A critical domain of *Sweet potato chlorotic fleck virus* nucleotide-binding protein (NaBp) for RNA silencing suppression, nuclear localization and viral pathogenesis

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SUMMARY

RNA silencing is an important mechanism of antiviral defence in plants. To counteract this resistance mechanism, many viruses have evolved RNA silencing suppressors. In this study, we analysed five proteins encoded by *Sweet potato chlorotic fleck virus* (SPCFV) for their abilities to suppress RNA silencing using a green fluorescent protein (GFP)-based transient expression assay in *Nicotiana benthamiana* line 16c plants. Our results showed that a putative nucleotide-binding protein (NaBp), but not other proteins encoded by the virus, could efficiently suppress local and systemic RNA silencing induced by either sense or double-stranded RNA (dsRNA) molecules. Deletion mutation analysis of NaBp demonstrated that the basic motif (an arginine-rich region) was critical for its RNA silencing suppression activity. Using confocal laser scanning microscopy imaging of transfected protoplasts expressing NaBp fused to GFP, we showed that NaBp accumulated predominantly in the nucleus. Mutational analysis of NaBp demonstrated that the basic motif represented part of the nuclear localization signal. In addition, we demonstrated that the basic motif in NaBp was a pathogenicity determinant in the *Potato virus X* (PVX) heterogeneous system. Overall, our results demonstrate that the basic motif of SPCFV NaBp plays a critical role in RNA silencing suppression, nuclear localization and viral pathogenesis.

Keywords: NaBp, nuclear localization, silencing suppressor, SPCFV, viral pathogenesis.

INTRODUCTION

RNA silencing is a conserved sequence-specific gene regulation system that operates in a wide variety of eukaryotic organisms (Baulcombe, 2004; Qu and Morris, 2005). The pathway is triggered by double-stranded RNA molecules (dsRNA) (Fire *et al.*, 1998), which are recognized and cleaved into 21–24-nucleotide small

interfering RNAs (siRNAs) by an RNase III-like enzyme named Dicer (Bernstein *et al.*, 2001; Grishok *et al.*, 2001; Knight and Bass, 2001). Subsequently, siRNAs are incorporated into an RNase complex named the RNA-induced transcriptional silencing complex (RISC) (Hammond *et al.*, 2000; Zamore, 2001), which contains the slicer endonuclease Argonaute (AGO) and serves as a guide for the cleavage of RNAs that have sequences identical to those of siRNAs (Carmell *et al.*, 2002).

In plants, RNA silencing is a natural defence mechanism against virus infection (Ding and Voinnet, 2007; Voinnet, 2001, 2005). During plant virus infection, dsRNAs derived from viral replication intermediates trigger RNA silencing (Li and Ding, 2006). Once RNA silencing is induced at one site in a plant, silencing signals spread from cell to cell through the plasmodesmata, from organ to organ through the vascular system and trigger systemic silencing of target RNAs (Himber *et al.*, 2003; Palauqui *et al.*, 1997; Voinnet and Baulcombe, 1997). The long-distance movement of virus-derived siRNA signals elicits viral defence responses in the whole plant. To counteract the anti-viral effect of host RNA silencing, many plant viruses have evolved suppressors of RNA silencing (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau and Carrington, 1998). To date, many silencing suppressors have been identified from plant viruses; these suppressors share no significant sequence similarity and interfere at different stages of the RNA silencing pathway (Incarbone and Dunoyer, 2013; Moissiard and Voinnet, 2004; Roth *et al.*, 2004). Some silencing suppressors target siRNAs to interfere with their entry into RISC. For instance, the p19 protein of *Tomato bushy stunt virus* (TBSV) suppresses RNA silencing through binding to siRNAs (Chapman *et al.*, 2004; Lakatos *et al.*, 2004). The helper component–proteinase (HC-Pro) of potyviruses suppresses RNA silencing through interference with the upstream production of siRNAs (Chapman *et al.*, 2004; Dunoyer *et al.*, 2004; Lakatos *et al.*, 2006). Some suppressors inhibit the functions of proteins of the RNA silencing pathway. The 2b protein encoded by *Cucumber mosaic virus* (CMV), the P25 protein of *Potato virus X* (PVX) and the P0 protein of *Enamovirus* can interact with AGO1 (Chiu *et al.*, 2010; Fusaro *et al.*, 2012; Ye *et al.*, 2009; Zhang *et al.*, 2006). The P38 protein of *Turnip crinkle virus* (TCV) inhibits DCL2 and DCL4 activities (Deleris *et al.*, 2006;

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Fig. 1 Identification of *Sweet potato chlorotic fleck virus* (SPCFV) nucleotide-binding protein (NaBp) as an RNA silencing suppressor using *Agrobacterium*-mediated infiltration assay. (A) Schematic representation of the genome of SPCFV showing the open reading frames (ORFs). CP, coat protein; TGB, triple gene block. (B) Green fluorescent protein (GFP) images of agroinfiltrated leaves of *Nicotiana benthamiana* line 16c under UV illumination at 5 days post-infiltration (dpi). The constructs used for agro-infiltration are indicated above the images. (C) Northern blot analysis of the accumulation of GFP mRNA and GFP siRNA extracted from the agroinfiltrated leaves at 5 dpi. Ethidium bromide staining of rRNA and tRNA is shown as a loading control.

Mérai *et al.*, 2006). Many silencing suppressors have been identified as pathogenicity determinants (Brigneti *et al.*, 1998). Therefore, the identification, subcellular localization and analysis of viral RNA silencing suppressors are necessary to better understand the mechanism of viral-mediated silencing suppression and virus–host interaction in plants.

Sweet potato chlorotic fleck virus (SPCFV) has recently been classified as a new member of the genus *Carlavirus* (family *Flexiviridae*) (Aritua *et al.*, 2007, 2009). However, SPCFV is distantly related to typical carlaviruses, as most of its putative gene products share amino acid sequence identities of less than 40% with those of typical carlaviruses (Aritua *et al.*, 2007). It has a single-stranded, positive-sense RNA genome containing six open reading frames (ORFs). ORF1 encodes a viral replicase, ORF2–ORF4 encode triple gene block (TGB) proteins (TGB1, TGB2 and TGB3) and ORF5 encodes the coat protein (CP). ORF6 encodes a putative nucleotide-binding protein (NaBp), also referred to as cysteine-rich protein (CRP) (Fig. 1A). Viruses of the genera *Allexivirus*, *Benyvirus*, *Carlavirus*, *Hordeivirus*, *Mandarivirus*, *Peduvirus*, *Pomovirus*, *Tobravirus* and *Vitivirus* can encode CRPs that do not exhibit significant similarity. However, all contain a characteristic motif consisting of multiple cysteine residues. A number of studies have demonstrated that these proteins play

important roles in viral infections, silencing suppression and function as virus pathogenicity determinants (Andika *et al.*, 2012; Dunoyer *et al.*, 2001, 2002; Galiakparov *et al.*, 2003; Ghazala *et al.*, 2008; Guilley *et al.*, 2009; Lukhovitskaya *et al.*, 2005, 2009; Martín-Hernández and Baulcombe, 2008; Senshu *et al.*, 2011; Sun *et al.*, 2013; Te *et al.*, 2005; Yelina *et al.*, 2002). However, little is known about the functions and properties of *Carlavirus* CRPs. The CRP encoded by carlaviruses *Potato virus M* (PVM) and *Potato virus H* (PVH) has been shown to act as a silencing suppressor to enhance viral pathogenicity (Li *et al.*, 2013; Senshu *et al.*, 2011). Interestingly, the CRP encoded by another carlavirus, *Chrysanthemum virus B* (CVB), does not suppress RNA silencing, but acts as a eukaryotic transcription factor to modulate the morphogenesis of plant cells (Lukhovitskaya *et al.*, 2009, 2013). These results suggest that there are some fundamental differences, even among homologous proteins encoded by the viruses in the same genus.

The CRP encoded by SPCFV is a characteristic protein (called NaBp in this study), as this protein shares only very low amino acid sequence identities (14–22%) with the majority of carlaviruses (Aritua *et al.*, 2007). In this study, we showed that SPCFV NaBp inhibits local and systemic silencing induced by sense RNA or dsRNA in green fluorescent protein (GFP)-expressing *Nicotiana*

benthamiana line 16c plants using an *Agrobacterium*-mediated transient assay. Our work also showed that NaBp of SPCFV accumulates predominantly in the nucleus of plant cells. Furthermore, NaBp can enhance PVX pathogenicity in *N. benthamiana* plants. Through deletion mutation analysis, we demonstrated that the basic motif (an arginine-rich region) is required for these activities of the protein.

RESULTS

Identification of SPCFV NaBp as an RNA silencing suppressor

To identify the potential RNA silencing suppressor of SPCFV, ORFs TGB1, TGB2, TGB3, CP and NaBp of this virus were tested. Each of the proteins was co-expressed with GFP into leaves of GFP transgenic *N. benthamiana* line 16c plants through the *Agrobacterium*-mediated infiltration procedure. Co-infiltration of 35S-GFP with either the empty PBI121 vector or a vector containing the well-characterized RNA silencing suppressor 2b from CMV was used as a negative or positive control.

GFP fluorescence was observed in the infiltrated leaves under UV light. Results showed that, by 2 days post-infiltration (dpi), strong GFP fluorescence was observed in all infiltrated leaves (not shown). By 5 dpi, however, leaves infiltrated with 35S-GFP plus the empty vector showed decreased GFP fluorescence, as a consequence of RNA silencing activation. In contrast, leaves co-infiltrated with 35S-GFP and 35S-2b showed intense GFP fluorescence, indicating that RNA silencing was suppressed. Among the five SPCFV genes tested, bright green fluorescence was maintained only in leaves co-infiltrated with 35S-GFP and 35S-NaBp at 5 dpi, whereas leaves infiltrated with 35S-GFP, together with TGB1, TGB2, TGB3 or CP, exhibited the disappearance of GFP fluorescence (Fig. 1B).

Northern blot analysis revealed that the concentration of GFP mRNA was significantly higher in leaves co-infiltrated with the GFP construct and NaBp or the 2b construct than in leaves co-infiltrated with the GFP construct and the empty vector or each of the other SPCFV ORFs, except NaBp, at 5 dpi (Fig. 1C). Further analysis of siRNA indicated that, by 5 dpi, GFP siRNA had accumulated to high levels in leaves co-infiltrated with the GFP construct and the empty vector. Similar results were also seen in leaves infiltrated with the GFP construct plus any SPCFV ORF tested, except NaBp. However, this accumulation was drastically reduced in leaves co-infiltrated with 35S-GFP and 35S-NaBp or 35S-2b (Fig. 1C). These results demonstrate that NaBp is an RNA silencing suppressor in SPCFV genomic RNA.

NaBp can suppress systemic RNA silencing

Once local silencing is initiated in plants, the silencing signal can spread systemically and induce the systemic silencing of homolo-

Fig. 2 Systemic RNA silencing suppression activities of *Sweet potato chlorotic fleck virus* (SPCFV) nucleotide-binding protein (NaBp) and TGB1. (A) Green fluorescent protein (GFP) images of the upper leaves of *Nicotiana benthamiana* line 16c plants infiltrated with 35S-GFP together with several constructs at 20 days post-infiltration (dpi); the 'Mock' plant was infiltrated with infiltration buffer. (B) Northern blot analysis of the accumulation of GFP mRNA extracted from the upper leaves at 20 dpi. Ethidium bromide staining of rRNA is shown as a loading control.

gous sequences in upper leaves (Himber *et al.*, 2003; Voinnet and Baulcombe, 1997). To determine whether SPCFV NaBp interferes with this process, GFP fluorescence was monitored in the upper leaves of *N. benthamiana* line 16c plants infiltrated with 35S-GFP together with the empty vector and 35S-NaBp. At 20 dpi, when 16c plants were infiltrated with 35S-GFP plus empty vector, GFP fluorescence disappeared extensively in upper non-infiltrated leaves, an indication of systemic silencing (Fig. 2A). In contrast, when 16c plants were infiltrated with 35S-GFP plus 35S-NaBp, the upper leaves of 91.7% (22 of 24) infiltrated plants remained brightly GFP fluorescent at 20 dpi (Fig. 2A and Table 1), indicating that NaBp functioned as a suppressor of systemic RNA silencing. A previous report (Senshu *et al.*, 2011) has shown that both TGB1

Table 1 Efficiency of systemic RNA silencing in *Nicotiana benthamiana* line 16c plants shown in Figs 2 and 3

Constructs infiltrated	Number of plants infiltrated	Number of plants showing systemic silencing	Efficiency of systemic silencing (%)
GFP + Vector	6	6	100
GFP + NaBp	24	2	8.3
GFP + TGB1	24	23	95.8
GFP + dsGFP + Vector	6	6	100
GFP + dsGFP + NaBp	24	4	16.7

ds, double-stranded; GFP, green fluorescent protein; NaBp, nucleotide-binding protein; TGB, triple gene block.

and CRP of a carlavirus PVM can interfere with systemic silencing; therefore, we also investigated whether TGB1 of SPCFV inhibits systemic silencing. In contrast with NaBp, the upper leaves of most (23 of 24) plants infiltrated with TGB1 exhibited the disappearance of GFP fluorescence (Fig. 2A and Table 1). Thus, SPCFV NaBp, but not TGB1, has systemic silencing suppression ability.

Northern blot analysis indicated that, at 20 dpi, the upper leaves of 16c plants infiltrated with GFP plus NaBp construct, which suppressed systemic silencing, contained significant levels of GFP mRNA. Conversely, the accumulation of GFP mRNA in the upper leaves of plants infiltrated with GFP plus empty vector or TGB1 construct was very low (Fig. 2B). These results demonstrate that SPCFV NaBp is able to inhibit the spread of silencing.

NaBp can suppress local and systemic RNA silencing triggered by dsRNA

The above-described transient co-infiltration assays showed that SPCFV NaBp can suppress local and systemic GFP silencing triggered by positive-sense RNA. Leaves infiltrated with vectors containing an inverted repeat GFP sequence (IR-GFP) lead to the expression of hairpin GFP RNAs that are considered to be strong inducers of RNA silencing, as they are rapidly processed into siRNAs.

To test whether NaBp could suppress GFP dsRNA-induced silencing, *N. benthamiana* line 16c plants were infiltrated with 35S-GFP plus 35S-dsGFP (IR-GFP) plus empty vector or vector expressing NaBp. As illustrated in Fig. 3A, leaves infiltrated with 35S-GFP plus 35S-dsGFP plus empty vector showed a loss of GFP fluorescence at 5 dpi, indicating strong local GFP RNA silencing. In contrast, enhanced GFP fluorescence was sustained in leaves infiltrated with 35S-GFP plus 35S-dsGFP plus vector expressing NaBp, indicating that NaBp could suppress local GFP silencing triggered by dsRNA. Higher GFP fluorescence was correlated with greater accumulation levels of GFP mRNA and lower accumulation of GFP siRNA (Fig. 3B). Northern blot analysis showed high accumulation of GFP mRNA and negligible accumulation of GFP siRNA in leaves co-infiltrated with 35S-GFP, 35S-dsGFP and 35S-NaBp. However, leaves co-infiltrated with 35S-GFP, 35S-dsGFP and empty vector showed strongly reduced accumulation of GFP mRNA and high accumulation of GFP siRNA.

We then investigated the suppression activity of SPCFV NaBp in *N. benthamiana* line 16c plants when systemic silencing was induced by infiltration with dsGFP. By 20 dpi, all 16c plants infiltrated with 35S-GFP plus 35S-dsGFP plus empty vector developed systemic GFP silencing in the upper leaves (Fig. 3C and Table 1). However, most plants infiltrated with 35S-GFP plus 35S-dsGFP plus vector expressing NaBp (20 of 24 infiltrated plants) remained brightly fluorescent (Fig. 3C and Table 1), indicating that NaBp interfered with systemic silencing induced by dsRNA. The GFP fluorescence intensities were positively correlated with the GFP mRNA levels in the systemic leaves. The increase in GFP mRNA levels in plants infiltrated with GFP plus dsGFP plus NaBp was evident relative to plants infiltrated with empty vector (Fig. 3D). Taken together, these results demonstrate that SPCFV NaBp can suppress either local or systemic RNA silencing triggered by GFP dsRNA.

RNA silencing activity of NaBp mutants

To determine which region(s) within NaBp is responsible for the RNA silencing suppressor activity, we constructed several NaBp mutants: NaBp (1–33), NaBp (34–63), NaBp (64–98) and NaBp (98–133) (relevant amino acid region of NaBp; Fig. 4A). Each of these mutants was co-infiltrated with 35S-GFP into *N. benthamiana* line 16c leaves. Leaves co-infiltrated with 35S-GFP and empty vector or 35S-NaBp served as a negative or positive control, respectively. At 5 dpi, GFP fluorescence decreased in leaves infiltrated with 35S-GFP plus the empty vector, 35S-NaBp (1–33), 35S-NaBp (64–98) or 35S-NaBp (98–133). In contrast, in leaves co-infiltrated with 35S-GFP and 35S-NaBp or 35S-NaBp (34–63), intense GFP fluorescence was observed (Fig. 4B). Hence, amino acids 34–63 are required for the RNA silencing suppressor activity of NaBp.

Previous studies have indicated that the basic motif (an arginine-rich region) is crucial for the activity of silencing suppression in CRP (Senshu *et al.*, 2011). Our experiments showed that NaBp (34–63), which contains a basic motif-like domain (⁵⁷RRARR⁶³), is required for silencing suppressor activity. This prompted us to determine whether this domain contributes to the suppression activity of NaBp. We also constructed mutants NaBp (34–63ΔB) and NaBp (ΔB) (deleting the basic motif). Leaves monitored under UV light at 5 dpi showed that co-expression of GFP and NaBp (34–63ΔB) or NaBp (ΔB) led to a loss of GFP fluorescence (Fig. 4B), indicating that

Fig. 3 *Sweet potato chlorotic fleck virus* (SPCFV) nucleotide-binding protein (NaBp) can suppress local and systemic RNA silencing triggered by double-stranded RNA (dsRNA). (A) Green fluorescent protein (GFP) images of *Nicotiana benthamiana* line 16c leaves infiltrated with 35S-GFP plus 35S-dsGFP plus empty vector or vector expressing NaBp at 5 days post-infiltration (dpi). (B) Northern blot analysis of the accumulation of GFP mRNA and GFP siRNA extracted from the agroinfiltrated leaves at 5 dpi. Ethidium bromide staining of rRNA and tRNA is shown as a loading control. (C) GFP images of the upper leaves of *Nicotiana benthamiana* line 16c plants infiltrated with 35S-GFP plus 35S-dsGFP plus empty vector or vector expressing NaBp at 20 dpi. (D) Northern blot analysis of GFP mRNA extracted from the upper leaves at 20 dpi. Ethidium bromide staining of rRNA is shown as a loading control.

Fig. 4 RNA silencing suppression activity of *Sweet potato chlorotic fleck virus* (SPCFV) nucleotide-binding protein (NaBp) and its mutants. (A) Schematic representation of six NaBp mutants: NaBp(1–33), deletion mutant containing amino acids 1–33 of NaBp and a stop codon; NaBp(34–63), deletion mutant containing a start codon, amino acids 34–63 of NaBp and a stop codon; NaBp(64–98), deletion mutant containing a start codon, amino acids 64–98 of NaBp and a stop codon; NaBp(98–133), deletion mutant containing a start codon and amino acids 98–133 of NaBp; NaBp(34–63ΔB), deletion of the basic motif (amino acids 57–63) in NaBp(34–63); NaBp(ΔB), deletion of the basic motif in NaBp. (B) Green fluorescent protein (GFP) images of agroinfiltrated leaves of *Nicotiana benthamiana* line 16c under UV illumination at 5 days post-infiltration (dpi). Each of these mutants was co-infiltrated with 35S-GFP.

they lost their silencing suppression activity. These results demonstrate that the basic motif in NaBp is indispensable for the suppression of RNA silencing.

The basic motif represents a NaBp nuclear localization signal

In order to investigate the subcellular localization of SPCFV NaBp, the NaBp sequence was fused with GFP to produce 35S-GFP-

NaBp. 35S-GFP-NaBp was transformed into *Arabidopsis thaliana* protoplasts, and these transformed protoplasts were observed at 16 h post-inoculation (hpi) by confocal laser scanning microscopy (CLSM). As shown in Fig. 5, GFP-NaBp was observed mostly in the nucleus, confirmed by 4',6-diamidino-2-phenylindole (DAPI) staining, which suggested that SPCFV NaBp targeted the nucleus.

To investigate whether the silencing suppression activity of NaBp plays a role in protein localization, the subcellular distribution of GFP fused with NaBp (34–63), NaBp (34–63ΔB) or NaBp (ΔB) was

Fig. 5 Subcellular distribution of nucleotide-binding protein (NaBp) and its mutants. Confocal laser scanning microscopy of *Arabidopsis thaliana* protoplasts expressing green fluorescent protein (GFP) fused with NaBp wild-type or mutants, chloroplast, GFP and 4',6-diamidino-2-phenylindole (DAPI) were observed at 16 h post-inoculation. Images are derived from single confocal sections. Bars, 10 μ m.

examined by CLSM. GFP-NaBp (34–63) accumulated clearly in the nucleus, in a similar manner to GFP-NaBp. In contrast, GFP-NaBp (Δ B) and GFP-NaBp (34–63 Δ B) were distributed in the cytoplasm without reaching the nucleus (Fig. 5). These observations strongly suggest that the basic motif domain is required for NaBp nucleus targeting. Collectively, the above results demonstrate that SPCFV NaBp has a nuclear localization capability, and the basic motif may represent the nuclear localization signal of the protein.

The basic motif of NaBp is essential to enhance the pathogenicity of PVX

The above results indicate that SPCFV NaBp is a strong RNA silencing suppressor. To investigate whether the silencing suppression activity of NaBp could increase viral pathogenicity, we utilized a PVX vector to express NaBp and its mutants (Fig. 6A). The empty PVX vector was used as a control.

As shown in Fig. 6B, disease symptoms were visible at 5 dpi in all inoculated *N. benthamiana* plants. However, the symptoms of leaves inoculated with PVX-NaBp were more severe than those of leaves infiltrated with PVX. Systemic symptoms could be observed in PVX-NaBp-inoculated plants as early as 8 dpi, with the development of chlorosis, necrotic mottling and whole plant stunting by 12 dpi. In contrast, plants inoculated with PVX exhibited faint chlorosis in the upper leaves at 10 dpi, and symptoms progressed to a mild mosaic at 12 dpi. These observations indicate that NaBp can enhance the pathogenicity of PVX. To examine whether the basic motif of the protein is essential for this function, PVX-NaBp (34–63), PVX-NaBp (34–63 Δ B) and PVX-NaBp (Δ B) were tested.

Similar to PVX-NaBp, PVX-NaBp (34–63) caused more rapid and more severe visible systemic symptoms, whereas plants inoculated with PVX-NaBp (34–63 Δ B) and PVX-NaBp (Δ B) showed mild systemic symptoms, similar to the plants inoculated with PVX alone.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) was used to examine the accumulation of PVX RNA in infiltrated leaves at 5 dpi and upper leaves at 12 dpi. As shown in Fig. 6C, D, enhanced virus virulence correlated with enhanced accumulation of PVX RNAs. Plants inoculated with PVX-NaBp or PVX-NaBp (34–63) showed a higher level of viral RNA than plants inoculated with PVX, PVX-NaBp (34–63 Δ B) or PVX-NaBp (Δ B) in both inoculated and upper leaves. Taken together, these results demonstrate that SPCFV NaBp enhances symptoms and replication levels of PVX, possibly through its activity as an RNA silencing suppressor.

DISCUSSION

Plants and viruses have engaged in an ongoing game of one-upmanship for many millions of years. In plant–virus interactions, RNA silencing is one of the most important defence mechanisms against viruses. To counteract the host antiviral mechanism, viruses have evolved silencing suppressors, which have the ability to inhibit different stages of the RNA silencing pathway (Burgýán and Havelda, 2011; Wu *et al.*, 2010). So far, about 40 RNA silencing suppressors have been identified from RNA and DNA viruses (Guo *et al.*, 2013).

In this study, we screened five SPCFV proteins using a classical *Agrobacterium tumefaciens* co-infiltration assay. Our results

Fig. 6 Effects of *Sweet potato chlorotic fleck virus* (SPCFV) nucleotide-binding protein (NaBp) and its mutants on *Potato virus X* (PVX) pathogenicity. (A) Schematic representation of recombinant PVX variants carrying NaBp or its mutants. (B) Symptoms of *Nicotiana benthamiana* plants infected with the recombinant PVX variants. Inoculated leaves (top panels) were photographed at 5 days post-infiltration (dpi); systemic leaves (bottom panels) were photographed at 12 dpi. (C) Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of PVX CP RNA accumulation in inoculated leaves at 5 dpi. (D) Quantitative RT-PCR analysis of PVX CP RNA accumulation in systemic leaves at 12 dpi. The error bars represent the standard deviations of three replicates. The significance of the differences was analysed by Student's *t*-test (**P* < 0.05).

demonstrated that the CRP (NaBp) encoded by SPCFV, but not the other viral proteins, could suppress RNA silencing efficiently. P25 (TGB1) is one of the well-characterized silencing suppressors in most members of the family *Flexiviridae* (Senshu *et al.*, 2009). Some members of the *Flexiviridae* (genera *Allexivirus*, *Carlavirus*, *Mandarinivirus*, *Vitivirus* and *Torichovirus*) encode CRPs in their genome. In some of these viruses, such as *Grape virus A*

(GVA; *Vitivirus*) (Zhou *et al.*, 2006), CRPs other than TGB1 have been identified as silencing suppressors. Furthermore, in some genus members outside the *Flexiviridae* family, CRPs also function as silencing suppressors (Sun *et al.*, 2013). Previous studies have indicated that CRPs of *Chinese wheat mosaic virus* (CWMV; *Furovirus*), *Tobacco rattle virus* (TRV; *Tobravirus*), *Beet necrotic yellow vein virus* (BNYVV; *Benyvirus*), PVM and PVH (*Carlavirus*)

exhibit weak silencing suppression activity (Andika *et al.*, 2012; Li *et al.*, 2013; Martín-Hernández and Baulcombe, 2008; Senshu *et al.*, 2011; Sun *et al.*, 2013). However, in this study, we found that SPCFV NaBp possesses a strong silencing suppression activity, similar to that of CMV 2b, a well-studied and efficient silencing suppressor. Interestingly, CRP of CVB also belongs to the genus *Carlavirus*, but has no suppressor activity (Lukhovitskaya *et al.*, 2009), thus suggesting that silencing suppression activity differs among CRPs encoded by homologous viruses.

The RNA silencing process has three steps, including silencing initiation, spread of the silencing signal and maintenance of silencing (Hammond *et al.*, 2000). Previous studies have suggested that 21- and 24-nucleotide siRNAs may act as signals for systemic RNA silencing in plants (Carmell *et al.*, 2002). Some RNA silencing suppressors have been reported to inhibit systemic RNA silencing, such as CMV 2b and *Apple chlorotic leaf spot virus* (ACLSV) P50 (Yaegashi *et al.*, 2007). In this study, we also demonstrated that SPCFV NaBp could inhibit systemic RNA silencing efficiently (Fig. 2A and Table 1). A possible explanation is that the suppression of systemic gene silencing by NaBp is mainly a result of its ability to bind siRNA and prevent the spread of these molecules in plants. Indeed, the levels of GFP-specific siRNA in leaves co-infiltrated with 35S-GFP and 35S-NaBp was significantly reduced (Fig. 1C). Senshu *et al.* (2011) reported that a carlavirus PVM, in which the CRP functioned as a silencing suppressor, possessed another suppressor protein TGB1. The PVM TGB1 suppressed systemic RNA silencing in the GFP transient co-expression assay, but did not inhibit local GFP silencing. As a member of carlaviruses, although SPCFV has both TGB1 and CRP, only CRP has been shown to suppress RNA silencing. In our experiments, it also appeared that the suppression activity of SPCFV CRP was stronger than that of PVM.

Previous studies have shown that inverted repeats (dsRNA) are strong inducers of RNA silencing (Cao *et al.*, 2005). Several viral suppressors have been reported to suppress local RNA silencing induced by dsRNA, whereas a few, such as TBSV P19, can suppress systemic silencing triggered by dsRNA (Chapman *et al.*, 2004). In this study, SPCFV NaBp showed strong suppression of local and systemic GFP silencing induced by 35S-dsGFP. This is also the first report of a CRP that suppresses dsRNA-induced silencing in the genus *Carlavirus*. As SPCFV NaBp could suppress RNA silencing triggered by sense RNA or dsRNA, we speculate that this suppressor probably interferes with RNA silencing downstream of dsRNA formation, either by binding siRNA or by interacting with components of RISC.

All CRPs encoded by carlaviruses contain two conserved regions: the basic motif (an arginine-rich region) and the zinc finger-like motif (a cysteine-rich region) positioned in the central part of the protein (Lukhovitskaya *et al.*, 2009). These two motifs are characteristic of eukaryotic transcription factors and possess nucleic acid-binding properties (Galiakparov *et al.*, 2003;

Lukhovitskaya *et al.*, 2013). Indeed, the CRP encoded by PVM has been shown to bind RNA and DNA *in vitro* (Gramstat *et al.*, 1990). Senshu *et al.* (2011) reported that both the basic motif and the zinc finger-like motif in PVM CRP were necessary for its silencing suppressor activity. However, in this study, we found that the suppression activity of SPCFV NaBp was determined by the basic motif (⁵⁷RRARR⁶³), whereas the zinc finger-like motif (located in amino acids 64–98 of NaBp) did not affect its suppression activity. In SPCFV NaBp, the zinc finger-like motif has a CX₂CX₄CX₄C signature, whereas, in PVM, it is CX₂CX₁₂CX₄C; this may be responsible for their different modes of action.

The determination of the subcellular localization of a protein is crucial for understanding its biological role. Our localization studies clearly showed that SPCFV NaBp could target the nucleus of *A. thaliana* cells. Further analysis of the NaBp mutant showed that its nuclear localization was determined by the basic motif, which could therefore represent part of the nuclear localization signal. As the basic motif is also responsible for the silencing suppression activity of NaBp, this indicates that nuclear localization of NaBp appears to be related to its suppression of RNA silencing. *Groundnut rosette virus* (GRV) ORF3 (Kim *et al.*, 2007; Ryabov *et al.*, 2004), *Potato virus A* (PVA) NIa (VPg) (Rajamäki and Valkonen, 2009), *Citrus tristeza virus* (CTV) P23 (Ruiz-Ruiz *et al.*, 2013) and CMV 2b proteins (González *et al.*, 2010) contain nuclear localization signals with a characteristic arginine-rich region similar to the basic motif of NaBp.

Many RNA silencing suppressors have also been identified as determinants of pathogenicity in viruses. In this study, we found that PVX-NaBp infected plants showed more severe symptoms and increased PVX genomic RNA levels compared with control plants. In addition, the ability of NaBp to increase PVX pathogenicity was consistent with the role of this protein as an RNA silencing suppressor, as NaBp (34–63ΔB) and NaBp (ΔB) mutants, which lacked detectable silencing suppression activity, induced mild PVX symptoms and low accumulation levels of PVX RNA in plants. Although the use of a PVX-based vector as a proxy of pathogenicity characterization is quite different from the natural context of an SPCFV infection, our results showed that NaBp enhanced the pathogenicity of heterologous viruses. On the basis of these results, we propose that NaBp acts as a pathogenicity determinant, at least partially as a result of its counter-RNA silencing effects.

SPCFV is very different from other members in the genus *Carlavirus*. The amino acid sequence identity between SPCFV and those of typical carlaviruses is less than 40%; in the case of CRP, the amino acid sequence identity is even lower (14%–22%) (Aritua *et al.*, 2007). The difference in CRPs between SPCFV and other carlaviruses contributes to the different functions of these proteins. Taken together, in this study, we have identified CRP, referred to as NaBp, of SPCFV as a strong RNA silencing suppressor. It can suppress local and systemic RNA silencing induced by

either sense RNA or dsRNA. We also demonstrate that SPCFV NaBp is localized to the nucleus and a viral pathogenicity determinant. Through deletion mutant analysis, we found that the basic motif in this protein plays a critical role in RNA silencing suppression, nuclear localization and viral pathogenesis. However, the role of NaBp in plant–virus interactions is still unknown; further studies are required to determine more biological roles of this protein.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

GFP transgenic *N. benthamiana* line 16c and wild-type *N. benthamiana* plants were grown in a glasshouse at 25 °C with a 16-h light/8-h dark cycle.

Plasmid construction

To determine which proteins encoded by SPCFV can suppress RNA silencing, five SPCFV ORFs, TGB1, TGB2, TGB3, CP and NaBp, were amplified by RT-PCR using total RNA extracted from sweet potato plants infected with SPCFV isolate SH1 (Sichuan Province, China) (Deng *et al.*, 2014). The resulting products were digested by the corresponding enzymes and inserted into pBI121 vectors to generate constructs 35S-TGB1, 35S-TGB2, 35S-TGB3, 35S-CP and 35S-NaBp, respectively. For the construction of 35S-GFP, the entire GFP gene was amplified from total RNA of *N. benthamiana* line 16c plants and inserted into the pBI121 vector. The CMV (FNY strain) 2b gene was inserted into the pBI121 vector to generate 35S-2b as a positive control in the experiment.

To determine which region(s) within NaBp is essential for the suppression of RNA silencing, we constructed several non-translatable deletion mutants (Fig. 4A) through overlap-extension PCR; the resulting products were then inserted individually into the pBI121 vector as described above. To generate 35S-dsGFP, an inverted repeat sequence of the GFP fragment (corresponding to the 99–473-nucleotide sequence of GFP) was cloned into the pRNAi-LIC vector as described previously (Xu *et al.*, 2010).

For subcellular localization studies, SPCFV NaBp was tagged at its N-terminus with GFP and then cloned into the PXDG vector using previously described procedures (Chen *et al.*, 2009), producing 35S-GFP-NaBp fusions. In a similar strategy to the wild-type NaBp, mutants NaBp(Δ B), NaBp(34–63) and NaBp(34–63 Δ B) were cloned into the above-mentioned vector to generate the three constructs expressing GFP-tagged NaBp mutants. To generate PVX vectors, wild-type NaBp and mutants NaBp(Δ B), NaBp(34–63) and NaBp(34–63 Δ B) were inserted into the *NotI/SalI* site in the PVX vector (kindly provided by Dr C.-G. Han, China Agricultural University, Beijing, China) (Fig. 6A).

All of the plasmids described above were verified by nucleotide sequencing. Each construct was transformed into *Agrobacterium tumefaciens* strain EHA105 by the freeze–thaw method (Jyothishwaran *et al.*, 2007). All primers used in this study are listed in Table S1 (see Supporting Information).

Agrobacterium infiltration

Agrobacterium tumefaciens carrying each construct was cultured overnight at 28 °C. The bacterial cells were harvested and resuspended in infiltration buffer containing 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 5.6), 10 mM MgCl₂ and 150 μ M acetosyringone to a final optical density at 600 nm (OD₆₀₀) of 1.0, and incubated for 3 h at room temperature. For the co-expression of GFP with different viral proteins, equal volumes of each suspension were mixed and infiltrated into the lower leaf surfaces of 4-week-old *N. benthamiana* line 16c plants with a syringe.

Protoplast preparation and transfection

Leaf mesophyll protoplasts were isolated from 3-week-old Col-0 *A. thaliana* plants. Protoplast isolation and polyethylene glycol (PEG)–calcium transfection were carried out by following previously described procedures (Yoo *et al.*, 2007). Transformed protoplasts were incubated in the dark for about 16 h at room temperature prior to imaging.

Fluorescence imaging

For co-expression assays, GFP fluorescence of infiltrated leaves and whole plants was observed using a 100-W long-wave UV lamp and photographed using a CANON ZOOM digital camera (Tokyo, Japan). For subcellular localization assay, GFP expression, chloroplast and DAPI staining in protoplasts were imaged using a confocal laser scanning microscope (Leica TCS SP5 II, Wetzlar, Germany). All images were edited using Adobe Photoshop CS6 software.

RNA isolation and analysis

Total RNA was extracted using Trizol Reagent (Invitrogen, California, USA) according to the manufacturer's recommendations. For Northern blot analysis of GFP mRNA, 5 μ g of total RNA for each sample was separated in a 1.2% denaturing agarose gel and transferred to Hybond-N⁺ membranes. The membranes were hybridized with digoxigenin (DIG)-labelled probe corresponding to the full-length GFP. Northern blot analysis was conducted by following the instructions described in the DIG system user's guide (Roche Diagnostics, Shanghai, China). For detection of GFP siRNAs, 30 μ g of total RNA was separated in a 15% polyacrylamide gel containing 7 M urea. The upper part of the gel was removed and the lower part was transferred to Hybond-N⁺ membranes. The hybridization and detection of siRNAs were performed as described previously (Goto *et al.*, 2003). The probe used in the analysis of siRNAs was the same as described above for the Northern blot of mRNA. For the detection of PVX, quantitative real-time RT-PCR analysis was performed using the comparative C_t (threshold cycle) method, as described previously (Senshu *et al.*, 2011); three replicates were performed for each experiment.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1 Sequences and restriction sites of polymerase chain reaction (PCR) primers used in this study.