing suppressor activities of potexvirus TGBp1s


