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Virus survival of RNA silencing without deploying protein-mediated suppression in *Nicotiana benthamiana*

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Abstract RNA silencing represents an evolutionarily conserved defence mechanism that plays a key antiviral role in protecting plants and animals against virus infection. To counterattack, plant, animal and fungal viruses produce proteins capable of suppressing RNA silencing. Here, we report an unprecedented phenomenon that *Potato virus X*, a single-stranded positive RNA virus, is able to survive RNA silencing without deploying protein-mediated anti-silencing by revealing an unexpected symptom re-emergence and re-accumulation of viral RNAs and proteins in plants maintaining strong RNA silencing. Our results provide evidence that a plant virus may have developed a getaway strategy to survive RNA silencing.

Key words: *Potato virus X*; RNA silencing; Suppressor; Survival

1. Introduction

RNA silencing including gene quelling in fungi, RNA interference in animals and post-transcriptional gene silencing in plants represents a sequence-specific RNA degradation mechanism against foreign RNA invasion [9,11,42]. RNA silencing is triggered by double-stranded RNA (dsRNA) that is processed into small interfering RNAs (siRNAs) of 21–25 nucleotides by Dicer, a member of the RNase III family of dsRNA-specific endonucleases [6]. The siRNAs become incorporated into an RNA-induced silencing complex via a Dicer-associated protein R2D2 that links the initiation and execution of RNA silencing [5,6,14–17,30]. As a consequence, degradation of specific target RNA sequences occurs. RNA silencing plays an active role in protecting plants and animals against viral infection. On the other hand, viruses across kingdoms have evolved a counterattack mechanism by encoding suppressor proteins that are capable of suppressing RNA silencing. RNA silencing suppressors that can target various stages of the RNA silencing process were identified from many plant RNA and DNA viruses [48,50]. More recently a suppressor of RNA silencing was also identified from *Flock house virus*, an RNA virus infecting insects [28], and from a fungal dsRNA hypovirus (van Wezel, Nuss and Hong, unpublished data).

Potato virus X (PVX), a member of the *Potexvirus* genus, has a positive sense single-stranded RNA genome encoding five open reading frames (ORFs) [21]. The first ORF at the 5′ terminus is the 166-kDa RNA-dependent RNA polymerase gene and the coat protein (CP) gene locates at the 3′ terminus; between them is the triple-gene block of three overlapping ORFs, which encode proteins of 25, 12 and 8 kDa. These three proteins and the CP are required for cell-to-cell movement, but dispensable for replication [4,25,26,34,45]. The p25 protein is also involved in suppression of the antiviral defence RNA silencing mechanism [49]. PVX has been modified as a vector that is widely used for foreign gene expression and as a functional tool to activate RNA silencing that specifically targets and suppresses gene expression and foreign RNA invading in plants [2,4,10]. Virus-induced RNA silencing, previously referred to as virus-induced gene silencing (VIGS) [40], occurs in plants if the virus possesses sequence similarity to either a transgene or an endogenous nuclear gene [27,29]. Many RNA and DNA viruses have been shown to be able to induce VIGS of a variety of transgenes and endogenous genes in monocot and dicot plants. For examples, tobacco mosaic virus (TMV) [27], PVX [40], tomato golden mosaic virus [24], tobacco rattle virus (TRV) [39], barley stripe mosaic virus [19], and turnip crinkle virus [41] have been developed as VIGS vectors to silence reporter genes encoding green fluorescent protein (GFP), β-glucuronidase, luciferase or neomycin phosphotransferase, and endogenous genes encoding polygalacturonase, phytoene desaturase, the small subunit of rubisco, cellulose synthase or a subunit of the magnesium chelatase (ChlH). Moreover, VIGS has been proved to be very useful in the assignment of function of genes involved in primary and secondary metabolism, development and disease resistance [3,8,23,31,36,39,43]. By a high throughput VIGS analysis, from 4992 *Nicotiana benthamiana* cDNAs, the gene encoding heat shock protein 90 was identified to be associated with viral and bacterial disease resistance in plants [32].

In this report, using PVX-based RNA silencing induction assay, we reveal that PVX is able to survive RNA silencing without deploying protein-mediated anti-silencing in plants.

2. Materials and methods

2.1. Virus infection, virus-induced RNA silencing and plant maintenance

The PVX genome organisation and strategy for replication and gene expression are outlined in Fig. 1. RNA transcripts were produced by in vitro transcription from PVX/GFP construct [47] after linearisation with *Spe*I, and mechanically inoculated onto non-transformed *N. benthamiana* or transgenic line 16c plants carrying a functional *gfp* gene expression cassette [7] as described [10]. Plants were

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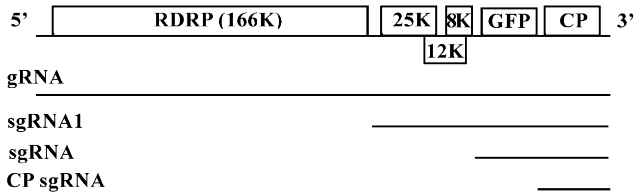

Fig. 1. PVX-based GFP expression vector (PVX/GFP) used for VIGS in plants. The 5′ proximal RNA-dependent RNA polymerase (RDRP, 166 kDa) is translated from viral genomic RNA (gRNA). The three proteins of 25, 12 and 8 kDa encoded by a triple-gene block and involved in cell-to-cell movement of viral RNA are translated from subgenomic RNA 1 (sgRNA1). The 3′ proximal CP is translated from the CP subgenomic RNA (CP sgRNA). Translation of GFP is from an extra sgRNA that is produced under the control of a duplicated CP sgRNA promoter.

maintained in an insect-free growth room at 25°C with continuous lighting to give a 12-h photoperiod. Symptom development was photographed under normal light using a Nikon digital camera Coolpix995. GFP expression and RNA silencing of *gfp* gene were routinely examined under long-wavelength UV and photographed through a yellow filter.

2.2. *RNA extraction and Northern blot analysis*

Total RNAs were extracted from leaf tissues using an RNeasy plant mini kit (Qiagen). To detect viral and *gfp* RNA accumulation, RNA aliquots (5 µg) were fractionated on a 1% formaldehyde agarose gel, transferred to nylon membrane, hybridised with digoxigenin-labelled probes specific to either PVX or *gfp* sequence as described [46] and immunodetected using a DIG DNA labelling and detection kit (Roche).

2.3. *Western blotting assays of PVX CP and GFP expression in plants*

To investigate PVX CP and GFP expression in plants, total protein was extracted from leaf tissues as described [20]. Western blot analyses of protein aliquots (10 µg) were performed with a polyclonal antiserum raised against PVX CP or GFP as described [13], and detected using a goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma) and BCIP/NBT substrates (Roche).

3. Results

3.1. *Dynamics of viral symptom development and virus inducing RNA silencing*

Viruses can be an inducer and a target of RNA silencing [1,2,19,24,27,39,40]. A feature associated with induction of virus-induced RNA silencing in plants is the 'symptom recovery' phenotype following initial viral infection, which results in silenced plant tissues 'free' of viruses [38]. However, during a course of RNA silencing induction, an unprecedented phenomenon was observed that a plant virus survived RNA silencing independent of the protein-mediated anti-silencing mechanism. Transgenic *N. benthamiana* line 16c plants carrying a constitutive GFP expression cassette were inoculated with PVX/GFP. PVX/GFP inoculation induced local chlorotic lesions on inoculated leaves 3–5 days post-inoculation (dpi) and systemic mosaic and chlorotic symptoms on younger leaves 6–7 dpi. In addition to *gfp* transgene expression, strong local and systemic expression of GFP from PVX/GFP was found in symptomatic tissues 10–14 dpi showing green fluorescence under long-wavelength UV illumination (Fig. 2A,D). At this stage, sporadic silencing of expression of the *gfp* transgene and the *gfp* gene in the PVX genome occurred in young leaves (Fig. 2D). Silencing became more pronounced and systemic silencing was complete at 18 dpi. *Gfp*-silenced line 16c plants displayed only red autofluorescence of chlorophyll under UV light. Consistent with the occurrence of RNA silencing, PVX/GFP-inoculated line 16c plants gradually recovered from the initial infection and were symptomless on newly emerging leaves at 18 dpi. However, the recovery endured only temporarily. Although strong *gfp* RNA silencing was maintained throughout whole plants, these 'symptom recovery' leaves re-developed chlorotic and mosaic lesions at 21 dpi (Fig. 2B,E), and severe mosaics and chlorosis with sporadic necrosis at 28 dpi (Fig. 2C,F). The sporadic necrosis on new symptomatic leaves produced bright green spots under long-wavelength UV light (Fig. 2F). They were not the manifesta-

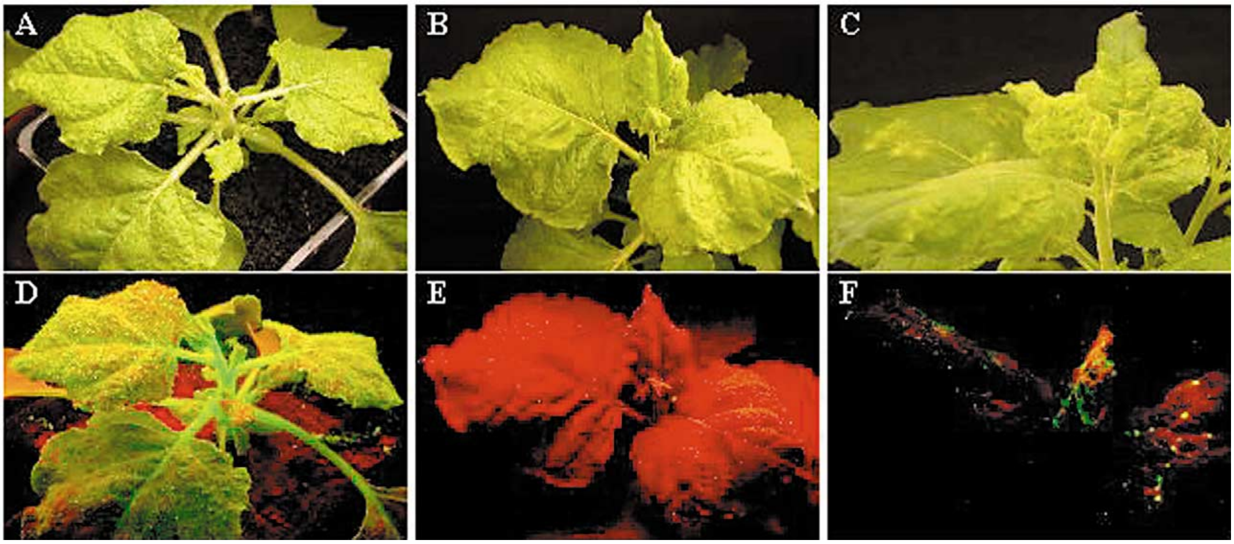

Fig. 2. Systemic symptom re-emergence and RNA silencing in line 16c plants inoculated with recombinant virus PVX/GFP. Plants were photographed 14, 21 and 28 dpi at different angles to show the effect of symptom development (A–C) and RNA silencing (D–F) under normal light or long-wavelength UV illumination through a yellow filter, respectively. *Gfp*-silenced plant tissues are red chlorophyll fluorescence and necrotic tissues bright autofluorescence.

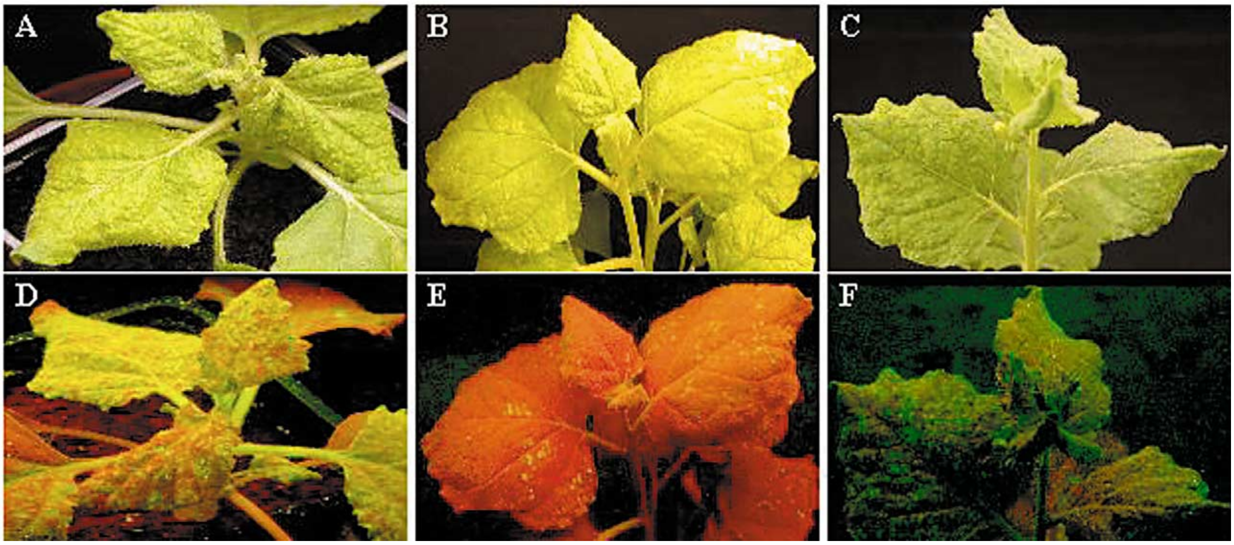

Fig. 3. Virus survival of RNA silencing in non-transformed *N. benthamiana* plants. Plants were inoculated with recombinant virus PVX/GFP and photographed at 14 (A,B), 21 (B,E) and 28 dpi (C,F) at different angles to show the effect of symptom development (A–C) and GFP expression (D–F) under normal light or long-wavelength UV illumination through a yellow filter, respectively. GFP fluorescence appears green and non-GFP plant tissues show red chlorophyll fluorescence.

tion of transient GFP expression from PVX/GFP or endogenous GFP reactivation, consistent with Western blot analysis of GFP production (Fig. 6B). In a parallel experiment, a similar pattern of initial development, then recovery, and later on re-emergence of symptoms (Fig. 3A–C) and of GFP expression (Fig. 3D–F) were observed in non-transformed *N. benthamiana* plants inoculated with PVX/GFP. The reduction of GFP production from PVX/GFP at 21 dpi was obvious and only a limited number of lesions on young leaves that were associated with PVX/GFP infection showed green fluorescence (Fig. 3B,E). It is worth noting that PVX encodes an RNA silencing suppressor, the p25 movement protein. However, this suppressor is unable to preclude PVX/GFP from inducing *gfp* RNA silencing due to its specific functional mode in preventing silencing signal spread in the transgenic line 16c plants [49]. Our results indicate that PVX was able to effectively adapt to its own induced and subsequently maintained host RNA silencing.

3.2. Re-accumulation of viral RNA in plants maintaining RNA silencing

To further reveal virus survival of RNA silencing in plants, viral RNAs and transgene *gfp* mRNA were analysed by Northern blot hybridisation (Fig. 4). In young leaves of PVX/GFP-inoculated non-transgenic *N. benthamiana* and line 16c plants at 6 dpi, viral genomic and subgenomic RNAs were readily detected by a PVX-specific probe. The amount of viral RNAs reached a peak at 10–14 dpi and declined significantly at 21 dpi. However, viral RNAs re-accumulated to substantially high levels at 28 dpi (Fig. 4A). In PVX/GFP-inoculated non-transgenic *N. benthamiana* plants, similar patterns of increase and decrease of PVX/GFP RNAs were also discovered using a *gfp*-specific probe (Fig. 4B). *Gfp* transgene mRNA was present in mock-inoculated line 16c plant and a similar viral RNA accumulation was observed in line 16c plants inoculated with PVX/GFP until 21 dpi, but no PVX/GFP RNAs were detected at 28 dpi. On the other hand, nor was transgene *gfp* mRNA detectable in PVX/GFP-inoculated line 16c plants at 21 and 28 dpi (Fig. 4B).

Western blot analysis of GFP and PVX CP expression further confirmed induction, maintenance and virus survival of RNA silencing in plants. Consistent with symptom development and viral RNA accumulation in non-transformed *N. benthamiana* plants inoculated with PVX/GFP, expression of viral CP and GFP reached the highest level at 10–14 dpi, decreased markedly at 21 dpi, but increased considerably at 28 dpi (Fig. 5). A same kind of oscillation of the CP protein

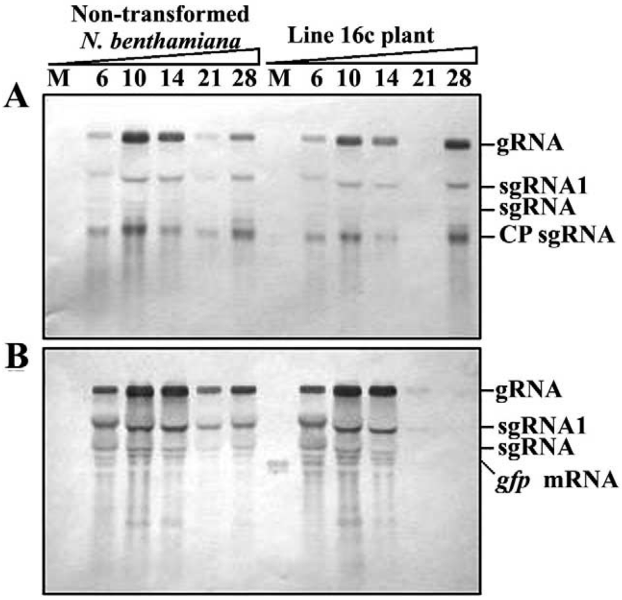

Fig. 4. Effect of RNA silencing on viral RNA accumulation. RNA samples (5 μg) extracted from young leaves harvested from non-transformed *N. benthamiana* and line 16c plants mock-inoculated or 6, 10, 14, 21, and 28 days post-inoculated with PVX/GFP were analysed. Blots were hybridised with DIG-labelled probes specific to PVX (A) and *gfp* (B) sequences. The positions of viral gRNA and sgRNA and *gfp* transgene mRNA are indicated.

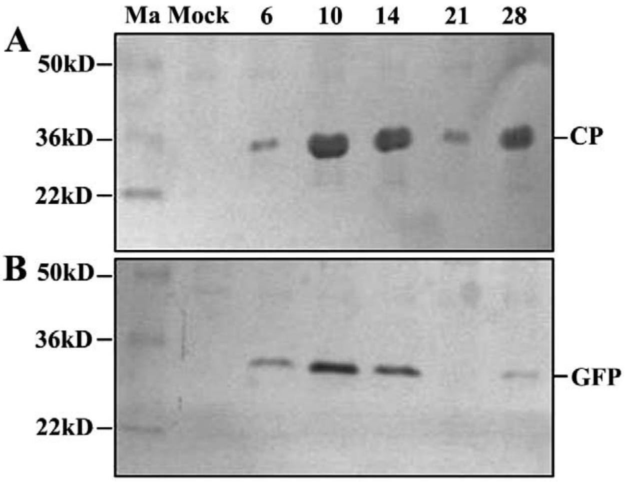

Fig. 5. PVX CP and GFP expression in non-transformed *N. benthamiana*. Protein samples (10 μg) extracted from young leaves harvested from *N. benthamiana* mock-inoculated or 6, 10, 14, 21, and 28 days post-inoculated with PVX/GFP were analysed. Blots were probed with antiserum specific to PVX CP (A) and GFP (B). The positions and sizes of protein markers (Ma), CP and GFP are indicated.

production was observed in line 16c plants inoculated with PVX/GFP (Fig. 6A). However, GFP expression from *gfp* transgene and *gfp* gene in PVX/GFP decreased persistently to an undetectable level at 28 dpi during the course of RNA silencing induction (Fig. 6B). Our data clearly demonstrated that a similar tendency associated with symptom development, viral RNA accumulation and protein production was closely co-related until 21 dpi, and later became independent of the induction and maintenance of RNA silencing in PVX/GFP-inoculated *N. benthamiana* and transgenic line 16c plants.

4. Discussion

VIGS in plants is often associated with a 'recovery' phenotype. In the early course of infection, RNA levels of the target gene (either an endogenous plant gene or a transgene) are not affected by the virus that possesses sequences similar to the target gene, and the plant developed normal viral symptoms. However, systemic symptoms later disappear in association with loss of homologous virus and reduction of target gene RNA. Such a recovery of virus disease also takes place in non-transgenic plants. *Brassica oleracea gongylodes* when infected with the DNA pararetrovirus cauliflower mosaic virus initially develops systemic symptoms from which it completely recovers later on [12]. Again, *Nicotiana clevelandii* inoculated with tomato black ring nepovirus initially shows symptoms and later recovers [37]. In both cases, virus infection of non-transgenic plants induces the RNA silencing defence mechanism. We now show that that 'recovery' phenomenon also occurred in non-transgenic *N. benthamiana* challenged with PVX/GFP. However, to our surprise, after the recovery, severe symptoms associated with re-accumulation of virus RNA re-occurred.

Analysis of the kinetics of viral and target gene RNA levels in plants has revealed that the mechanism of VIGS involves separate initiation and maintenance stages [40]. In time course experiments, Ruiz et al. [40] demonstrated that in transgenic GFP plants inoculated with PVX-GF(P) containing partial or full-length GFP coding sequence, at 13 dpi, viral RNA was abundant and readily detectable. In PVX-GF(P)-infected non-transgenic plants, viral RNA accumulated to a peak level at 13 dpi and then remained abundant. However, by 20 dpi, the level of viral RNA decreased significantly in the transgenic plants that recovered from initial infection, and the viral RNA level further decreased to below the limit of detection until 41 dpi. In our study, a similar phenomenon of an early virus infection and VIGS induction and maintenance associated with the 'recovery' phenotype in transgenic plants until 21 dpi was observed. On the other hand, our findings of symptom re-occurrence and viral RNA re-accumulation in plant tissues that maintained strong RNA silencing after 28 dpi did not conform with those previously reported results [40]. Such contradictions may be explained by the physical conditions applied in these studies. For example, various temperatures and lighting periods may affect the outcome of VIGS induction and maintenance as well as the fate of virus in plants. It has been reported that at low temperature both VIGS and transgene-triggered RNA silencing can be inhibited [44].

Recently, using a TMV-based vector, the endogenous *Chl* gene was silencing in *N. benthamiana* plants by VIGS [18]. Strong RNA silencing of the *ChlH* gene and recombinant TMV-ChlH occurred in the apical tissues. Interestingly, although TMV-ChlH was initially targeted by VIGS, virus accumulated in a fluctuating manner in the plant apex and was not permanently inhibited, indicating that TMV was able to effectively escape RNA silencing. This differs from PVX- and TRV-triggered VIGS that causes an enduring inhibition of virus in silenced plant tissues [39,40]. On the other hand, the ability of TMV to escape from RNA silencing reinforces the conclusion drawn from our work described in this report. However, the mechanisms involved in escaping or surviving RNA silencing for TMV or PVX respectively may be different. It is proposed that in the apical tissues, the VIGS of

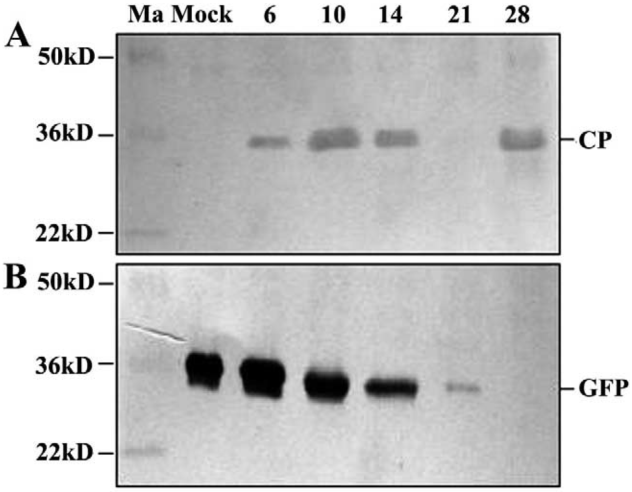

Fig. 6. PVX CP and GFP expression in transgenic line 16c plants. Protein samples (10 μg) extracted from young leaves harvested from line 16c plants mock-inoculated or 6, 10, 14, 21, and 28 days post-inoculated with PVX/GFP were analysed. Blots were probed with antiserum specific to PVX CP (A) and GFP (B). The positions and sizes of protein markers (Ma), CP and GFP are indicated.

TMV-ChlH and the endogenous *ChlH* gene mRNA may lead to a reduction of the silencing pressure. As a consequence, the newly growing tissues partially recover from VIGS, i.e. no RNA silencing occurs in the new growth. During this transient period, the levels of virus RNA and *ChlH* gene mRNA increase to reach a threshold that would trigger VIGS, and consequently, both TMV-ChlH and *ChlH* mRNA will be targeted and degraded again. That means TMV-ChlH escapes to the apical tissues in which no RNA silencing is induced and maintained [18]. However, in the PVX-GFP silencing system, PVX/GFP survived RNA silencing in plant tissues maintaining strong RNA silencing that was initially triggered by the PVX/GFP.

RNA silencing can be induced by viruses and it is subsequently targeted against viral RNA and endogenous or transgene mRNA. Our results further demonstrated that virus-induced RNA silencing could only inhibit virus (PVX/GFP) accumulation for a certain period of time. Afterwards, virus may survive the RNA silencing defence mechanism. Although strong *gfp* RNA silencing was induced and not suppressed, severe symptoms re-appeared in consistency with re-accumulation of viral RNA and CP in plants soon after RNA silencing-associated symptom recovery from initial viral infection occurred. There may be at least two different models that could account for this unexpected re-infection of recovered plants. Firstly, a lower virus level resulting from the silencing-associated 'recovery' may lead to weakening of RNA silencing in recovered leaves and the residual virus could then re-establish a secondary infection from old symptomatic leaves. This is consistent with the 'feedback regulation' proposed for the TMV-mediated induction of and escape from silencing [18]. Intriguingly, the insertion of *gfp* RNA in the PVX/GFP genome was stable in non-transformed *N. benthamiana*, but not in line 16c plants (Fig. 4). This would suggest that in transgenic plants, the strong *gfp* silencing which was manifested in red autofluorescence was likely due to systemic spread of transgene *gfp* silencing and may not indicate that strong silencing against PVX was maintained. It could be argued that maintenance of virus and *gfp* silencing was different. In this regard, PVX lacking GFP could infect, whereas recombinant PVX/GFP failed to re-infect, the *gfp* silenced leaves. Thus, the loss of *gfp* sequence may at least explain the genetic context of virus survival in transgenic plants. However, that was not the case in non-transgenic plants on which re-infecting virus (PVX/GFP) maintained the *gfp* sequence.

Alternatively, our data may suggest that reciprocal silencing of transgene and virus-carrying *gfp* RNA was more selectively targeted in transgenic than in non-transgenic plants. It would be interesting to elucidate whether viruses re-emerging in secondary infections in plants that maintained an already induced and active RNA silencing are genetically different from the original strain and more silencing-resistant. Nevertheless, our results provide direct evidence that PVX, a single-stranded positive RNA virus, can effectively escape an RNA silencing defence mechanism induced by its own that has or does not have significant homology to host endogenous genes without suppressing RNA silencing by a protein suppressor. This view is supported by recent findings that several chloroplastic and nuclear viroids, small circular single-stranded RNA pathogens that encode no protein, can efficiently induce RNA silencing in plants developing systemic viroid infection [22,33,35]. Thus, virus can rapidly and efficiently escape/survive RNA silencing, which may lead to a re-evaluation of the concept of using RNA silencing to combat plant, animal and human virus diseases.

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