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Identification of Pns12 as the second silencing suppressor of *Rice gall dwarf virus*

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RNA silencing is a conserved mechanism found ubiquitously in eukaryotic organisms. It has been used to regulate gene expression and development. In addition, RNA silencing serves as an important mechanism in plants' defense against invasive nucleic acids, such as viruses, transposons, and transgenes. As a counter-defense, most plants, and some animal viruses, encode RNA silencing suppressors to interfere at one or several points of the silencing pathway. In this study, we showed that Pns12 of RGDV (*Rice gall dwarf virus*) exhibits silencing suppressor activity on the reporter green fluorescent protein in transgenic *Nicotiana benthamiana* line 16c. Pns12 of RGDV suppressed local silencing induced by sense RNA but had no effect on that induced by dsRNA. Expression of Pns12 also enhanced *Potato virus X* pathogenicity in *N. benthamiana*. Collectively, these results suggested that RGDV Pns12 functions as a virus suppressor of RNA silencing, which might target an upstream step of dsRNA formation in the RNA silencing pathway. Furthermore, we showed that Pns12 is localized mainly in the nucleus of *N. benthamiana* leaf cells.

***Rice gall dwarf virus*, Pns12, silencing suppressor**

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RNA silencing is a conserved mechanism used by plants and other eukaryotes to defend themselves against viruses and transposons [1]. Generally, RNA silencing is triggered by dsRNAs. The dsRNAs are recognized by specific cellular enzymes and cleaved into small interfering RNAs (siRNAs) of 20–24 nt in length. The siRNAs function as guides for a silencing complex that can block gene expression at transcriptional, post-transcriptional, and translational levels [2–6]. Most viruses produce dsRNAs during their life cycles. This means that viruses can be both triggers and targets of RNA silencing [7,8]. As a counter-defense, most plant viruses have evolved to encode a protein that can suppress RNA silencing [1,9–13].

A number of studies have shown that VSRs (viral suppressors of RNA silencing) target distinct steps of RNA silencing: from the production of dsRNAs to the incorporation of siRNAs into the RNA-induced silencing complex (RISC) [1,14,15]. In addition, some VSRs have been shown to interfere with the movement of silencing signals. A number of VSRs have been identified from plant and animal viruses [15–25]. Although most plant viruses only have one VSR, some plant viruses encode more than one silencing suppressor, which target distinct steps of RNA silencing [26,27].

Rice gall dwarf virus (RGDV) is a member of the genus

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Phytoreovirus under the family Reoviridae. Besides RGDV, viruses of the genus *Phytoreovirus* include *Rice dwarf virus*, *Wound tumor virus* [28], *Rice bunchy stunt virus* [29], *Tobacco leaf enation virus* [30] and *Homalodisca vitripennis virus* (HVP) [31]. RGDV has a double-shelled icosahedral particle. Its genome consists of 12 double-stranded RNAs, which encode six structural (P1, P2, P3, P5, P6, and P8) and six non-structural (Pns4, Pns7, Pns9, Pns10, Pns11, and Pns12) proteins. The core capsid is composed of P3, the major structural protein, which encloses P1, P5, and P6. The outer layer consists of two proteins, P2 and P8 [32]. RGDV is transmitted by *Recilia dorsalis* and *Nephotettix cincticeps* in a persistent and circulative manner [33]. The complete sequences of RGDV Thai [34,35] and Guangxi [36,37] isolates have been obtained, which paved the way for molecular studies on the functions of RGDV gene products and the RGDV-rice interaction.

S12, the smallest RNA segment of RGDV, is 853 bp long. It contains an ORF of 620 bp, from 31 to 650 nt [38]. To date, the function of the protein encoded by S12 and Pns12 remains unknown. A previous report showed that RGDV S11 encodes a silencing suppressor [21]. In this study, we present evidence that Pns12 of RGDV also has silencing suppressor activities. Pns12 of RGDV exhibited silencing suppressor activity in coinfiltration assays with the reporter green fluorescent protein (GFP) in transgenic *Nicotiana benthamiana* line 16c. It suppressed local silencing induced by sense RNA, but had no effect on that induced by dsRNA. Expression of Pns12 also enhanced *Potato virus X* pathogenicity in *N. benthamiana*. In addition, we showed that Pns12 localizes mainly to the nucleus in *N. benthamiana* cells, which suggested that Pns12 might function in the nucleus.

1 Materials and methods

1.1 Plasmids and *Agrobacterium*

Rice plants infected by RGDV, which were collected from Xinyi, Guangdong Province, were used as the source of RGDV. The viruses were transmitted artificially using its insect vectors to rice seedlings and the seedlings were grown and maintained in the greenhouse of the Institute of Plant Virology, Fujian Agriculture and Forestry University.

The primers for S12, 5′-CGC*AAGCTT*ATGACGAGCAACGAGGAAAAC-3′ and 5′-GAG*GAGCTC*TTACCTCGGTCTTCGTTTAC-3′, were designed according to sequences in NCBI (GenBank accession No. EF177263; restriction enzyme cleavage sites are underlined). The full open reading frame of Pns12 was amplified from pMD-18T vector containing RGDV S12. The PCR products were cleaved using *Hin*d III and *SaC* I and then transferred into the binary vector pPZP212 [39] (a kind gift from Dr. Powers), creating pPZP212-S12 (named 35S:S12 for simplicity).

The primer 5′-CGC*AAGCTT*TGACGAGCAACGAGGAAAAC-3′ was used to obtain ΔS12. PCR amplification using this primer results in the deletion of the first nucleic acid of the S12 ORF. ΔS12 was transferred into the same binary vector as S12, resulting in pPZP212-ΔS12 (named 35S:ΔS12 for simplicity). Deletion of the first nucleic acid of the S12 ORF leads to a construct that can be transcribed but cannot be translated into a protein. Similarly, the primer 5′-ATA*CCCGGG*TGACGAGCAACGAGGAAAAC-3′ was used to create PVX:ΔS12 (see below).

The ORF of S12 was amplified using primers 5′-ATA*CCCGGG*ATGACGAGCAA CGAGGAAAAC-3′ and 5′-ACG*GTCGAC*TTACCTCGGTCTTCGTTTAC-3′, which contains recognition sites for the enzymes *Sma* I and *Sal* I. The PCR products were ligated into the vector pGR107 (a kind gift from David Baulcombe) after double digestion using *Sma* I and *Sal* I. The recombinant plasmids were named PVX:S12. PVX:ΔS12 was obtained by using the primer 5′-ATA*CCCGGG*TGACGAGCAACGAGGAAAAC-3′.

The S12 ORF lacking the stop codon was obtained using the primers 5′-ATA*CCATGG*AAATGACGAGCAACGAGGAAA-3′ and 5′-ATA*CCATGG*ACCTCGGTCTTCGTTTACTTT-3′, which contained the recognition sites for the enzyme *Nco* I. The PCR products were ligated into the vector pRTL2-GFP, resulting in pRTL2-S12:GFP, which, after digestion with the enzyme *Hin*d III, was transferred into the binary vector pCAMBIA1301, creating pCAMBIA1301-S12:GFP (35S:S12:GFP for simplicity).

The constructs pPZP212, 35S:S12, 35S:ΔS12, 35S:ssGFP, 35S:dsGFP, 35S:TAV 2b, PVX, PVX:S12, PVX:ΔS12, and 35S:S12:GFP were sequenced to verify their correct construction and transformed into *Agrobacterium tumefaciens* GV3101 (kindly provided by David Baulcombe) by electroporation.

1.2 Agroinfiltration and GFP imaging

The *Nicotiana benthamiana* plant constitutively expressing a GFP transgene (line 16c; a gift from David Baulcombe) and the *Agrobacterium* infiltration operation have been described previously [40]. The *N. benthamiana* line 16c plants were cultured in growth chambers at 22–24°C before and after infiltration. GFP fluorescence was observed under long wavelength UV light (Black Ray model B 100A; UV Products) and photographed using a Nikon D70 digital camera with a Y48 yellow filter.

1.3 RNA extraction and Northern blotting

Total RNAs were extracted from leaves with TRIzol reagent (Invitrogen) in accord with the manufacturer's instructions. Northern blotting analysis was conducted according to instructions described in "the DIG system user's guide for

filter hybridization". For Northern blot analysis of siRNAs, low-molecular-weight RNAs were enriched from total RNAs by eliminating high-molecular-weight RNA using 5% polyethylene glycol (PEG 8000) plus 0.5 mol L^{-1} NaCl, separating on a 15% polyacrylamide-7 mol L^{-1} urea gel, and transferring to Hybond-N membranes. The hybridization and detection of siRNAs were performed as described previously [41]. The probes used in the analysis of siRNA were the same as those for Northern blots of mRNA.

1.4 Localization of Pns12

The leaves of four-week-old *N. benthamiana* plants were agroinfiltrated with *A. tumefaciens* harboring pCAMBIA1301-S12:GFP and pCAMBIA1301-GFP, respectively. By 3 dpi (days postinfiltration), the GFP expression in the leaves was observed at 488 nm using a confocal microscope (Leica TCS STED, Germany) and DAPI (2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride) to counterstain the nuclei. Images were captured using LAS AF Lite software and converted to tagged image file format for export.

2 Results

2.1 RGDV Pns12 inhibits local silencing induced by ssGFP

To determine whether RGDV Pns12 has silencing suppressor activities, a method named the agroinfiltration bioassay was used. A transformed *Agrobacterium* strain carrying RGDV Pns12 was mixed with a strain that carried 35S-GFP at a ratio of 3:1 and infiltrated into leaves of *N. benthamiana* line 16c. Agrobacteria harboring only the GFP gene or the 2b gene of *Tomato aspermy cucumovirus* (TAV) was used as negative and positive controls, respectively. In each treatment, 15 plants were infiltrated and the experiments were repeated at least three times. GFP fluorescence became visible at 2 dpi in localized regions. However, the green fluorescence intensity in the patches infiltrated with GFP alone or with GFP plus ΔS12, in which the first nucleic acid of the S12 ORF was deleted, became invisible after 7 dpi. By contrast, the green fluorescence intensity remained strong at 7 dpi in the patches coinfiltrated with 35S-GFP plus 35S-S12 and 35S-GFP plus 35S-TAV 2b, respectively (Figure 1A–F). These results suggest that Pns12 could suppress the RNA silencing induced by sense-GFP, presumably in a similar manner to TAV 2b. In addition, the fact that ΔS12, which could not produce an intact Pns12, had no effect on RNA silencing indicated it was the protein expressed from S12, but not the RNA, that could function as a silencing suppressor.

To confirm that the above observations resulted from differential accumulation of GFP mRNA, Northern blot analyses were conducted to detect steady-state levels of GFP mRNA using digoxin labeled probes. The results showed that GFP mRNA accumulated in tissues expressing 35S:GFP plus 35S:TAV 2b or 35S:GFP plus 35S:S12 and were high at 7 dpi, although the accumulated levels of GFP mRNA in tissues expressing 35S:GFP plus 35S:S12 were much lower than in tissues expressing 35S:GFP plus 35S:TAV 2b. By contrast, GFP mRNA could not be detected in tissues expressing GFP alone or 35S:GFP plus 35S:ΔS12 at the same time point (Figure 1G).

To further test whether RNA silencing suppressor activities of PnS12 were responsible for the above observations, Northern blot analyses were conducted to detect GFP-specific siRNAs. In all treatments, higher accumulation of GFP mRNA was correlated to lower accumulation of GFP-specific siRNAs, and lower accumulation of GFP mRNA was accompanied by higher accumulation of GFP-specific siRNAs (Figure 1G). These results indicated that transcription of the exogenously introduced sense-GFP can induce co-suppression or RNA silencing of both the exogenous and the endogenous GFP. RGDV PnS12 can suppress the RNA silencing induced by sense-GFP, in a similar manner to TAV 2b, although its silencing suppressor activities were much weaker than the latter.

2.2 Pns12 does not suppress local RNA silencing triggered by GFP dsRNA

DsRNA, as found by Fire *et al*. [7] in 1998, is the initiator of RNA silencing. RNA silencing involves three distinct stages: the generation of dsRNA, the cleavage of the dsRNAs into siRNAs 20–26 nt in length, and the incorporation of the siRNAs into an effector complex [12,42]. Many studies have used dsGFP in co-infiltration assays to determine the molecular targets of a particular VSR. It was found that VSRs, including Pns10 of *Rice dwarf virus* [20], P69 of *Turnip yellow mosaic virus* (TYMV) [43], and P25 of *Potato virus X* (PVX) [44] could not inhibit RNA silencing induced by dsRNAs, suggesting that these VSRs targeted a step upstream of dsRNA formation in RNA silencing. Our results indicated that Pns12 could inhibit local silencing of GFP triggered by ssGFP. However, the targets of Pns12 in the RNA silencing were unknown.

The effect of Pns12 on dsGFP-triggered silencing was tested, using 35S:TAV 2b plus 35S:ssGFP plus 35S:dsGFP and 35S:ssGFP plus 35S:dsGFP plus 35S:ΔS12 or the empty vector as positive and negative controls, respectively. As shown in Figure 2, leaves infiltrated with 35S-ssGFP plus 35S-dsGFP or with 35S-ssGFP plus 35S-dsGFP plus 35S-S12 (or 35S-ΔS12) lost GFP fluorescence at 7 dpi, indicating strong local GFP RNA silencing. This indicated that Pns12 could not suppress local silencing induced by dsRNA. As expected, strong GFP fluorescence was main-

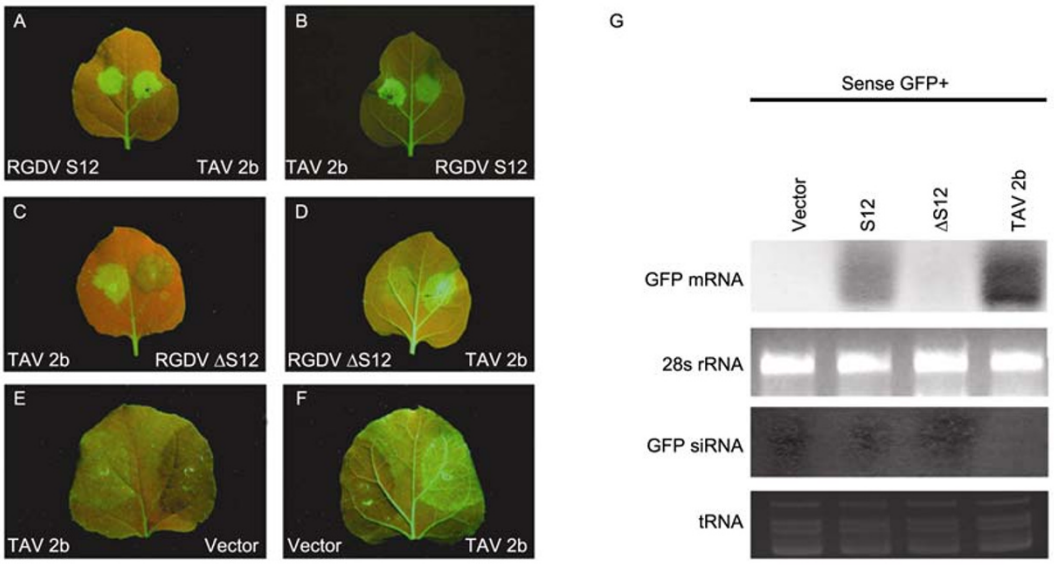

Figure 1 Suppression of local GFP silencing by RGDV Pns12 (A–F). *N. benthamiana* line 16c plants were coinfiltrated with *Agrobacterium* spp. (Agro.) mixtures carrying 35S-GFP and the individual constructs indicated in each image. GFP fluorescence was viewed under long wavelength UV light at 7 d postinfiltration (dpi). G, Northern blot analysis of the steady-state levels of GFP mRNA and siRNA extracted from different infiltrated patches shown in A–F. rRNA and tRNA were used as loading controls for detection of GFP mRNA and GFP siRNA, respectively.

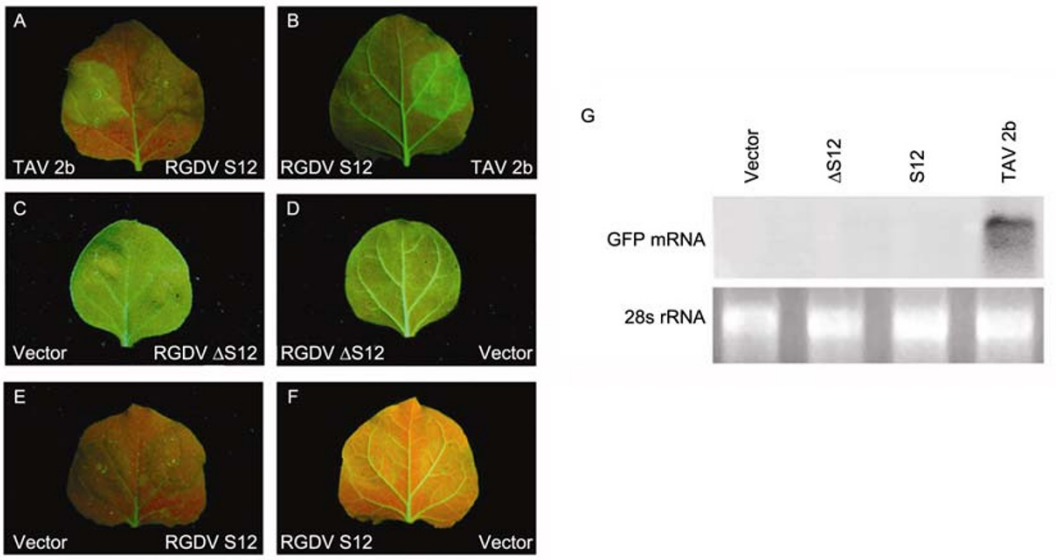

Figure 2 RGDV Pns12 did not inhibit local silencing induced by dsRNA (A–F). *N. benthamiana* line 16c plants were coinfiltrated with *Agrobacterium* spp. (Agro.) mixtures carrying 35S:dsGFP and the individual constructs indicated in each image. GFP fluorescence was viewed under long wavelength UV light at 7 d postinfiltration (dpi). G, Northern blot analysis of the steady-state levels of GFP mRNA extracted from different infiltrated patches shown in A–F. The bottom gel shows 28s rRNA with ethidium bromide staining as a loading control.

tained in leaves infiltrated with 35S-ssGFP plus 35S-dsGFP plus 35S:TAV 2b, indicating that TAV 2b suppressed local GFP RNA silencing triggered by dsRNA (Figure 2A–F). Northern blot analyses showed negligible accumulation of GFP mRNA and high accumulation of GFP-specific siRNAs in leaves infiltrated with 35S-ssGFP plus 35S-dsGFP and 35SssGFP plus 35S:dsGFP plus 35S:S12/35S:ΔS12 (Figure 2G), further demonstrating that Pns12 did not suppress local RNA silencing triggered by GFP dsRNA. By contrast, leaves infiltrated with 35S:ssGFP plus 35S:dsGFP plus 35S:TAV 2b showed high accumulation of GFP mRNA and much reduced accumulation of siRNA (Figure 2G). These results suggested that Pns12 targets a step upstream of dsRNA synthesis in the RNA silencing pathway.

2.3 Pns12 enhances PVX pathogenicity

Studies of Voinnet *et al*. [44] suggested that the RNA si-

lencing triggered by ssRNA and viruses might involve different mechanisms. P25 of PVX can inhibit ssRNA induced silencing. However, it had no apparent effects on silencing induced by viruses [44]. Synergism refers to a phenomenon in which plants infected with two unrelated viruses displayed increased disease symptoms compared with plants infected with either of the two viruses alone [45]. Currently, it is generally accepted that synergism is the result of very effective suppression of silencing by one virus, resulting in a dramatic increase in the accumulation of the co-infecting virus [46]. The above results showed that PnS12 of RGDV can suppress local silencing induced by ssRNA when transiently expressed in *N. benthamiana*. However, the function of the silencing suppressor activity of PnS12 in natural viral infection is unknown. As a dsRNA virus with a large genome, no infectious clones for reverse genetic studies of RGDV were available, making a direct test of the role of PnS12 in RGDV infection of rice impossible.

To study the role of Pns12 in viral infection in the context of plant-virus interaction, we utilized a PVX vector to express S12 and an ORF frame-shift mutant (ΔS12). Seedlings of *N. benthamiana* plants (four- to six-leaf stage) were inoculated with PVX, PVX-S12, and PVX-ΔS12, respectively. All inoculated leaves were asymptomatic. However, symptoms were visible in systemically infected leaves inoculated with PVX or PVX-ΔS12 as early as 6 dpi. Generally, the symptoms caused by PVX or PVX-ΔS12 developed initially as veinal chlorosis between 6 and 9 dpi but subsequently (9–18 dpi) as mild chlorotic spots in some leaves. In addition, some newly developed leaves became asymptomatic, a phenomenon reminiscent of recovery from viral infection. The symptoms caused by PVX-S12 developed one to two days later than those caused by PVX or PVX-ΔS12. However, the symptoms caused by PVX-S12 became indistinguishable from those caused by PVX or PVX-ΔS12 at 9 dpi. At 12 dpi, it became evident that the symptoms caused by PVX-S12 were more severe than those caused by PVX or PVX-ΔS12. This became even more obvious at 18 dpi. At this time, symptoms caused by PVX-S12 were still visible, whereas only very slight symptoms could be observed in some of the leaves of PVX or PVX-ΔS12 inoculated plants (Figure 3A).

To investigate the mechanisms by which Pns12 caused increased disease symptoms in the heterologous system, Northern blots were carried out to detect the accumulated levels of the genomic RNAs of PVX. As shown in Figure 3B, PVX mRNAs accumulated to high levels in systemic leaves of *N. benthamiana* infected by either PVX, PVX:ΔS12 or PVX-S12 at 6 dpi. However, the accumulation of PVX mRNAs decreased significantly in plants infected by either PVX or PVX:ΔS12 at 18 dpi. By contrast, the accumulated level of PVX mRNAs remained very high in plants infected by PVX-S12 at this time. Taken together, these results indicated that the presence of Pns12 resulted in increased disease symptoms in PVX infected *N. benthamiana.* The increased disease symptoms were probably a result of enhanced replication of PVX, which in turn might be caused by the suppression of RNA silencing by Pns12.

2.4 Cellular localization of RGDV Pns12

To determine the subcellular localization of Pns12, the S12 coding sequence was fused in-frame to the 5' terminus of the GFP gene driven by a Cauliflower mosaic virus 35S promoter. The constructs were introduced into *N. benthamiana* leaf cells via agroinfiltration. GFP fluorescence was observed using confocal microscopy 3 dpi. As shown in Figure 4, the fluorescence occurred in the nuclei of leaves expressing the S12:GFP fusion protein. However, an examination of leaves expressing free GFP by confocal microscopy showed fluorescence distributed evenly in the cytoplasm and nuclei. The nuclear localization of Pns12 was consistent with our predications based on sequence analysis using PSORT II Prediction (http://psort.ims.u-to-kyo.ac.jp/form2.html), which showed that a region located in the C terminus of Pns12 was rich in basic amino acids and might function as a nuclear localization signal (NLS). The relationship between the nuclear localization and the silencing suppressor activities of Pns12 warrants further investigation.

3 Discussion

Recent studies have shown that a specific plant virus can encode more than one silencing suppressors. For example, *Citrus tristeza virus* (CTV) encodes three VSRs. The three VSRs, namely p20, p23, and CP, can function independently at the intracellular or intercellular levels [4,26]. In addition, Cui *et al*. [47] demonstrated that a Geminivirus, *Tobacco curly shoot virus* Y35 (TbCSV-Y35), could process three VSRs, namely AC2, AC4, and βC1. Liu *et al*. [21] have reported that RGDV Pns11 was a silencing suppressor. Here, we showed that Pns12 might be another silencing suppressor of RGDV. Pns12 could suppress silencing induced by sense-GFP. It can reduce, but not eliminate, the accumulation of siRNAs arising from local silencing of GFP. These results indicated that Pns12 might target an initial step of RNA silencing, having a similar mode of action to 2b of CMV [48] and p69 of TYMV [43]. Supporting this, we showed that Pns12 could not inhibit silencing induced by GFP dsRNA (Figure 2). In addition, Pns12 could enhance the pathogenicity of PVX in a heterologous system. The introduction of Pns12 into the genome of PVX resulted in increased viral accumulation in infected *N. benthamiana* (Figure 3B). This suggested that Pns12 might have an important role in RGDV infection of rice.

Our observation indicates that the silencing suppressor activities of Pns12 are weaker than those of Pns11. Both Pns12 and Pns11 can inhibit ssRNA-induced silencing and

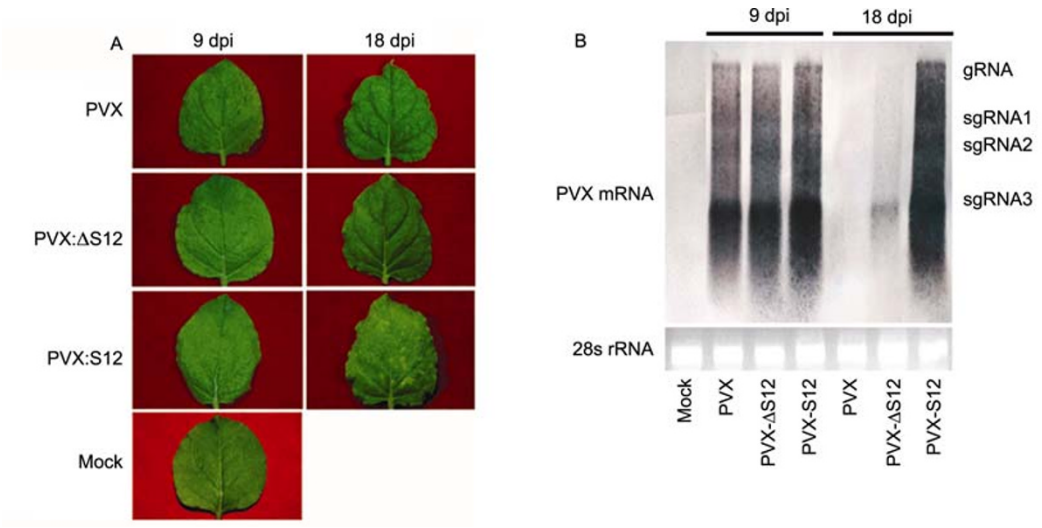

Figure 3 RGDV Pns12 enhances pathogenicity of chimeric PVX. A, Plants infected by PVX or PVX:ΔS12 show mild disease symptoms as a few scattered chlorotic speckles, whereas leaves infected with PVX:S12 show more severe symptoms. B, RNA gel blot analysis of accumulation of PVX genomic (gRNA) and subgenomic mRNAs (sgRNA1 to sgRNA3) at 9 and 18 dpi. The bottom gel shows rRNA with ethidium bromide staining as a loading control.

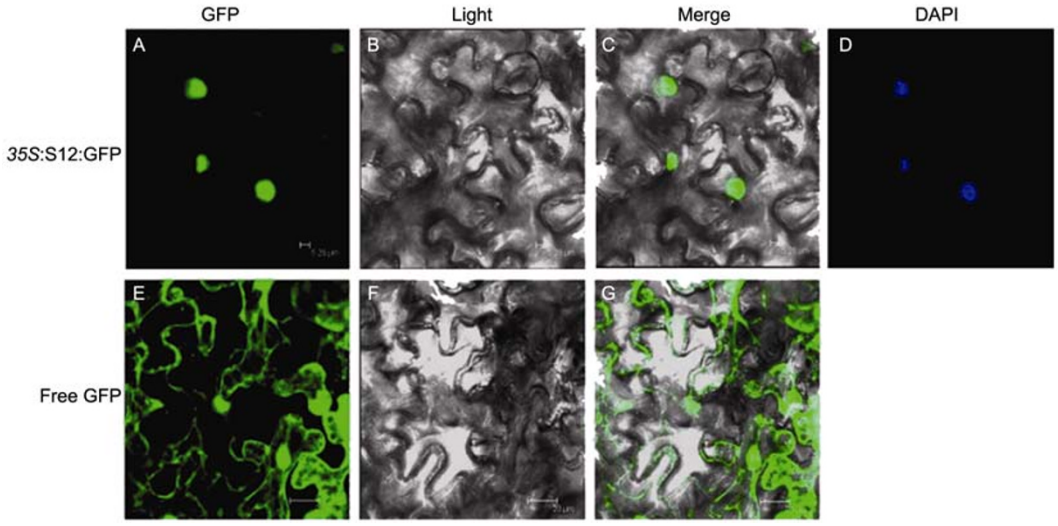

Figure 4 Subcellular localization of 35S:S12:GFP fusion protein transiently expressed in tobacco epidermis cells. A, Fluorescence from the Pns12 tagged with GFP accumulated in the nucleus. B, The same cell as in A under bright-field illumination (C) is an overlay of images A and B. D, DAPI stained nucleus. E, Accumulation of GFP in the cytoplasm and nucleus. F, The same cell as in E under bright-field illumination. G, An overlay of bright and fluorescent illumination of E and F.

enhance the pathogenicity of PVX in a heterologous system. However, unlike Pns11, Pns12 could not suppress systemic silencing triggered by ssRNAs (data not shown). However, the effects of Pns11 on silencing induced by dsRNAs are unknown at present. Thus, we suggest that Pns12 and Pns11 target different steps in the RNA silencing pathway.

Transient expression of Pns12 in *N. benthamiana* showed that this protein mainly accumulated in the nucleus. Nuclear localization is essential for the silencing suppressor activities of some VSRs. For example, the nuclear localization of the protein 2b, a VSR of *Cucumber mosaic virus* (CMV), was closely related to its pathogenicity [49]. Studies on 2b indicated that it could interact with AGO1, a major protein of the RNA-induced silencing complex (RISC). The interaction resulted in the suppression of the slicer activity of the RISC and thus compromised the miRNA pathway [50]. Nuclear accumulation was also required for these activities of 2b [51]. Nuclear accumulation was also required for the silencing suppressor activity and induction of necrosis of *Tomato yellow leaf curl virus*-China (TYLCV-C) C2 [52,53]. In addition, a recent study of Xiong *et al*. [23] showed that NS3, the VSR of *Rice stripe virus* (RSV) localized to the nuclei. Deletion of the NSL of NS3 resulted in reduced silencing suppressor activity. We hypothesize that Pns12 enters the nucleus and inactivates certain protein components of the RNA silencing pathway through physical interactions

with them. However, further studies are required to correlate the nuclear accumulation of Pns12 with its silencing suppressor activities. Additionally, it will be interesting to further characterize the role of Pns12 in RGDV infection of rice.

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