A viral transcription factor exhibits antiviral RNA silencing suppression activity independent of its nuclear localization

Nina I. Lukhovitskaya,† Ramesh R. Vetukuri,† Indu Sama, Srinivas Thaduri, Andrey G. Solovyev and Eugene I. Savenkov

1Department of Plant Biology, Uppsala BioCenter, Swedish University of Agricultural Sciences, Linnean Center for Plant Biology, Box 7080, 75007 Uppsala, Sweden
2A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, 119992 Moscow, Russia

Viral suppressors of RNA silencing (VSRs) are critical for the success of virus infection and efficient accumulation of virus progeny. The chrysanthemum virus B p12 protein acts as a transcription factor to regulate cell size and proliferation favourable for virus infection. Here, we showed that the p12 protein suppressed RNA silencing and was able to complement a VSR-deficient unrelated virus. Moreover, p12 counter-silencing activity could be uncoupled from its function as a transcription factor in the nucleus. The altered p12 protein, which lacked a nuclear localization signal and was not imported into the nucleus, was able to suppress RNA silencing as efficiently as the native protein. The data revealed new aspects of p12 functioning and identified a novel role for this viral zinc-finger transcription factor. The results provided a general insight into one of the activities of the p12 protein, which appeared to possess more than one function.

RNA silencing functions as a major host defence mechanism against viruses in plants, fungi and invertebrates (Ding, 2010). Positive-strand RNA viruses, which comprise the vast majority of plant viruses, are potent inducers of RNA silencing through the formation of double-stranded intermediates either via internal self-complementarity, or as a result of eukaryotic RNA-directed RNA polymerase or viral RNA-dependent RNA polymerase activities. Viral dsRNAs are processed by Dicer-like (DCL) proteins, mostly DCL4, into viral short interfering RNAs (vsiRNAs), which interact with Argonaute (AGO) proteins AGO1 and AGO2 to direct silencing (degradation) of viral RNA progeny (Pumplin & Voinnet, 2013; Szittya & Burgyan, 2013). To counteract silencing-based plant defence, viruses have evolved viral suppressors of RNA silencing (VSRs) (Burgyan & Havelda, 2011; Incarbone & Dunoyer, 2013; Pumplin & Voinnet, 2013). VSRs encoded by different viruses employ diverse strategies to interfere with antiviral silencing, including dsRNA binding and protection from DCL processing, vsiRNA binding and sequestration, cleavage of vsiRNAs into dysfunctional smaller products, interaction with AGO1 and inhibition of its activity, targeting of AGO1 for degradation, and downregulation of AGO1 expression (Szittya & Burgyan, 2013).

A number of positive-strand RNA viruses encode cysteine-rich proteins (CRPs) and the CRPs have been classified into several groups according to their sequence conservation. The first group includes proteins encoded by the genera Hordeivirus, Peclavivirus, Furovirus and Tobravirus; the second group includes proteins encoded by the genera Carlaviruses and Allexiviruses; another group is represented by the 8K protein of potato mop-top virus (PMTV) (Lukhovitskaya et al., 2005). In general, these CRPs are virulence factors (Donald & Jackson, 1994; Dunoyer et al., 2001; Liu et al., 2002; Lukhovitskaya et al., 2005, 2009) and function as VSRs (Dunoyer et al., 2002; Yelina et al., 2002, 2005; Bragg & Jackson, 2004; Martín-Hernández & Baulcombe, 2008; Martínez-Priego et al., 2008; Senshu et al., 2011; Lukhovitskaya et al., 2013a).

The chrysanthemum virus B (CVB) p12 protein is localized in the nucleus, and binds both RNA and DNA in vitro, exhibiting a preference for DNA in the presence of Zn$^+$ ions (Lukhovitskaya et al., 2009). Mutational analysis of CVB p12 confirmed the presence of the nuclear localization signal (NLS) in the N terminus of p12, whereas a predicted zinc-finger motif, another structural element conserved in CRPs of this group, is needed for Zn$^{2+}$-dependent DNA binding (Lukhovitskaya et al., 2009). Nuclear import of p12 is required for induction of a hypersensitive response upon expression from potato virus X (PVX). Moreover, a hypersensitive response was shown to be preceded by induction of a number of plant genes involved in responses...
to various pathogens (Lukhovitskaya et al., 2009). These findings established that CVB p12 functions in the nucleus and modulates expression of the host genes. Furthermore, CVB p12 was found to act as a genuine transcription factor to upregulate expression of a host gene designated upp-L (upregulated by p12). The induction of upp-L requires p12 to enter the nucleus and a specific interaction with the conserved regulatory region in the upp-L promoter involving the p12 zinc-finger domain (Lukhovitskaya et al., 2013b, c). The Upp-L transcription factor, in turn, when ectopically expressed, affects expression of a number of key cell cycle regulators, which results in cell proliferation and severe leaf malformation – a typical phenotypic manifestation of the carlavirus infection (Lukhovitskaya et al., 2013b, c). Thus, CVB p12 was proved to act as a transcription factor, i.e. as a transcriptional activator of upp-L involved in regulation of cell size and tissue growth.

Our previous attempts to demonstrate counter-silencing activity of p12 in the conventional GFP transient co-expression assay did not reveal such a function (Lukhovitskaya et al., 2009). However, we have recently been able to demonstrate silencing suppression activity for a weak VSR, the 8K protein encoded by PMTV (Lukhovitskaya et al., 2013a), using a novel complementation assay (Powers et al., 2008). In this experimental setup, the identification of VSR activity was achieved using a versatile assay based on complementation of cell-to-cell movement of turnip crinkle virus (TCV), the genome of which was modified by swapping a capsid protein (CP) gene of the virus with a reporter gene for a synthetic GFP (sGFP) (Powers et al., 2008). The cell-to-cell movement of TCV in host plants belonging to Nicotiana spp. requires expression of its CP, a well-characterized VSR. Thus, in the absence of CP expression, TCV-sGFP is not able to move efficiently to neighbouring cells and is confined to single cells or foci consisting of two or three cells due to induction of antiviral silencing. This system provides a convenient assay for the suppression of antiviral silencing through complementation in trans.

To evaluate whether the TCV-sGFP complementation assay could be used to analyse RNA suppression activity of p12, we performed experiments in Nicotiana benthamiana leaves with Agrobacterium-launched p12 expression and subsequent inoculation with in vitro generated infectious TCV-sGFP transcripts 3 days later. In this assay, infiltration of Agrobacterium strains carrying an empty plasmid (EP) or a plasmid directing expression of the HcPro silencing suppressor from potato virus A (PVA) (Savenkov & Valkonen, 2001) was used as negative or positive controls, respectively. The pre-infiltrated leaves for expression of EP, p12 or HcPro were rub-inoculated with the TCV-sGFP reporter construct. Subsequently, movement of TCV-sGFP was monitored, the fluorescent foci consisting of one, two, three or more than four cells counted (Table 1), and the diameter of the resulting fluorescent foci measured (Fig. 1a, b, Table S1, available in the online Supplementary Material). As expected, PVA HcPro, a well-characterized VSR, efficiently suppressed antiviral RNA silencing, resulting in well-pronounced TCV-sGFP fluorescent foci consisting of four to 60 cells (Fig. 1a) as compared with a negative control represented mostly by single cells (Table 1, Fig. 1a). Co-expression of p12 and TCV-sGFP also resulted in foci of four to 30 cells, demonstrating that CBV p12 was a suppressor of RNA silencing. However, TCV-Δ92-sGFP, a movement-deficient construct lacking a viral gene for a movement protein, was found not to be complemented by p12 as GFP fluorescence was confined to single cells (Fig. 1a). This experiment ruled out the possibility that the p12 protein assisted in cell-to-cell movement rather than being a VSR.

Table 1. Agroinfiltration of HcPro, p12 and its derivatives, and subsequent inoculation with TCV-sGFP or TCV-Δ92-sGFP established the validity of the leaf-based VSR complementation assay

<table>
<thead>
<tr>
<th>Assay</th>
<th>GFP-tagged TCV*</th>
<th>Co-expressed proteins†</th>
<th>Total foci‡§</th>
<th>No. of foci [n (%)]§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>One cell</td>
<td>Two cells</td>
</tr>
<tr>
<td>1</td>
<td>TCV-sGFP</td>
<td>–</td>
<td>106</td>
<td>80 (75.5)</td>
</tr>
<tr>
<td>2</td>
<td>TCV-sGFP</td>
<td>p12</td>
<td>139</td>
<td>45 (32.4)</td>
</tr>
<tr>
<td>3</td>
<td>TCV-sGFP</td>
<td>p12ZF</td>
<td>61</td>
<td>40 (65.6)</td>
</tr>
<tr>
<td>4</td>
<td>TCV-sGFP</td>
<td>p12NLS</td>
<td>70</td>
<td>25 (35.7)</td>
</tr>
<tr>
<td>5</td>
<td>TCV-sGFP</td>
<td>HcPro</td>
<td>88</td>
<td>18 (20.5)</td>
</tr>
<tr>
<td>6</td>
<td>TCV-Δ92-sGFP</td>
<td>p12</td>
<td>51</td>
<td>42 (82.4)</td>
</tr>
</tbody>
</table>

*GFP expressed from the TCV genome. For each assay, Agrobacterium strain GV3060, expressing each of the constructs indicated, at an OD₆₀₀ of 0.6 was infiltrated onto N. benthamiana leaves and the leaves were inoculated with TCV-sGFP (or TCV-Δ92-sGFP) 3 days after infiltration.
†GV3060 expressing each of the indicated constructs.
‡Total number of fluorescent foci counted.
§Number of foci in which TCV-sGFP or TCV-Δ92-sGFP was confined to a single cell, or was found in two adjacent cells, in a cluster of three cells or in a cluster of four or more cells.
||GV3060 transformed with an EP, pLH7000.
Suppression of antiviral RNA silencing

(a) Images showing viral focius diameters.

(b) Graph showing mean focus diameter (µm) with genetic interactions.

(c) Graph showing GFP fluorescence (U [µg protein]⁻¹) with genetic interactions.
Next, we tested whether p12 expression resulted in increased GFP accumulation, and was thus indicative of silencing suppression. To this end, we repeated the TCV-sGFP complementation assay, using the PZP-TCV-sGFP construct, in which TCV-sGFP is driven by the 35S promoter of cauliflower mosaic virus (Powers et al., 2008). Leaf samples co-infiltrated with PZP-TCV-sGFP and the different constructs were collected 5 days post-inoculation from the infiltrated N. benthamiana leaves and analysed for evidence of enhanced GFP accumulation compared with an EP control. Also under these conditions, CVB p12 suppressed silencing of the GFP reporter as was determined by quantification of GFP fluorescence using a fluorometric analysis as described previously (Zamyatnin et al., 2006) (Fig. 1c). Both PVA HcPro and CVB p12 showed statistically significant (P<0.05, Student’s t-test) increases in GFP fluorescence as compared with PZP-TCV-sGFP co-infiltration with an EP control. Thus, using this approach we found that p12 was able to suppress antiviral RNA silencing and complement cell-to-cell movement of VSR-deficient TCV-sGFP, but not TCV-Δ92-sGFP, which lacked a gene encoding a movement protein of the virus (Table 1, Fig. 1).

To clarify whether nuclear localization was needed for the p12 protein to act as a VSR, we made use of previously characterized p12 mutants, one of which, designated p12NLS, expressed an altered p12 protein with four alanines replacing arginine residues in the NLS, which rendered the protein unable to enter the nucleus (Lukhovitskaya et al., 2009), whilst another mutant, p12ZF, produced an altered p12 protein with two alanine residues replacing cysteines of the zinc-finger domain, which rendered the protein unable to bind nucleic acids, including DNA (Lukhovitskaya et al., 2009). The anti-silencing properties of these two mutants were evaluated using the experimental setup described above. We observed that whereas the NLS-deficient p12 protein was able to complement TCV-sGFP indicative of the VSR activity (Table 1, Fig. 1a, b), the p12ZF protein’s ability to inhibit antiviral silencing was compromised (Table 1, Fig. 1a, b). Consistent with its RNA silencing suppressive activity, p12NLS mutant co-infiltration with PZP-TCV-sGFP resulted in statistically significant (P<0.05, Student’s t-test) increased GFP accumulation (Fig. 1c), whereas the p12ZF mutant was inactive in this respect at the 0.05 significance level (P>0.05, Student’s t-test; Fig. 1c). Interestingly, we observed consistently larger TCV-sGFP foci in the presence of p12NLS as compared with native p12. Indeed, statistical analysis of the size of fluorescent foci in the TCV-sGFP complementation assay (Fig. 1b) as well as enhanced GFP accumulation in the PZP-TCV-sGFP co-infiltration assay (Fig. 1c) confirmed these visual observations (P<0.05, Student’s t-test).

Having established that p12 displayed RNA silencing suppressive activity in planta, we next analysed the effect p12 and its two mutants had on viral pathogenicity in Nicotiana tabacum cv. Samsun nn. As VSRs typically enhance viral virulence, we introduced haemagglutinin (HA)-tagged p12, p12NLS and p12ZF into the PVX genome, and assessed their effect on the infection phenotype. In each case, the identity and high levels of protein accumulation were confirmed by immunoblotting with HA-specific antisera (Fig. 2a). Moreover, reverse transcription (RT)-PCR performed on total RNA isolated from upper leaves consistently revealed the presence of the p12 sequences in the modified PVX genome, suggesting the integrity and stability of the PVX constructs in systemically infected leaves (Fig. 2b). Consistent with our previous report (Lukhovitskaya et al., 2013b), N. tabacum plants infected with PVX-HAp12 showed typical leaf malformations due to mesophyll tissue growth accompanied by necrosis at later stages of viral infection (Fig. 2c), as was previously shown for a p12 un-tagged construct, PVX-p12 (Lukhovitskaya et al., 2013b). The data suggested that the N-terminal HA-tag does not interfere with p12 function. Unlike PVX-HAp12, both PVX-HAp12NLS and PVX-HAp12ZF caused weak disease symptoms similar to WT PVX (Fig. 2c), suggesting that the transcriptional activator activity (Lukhovitskaya et al., 2013b), but not the RNA silencing suppression activity of p12 is responsible for facilitating PVX infection. Real-time RT-PCR experiments employing PVX-specific primers annealing to the 5’-proximal part of the PVX genome revealed 4.0- and 3.1-fold increases in the viral genomic RNA levels in upper leaves of the plants infected with PVX-HAp12 and PVX-HAp12NLS, respectively, as compared with WT PVX, whereas the increase was less pronounced in the case of PVX-HAp12ZF (Fig. 2d). Although these results indicated that p12 enhanced viral pathogenicity,
they failed to show that this effect depended on its VSR activity as the altered p12 protein, p12NLS, whilst being able to suppress RNA silencing and enhance accumulation of the viral RNA, was not capable of enhancing the severity of PVX symptoms. As viral proteins are often multifunctional, the effect of p12 on symptom induction may be attributed to another activity of p12, i.e. to its function as a transcriptional activator. The results therefore were consistent with p12 being a rather weak VSR.

The most obvious parallels in terms of suppression of antiviral silencing can be drawn between CVB p12 and CMV 2b proteins. Both CVB p12 and CMV 2b are nuclear-localized, and their nuclear import depends on the presence of arginine-rich NLSs (Wang et al., 2004; Lukhovitskaya et al., 2009). Similar to p12, the VSR activity of CMV 2b protein is exerted predominantly by a cytoplasmic 2b pool and does not require entry into the nucleus (González et al., 2004).
Interestingly, CMV 2b acts as a transcriptional activator suppression activity of p12 enhances viral virulence. {
\textit{et al.}} that the transcriptional activator activity in the nucleus{
\textit{et al.}}. Again, this situation resembles our findings showing that the nuclear-localized 2b protein may contribute to CMV pathogenicity by mis-regulation of transcription of host genes regulating plant development and programmed cell death (Du {
\textit{et al.}}, 2014), perhaps as a transcriptional activator/repressor similar to p12.

Our findings suggest that in CVB, where the p12 protein appears to be multifunctional, the activity of p12 as a VSR and its function as a transcription factor are mechanistically different: the activity of the p12 protein as a transcriptional activator requires the presence of a NLS and zinc-finger domain and, thus, is dependent on entry into the nucleus and binding to DNA, whereas the silencing suppressor activity of CVB seems to be dependent on the portion of the p12 protein residing in the cytoplasm. Consistent with this notion, the NLS-deficient altered p12 protein excluded from the nucleus was able to suppress RNA silencing as efficiently as the native protein.

In summary, our results show that a viral transcription factor possesses VSR activity. Further experiments will be required to investigate whether our findings can be applied to cellular transcription factors as well. We anticipate the discovery of further levels of gene regulation not only through transcriptional control, microRNA (miRNA) and tissue-specific miRNA expression, but, possibly, via tissue-specific suppression of cognate miRNA activity by a miRNA-regulated transcription factor through a positive feedback loop.

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References


