Rice yellow stunt rhabdovirus Protein 6 Suppresses Systemic RNA Silencing by Blocking RDR6-Mediated Secondary siRNA Synthesis

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The P6 protein of *Rice yellow stunt rhabdovirus* (RYSV) is a virion structural protein that can be phosphorylated in vitro. However its exact function remains elusive. We found that P6 enhanced the virulence of *Potato virus X* (PVX) in *Nicotiana benthamiana* and *N. tabacum* plants, suggesting that it might function as a suppressor of RNA silencing. We examined the mechanism of P6-mediated silencing suppression by transiently expressing P6 in both *N. benthamiana* leaves and rice protoplasts. Our results showed that P6 could repress the production of secondary siRNAs and inhibit systemic green fluorescent protein RNA silencing but did not interfere with local RNA silencing in *N. benthamiana* plants or in rice protoplasts. Intriguingly, P6 and RDR6 had overlapping subcellular localization and P6 bound both rice and *Arabidopsis* RDR6 in vivo. Furthermore, transgenic rice plants expressing P6 showed enhanced susceptibility to infection by *Rice stripe virus*. Hence, we propose that P6 is part of the RYSV's counter-defense machinery against the plant RNA silencing system and plays a role mainly in affecting RDR6-mediated secondary siRNA synthesis. Our work provides a new perspective on how a plant-infecting nucleorhabdovirus may counteract host RNA silencing-mediated antiviral defense.

In plants, RNA silencing plays prominent roles in defense against invading nucleic acids such as viruses and transposons (Csorba et al. 2009). Briefly, antiviral silencing is initiated when viral double-stranded RNAs (dsRNAs) or highly structured regions of single-stranded viral RNAs are processed into 21- to 24-nucleotide (nt) small interfering RNA (vsiRNA) duplexes by dsRNA-specific RNaseIII-type Dicer-like (DCL) enzymes (Ding and Voinnet 2007). One strand of the vsiRNA duplex is then incorporated into the RNA-induced silencing complex (RISC) containing the core component Argonaute (AGO) proteins to guide the degradation of complementary viral RNA targets (Pantaleo et al. 2007). Recently published data

demonstrated that, in plants, viral secondary siRNAs, derived from dicing of secondary dsRNA synthesized by the host RNA-dependent RNA polymerase (RDR)1 or RDR6, are also necessary for successful resistance to virus infection (Qu et al. 2005; Yang et al. 2004). Finally, the amplified silencing signal can spread locally, between neighboring cells through plasmodesmata, and systemically, via the vascular system, persisting for a long time (Melnik et al. 2011).

Viruses have evolved counterdefense mechanisms to thwart the host silencing system. In plants, many, if not all, viruses with positive- or negative-sense RNA or DNA genomes express silencing suppressor proteins to guarantee their successful invasion (Ding and Voinnet 2007; Roth et al. 2004). It is believed that the mode of action differs between suppressors. For example, the P19 protein of *Tomato bushy stunt virus* (TBSV) binds the siRNA duplexes and prevents their entry into the RISC (Silhavy et al. 2002). The 2b protein of *Cucumber mosaic virus* interacts with AGO1 and binds siRNA (Duan et al. 2012; González et al. 2010; Zhang et al. 2006) and may also interfere with the production of RDR1-dependent vsiRNAs (Diaz-Pendon et al. 2007). The coat protein (CP) of *Turnip crinkle virus* could bind dsRNAs to prevent them from being processed by the Dicer-like RNase (Qu et al. 2003). P6 of *Cauliflower mosaic virus* (CaMV) interacts with the dsRNA-binding protein 4 (DRB4) and thereby decreases the activity of DCL4 (Haas et al. 2008). The P protein of *Sonchus yellow net virus*, the only suppressor protein identified in plant rhabdoviruses, was reported to suppress gene silencing via unknown mechanisms (Jackson et al. 2005).

Two major classes of endogenous small RNAs, miRNA and siRNA, have been extensively studied in plants. miRNAs are approximately 21-nt noncoding RNAs produced by DCL-catalyzed excision from stem-loop precursors (Meyers et al. 2008), while siRNAs are processed from long dsRNAs produced by RDR. In addition to the differences in their biogenesis, these two classes of small RNAs also differ in their functions (Carthew and Sontheimer 2009). Interestingly, *trans*-acting siRNAs (ta-siRNAs), one class of endogenous siRNAs, are generated through the suppressor of gene silencing 3 (SGS3)/RDR6/DCL4 pathway after miRNA-guided cleavage of the RNA transcribed from *TAS* genes (Peragine et al. 2004; Vaucheret 2005; Yoshikawa et al. 2005). Among them, *TAS3* ta-siRNAs, which depend on the miR390-AGO7 complex and require two miR390 complementary sites for successful biogenesis, are conserved in plants, as indicated by bioinformatic analysis (Allen et al. 2005; Axtell et al. 2006; Montgomery et

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al. 2008). Ta-siRNAs function as negative regulators via interacting with the target transcripts and guiding their cleavage, mimicking the miRNA mechanism (Fahlgren et al. 2006; Vazquez et al. 2004).

Rice yellow stunt virus (RYSV) is a member of the *Nucleorhabdovirus* genus in the *Rhabdoviridae* family. It has a negative-sense, nonsegmented single-stranded RNA genome (Walker et al. 2000). The 12-kb genome contains a 5' trailer, a 3' leader, and seven open reading frames (ORF) ordered as 3'-le-N-P-3-M-G-6-L-5'-tr (Huang et al. 2003). The 3' proximal ORF-encoded N is a nucleocapsid protein (Fang et al. 1994) and P, M, G, and L are the viral phosphoprotein (Zhu et al. 1997), matrix protein (Luo et al. 1998), glycoprotein (Luo and Fang 1998), and RNA-dependent RNA polymerase (RdRp) (Huang et al. 2003), respectively. ORF3 has been identified as encoding a movement protein (Huang et al. 2005). ORF6 is located between the G and L genes and encodes a 93-aa, acidic protein (designated P6) (Huang et al. 2003). Similar ORF have also been found in the equivalent locations in the genomes of *Strawberry crinkle virus* and *Northern cereal mosaic virus* (Jackson et al. 2005; Tanno et al. 2000). However, their encoded proteins share little sequence similarity with RYSV P6 (Huang et al. 2003). As a virion protein, P6 can be phosphorylated in vitro but its functions remain unclear (Huang et al. 2003).

Agrobacterium-mediated transient assay in *Nicotiana benthamiana* has been extensively exploited as an approach to investigate the mechanisms underlying the functions of viral suppressors of RNA silencing (VSR) (Qu et al. 2003). However, a similar transient expression system is not available for monocots like rice. Accordingly, the protoplast system has been developed as a transient assay to complement the existing approaches in such plants. Recent studies demonstrated that RNA silencing could be induced in protoplast systems by the delivery of silencing inducers, such as synthetic siRNAs, in vitro-transcribed dsRNAs, or plasmids encoding dsRNA (Chen et al. 2006; Qi et al. 2004; Vanitharani et al. 2003). Here, the rice protoplast system was employed to evaluate the RNA-silencing suppressor function of the RYSV P6 protein.

In this study, we showed that RYSV P6 enhanced the virulence of *Potato virus X* (PVX) and inhibited systemic silencing induced by sense RNA or dsRNA in nonhost plants. Transiently expressing P6 in *N. benthamiana* leaves caused diminished accumulation of secondary siRNAs. In addition, we showed that P6 and RDR6 were colocalized in the nuclei and P6 can bind OsRDR6 and AtRDR6 in coimmunoprecipitation experiments. Furthermore, the transgenic rice plants expressing P6 became more sensitive to the infection of a tested virus, *Rice stripe virus* (RSV). Together, our results suggest that P6 is part of the RYSV's counter-defense system and interferes with RNA silencing by affecting the function of RDR6 during secondary siRNA synthesis.

RESULTS

P6 enhanced virulence of PVX.

To determine if RYSV P6 is related to the viral pathogenicity, we used a heterologous gene expression system based on an infectious PVX clone. *N. benthamiana* and *N. tabacum* SR1 plants were agroinfiltrated with plasmids expressing PVX, PVX-P6, or PVX-PA6, which contained a nontranslatable form of P6 mRNA due to a premature stop codon. Leaves of plants infiltrated with PVX or PVX-PA6 showed mild mosaic symptoms at 12 days postinfiltration (dpi), while those infiltrated with PVX-P6 showed more severe symptoms, including necrotic and chlorotic spots on leaves, leaf curling, and plant stunting (Fig. 1A to E; data in *N. tabacum* SR1 not shown).

These results showed that RYSV P6 could enhance pathogenicity when expressed from an engineered heterologous virus.

The accumulation of the PVX genomic and subgenomic RNAs (gRNAs and sgRNAs, respectively) in the plants infected with PVX, PVX-P6, or PVX-PA6 was checked. Total RNAs were extracted from systemic leaves of plants at 12 dpi. As shown in Figure 1F, the PVX gRNA mildly increased in the PVX-P6-infected *N. benthamiana* and *N. tabacum* SR1 plants. These results suggest that RYSV P6 acts as a pathogenicity determinant and this pathogenicity-enhancing activity is somewhat associated with viral RNA accumulation in the systemic leaves.

P6 could not suppress local silencing in *N. benthamiana*.

The ability of enhancing PVX virulence implied P6 might play a role in suppression of antiviral silencing pathway. In order to determine the activity of the P6 protein against local silencing, the agroinfiltration transient expression assay was used. An *Agrobacterium* strain harboring pBin61-P19 expressing TBSV silencing suppressor P19 was used as a positive control. When leaves of *N. benthamiana* line 16c plants were infiltrated with *Agrobacterium* strains harboring the plasmid pCambia1302 that expresses the green fluorescent protein (GFP), the infiltrated area appeared as a uniform red patch under UV light at 6 dpi, because the GFP gene was silenced locally (Fig. 2A). When the leaves of 16c were infiltrated with a mixture of *Agrobacterium* strains carrying GFP and P19, there was a marked increase in GFP fluorescence (Fig. 2A) and the high-level fluorescence persisted for at least 14 days. When 16c was infiltrated with *Agrobacterium* strains carrying GFP along with P6 or its mutant (pBA002-3HA-P6 or pBA002-3HA-PA6), there was no significant increase in the GFP fluorescence intensity in the infiltrated areas (Fig. 2A).

The results obtained from Northern blot analysis of the GFP mRNA were consistent with the observations of GFP fluorescence. There was a marked reduction in the accumulation of the GFP mRNA in leaves infiltrated with the *Agrobacterium* strain harboring GFP, and similar GFP mRNA reduction was observed in leaves infiltrated with GFP plus P6 or PA6. In contrast, there was increased accumulation of the GFP mRNA in leaves infiltrated with GFP plus P19 (Fig. 2B).

Abundant GFP-specific siRNAs of 21 to 24 nt were detected in leaves infiltrated with GFP alone, with GFP plus P6, or with GFP plus PA6, but not in those infiltrated with GFP plus P19 (Fig. 2B). To determine whether P6 was expressed in leaves infiltrated with GFP plus P6, total proteins were extracted and were analyzed by Western blot using monoclonal antibodies against hemagglutinin (HA) tag. The result showed that P6 was indeed present (Fig. 2C).

P6 could prevent systemic silencing in *N. benthamiana* plants.

After local silencing is initiated, the silencing signal can spread systemically throughout the plant (Dunoyer et al. 2010; Molnar et al. 2010). To investigate whether P6 affects this process, GFP fluorescence was monitored in upper leaves of 16c plants infiltrated with *Agrobacterium* strains carrying either GFP only (pCambia1302) or GFP along with P6 or its mutant PA6 (pBA002-3HA-P6, pBA002-3HA-PA6, respectively). After 16 dpi, we observed red fluorescence in upper uninfiltrated leaves of GFP-infiltrated plants, an indication of systemic silencing. The red fluorescence was also visible in plants infiltrated with GFP plus PA6 (Fig. 2D). In contrast, upper leaves of plants infiltrated with GFP plus P6 or control TBSV suppressor P19 showed green fluorescence (Fig. 2D). As shown in Table 1, the efficiency of P6 to inhibit systemic silencing was 85.7% (18 of 21 plants showed inhibition of systemic silencing). PA6 could not suppress systemic silencing, implying that

the P6 protein but not its mRNA was responsible for the suppression function. Northern blot showed that new upper leaves of the 16c plants that emerged after infiltration with GFP plus P6 or P19 contained significant levels of GFP mRNA and undetectable GFP-specific siRNAs (Fig. 2E). Conversely, GFP siRNAs were detected and no GFP mRNA was found in the upper leaves when GFP alone or with PΔ6 were used.

P6 diminished accumulation of secondary siRNAs.

The systemic spread of gene silencing mostly depends on the amplification of a silencing signal that likely contains siRNAs (Dunoyer et al. 2010; Molnar et al. 2010). The amplification process needs the RDR to duplicate the single-strand RNA templates to dsRNAs, which are subsequently processed into secondary siRNAs by DCL, a phenomenon known as transitivity (Moissiard et al. 2007). To study the underlying mechanism of inhibition of systemic silencing by P6, we explored its effect on transitivity, using the GFP gene as a test sample. An inverted-repeat construct corresponding to the 5' part of the *mgfp5* gene (covering 1 to 392 nt, named GF) was created. This construct, predicted to produce a dsRNA transcript composed of the sense strand GF and its complementary strand FG, was denoted as dsGF. Leaves of *N. benthamiana* were coagroinfiltrated with GFP, dsGF, and P6. Secondary siRNAs were visualized using probe P, which recognizes the 3' part of *mgfp5*, the region outside of the GF. As expected, the

patches infiltrated with GFP, dsGF, and P19 together displayed bright green fluorescence, while the patches infiltrated with GFP, dsGF, and P6 or PΔ6 displayed red fluorescence, which was consistent with the levels of GFP mRNA (Fig. 3A and B). dsGF-induced post-transcriptional gene silencing was verified by analyses of both GF primary and P secondary siRNAs at 3 and 6 dpi. The levels of primary siRNAs in P6-expressing patches were similar to those observed in mock and PΔ6-expressing patches at 3 dpi (Fig. 3B). At 6 dpi, the level of primary siRNA in P6 expression patches seemed lower than that in the mock and PΔ6-expressing tissues. Interestingly, neither P19- nor P6-expressing tissues showed visible accumulation of secondary siRNA at 3 dpi (Fig. 3B). Taken together, these results suggest that RYSV P6 inhibited systemic silencing triggered by dsRNA through restricting the transitivity of the silencing signals.

P6 interacted with RDR6 in vivo and they colocalized in the nuclei.

The ability of P6 to inhibit the formation of secondary siRNAs suggested its interference with dsRNA processing, which involves the RDR6 activity. To test whether P6 interacts with RDR6, we infiltrated leaves of *N. benthamiana* with *Agrobacterium* strains expressing 3HA-P6 along with 6myc-AtRDR6, 6myc-OsRDR6, or other control proteins. The results showed 3HA-P6 specifically coimmunoprecipitated with

Fig. 1. Effect of Rice yellow stunt rhabdovirus P6 on virulence of Potato virus X (PVX) on *Nicotiana benthamiana* and *N. tabacum* SR1. **A to E**, *N. benthamiana* were agroinoculated with PVX (**A**, **D** left, and **E** left), PVX-P6 (**B**, **D** right, and **E** right), or PVX-PΔ6 (**C**). Photographs were taken at 12 days post-infiltration (dpi) (**A** to **C**) and 28 dpi (**D** and **E**). **F**, Northern blot analysis of accumulation of PVX genomic and subgenomic RNAs (gRNA and sgRNAs, respectively) in plants infected with PVX (lane 1), PVX-P6 (lane 2), or PVX-PΔ6 (lane 3) at 12 dpi, using a probe corresponding to the PVX coat protein gene. Methylene blue-stained rRNA served as a loading control.

OsRDR6 and AtRDR6 but not with AtSGS3 or AtSDE3. All of these proteins were tagged with 6xmyc at their N termini (Fig. 4A).

The specific interaction between P6 and RDR6 was further confirmed by a subcellular colocalization experiment. *Agrobacterium* cells containing P6-enhanced cyan fluorescent protein (eCFP), and those containing enhanced yellow fluorescent protein (eYFP)-AtRDR6 were co-infiltrated into *N. benthamiana* leaves. The P6 protein exhibited localization in the nucleus and cell periphery, whereas eYFP-AtRDR6 was distributed throughout the nucleus and cytoplasm. Colocalization of P6-eCFP and eYFP-AtRDR6 was found mainly in the nuclei but not at the cell periphery (Fig. 4B).

P6 expression did not alter the expression level of ta-siRNAs in rice plants.

To gain further insights into the mechanism of P6 function, we examined whether the presence of P6 affected the activity of RDR6 in rice, the natural host of RYSV. Previous reports showed that ta-siRNAs are generated through the SGS3/RDR6/DCL4 pathway (Peragine et al. 2004; Yoshikawa et al. 2005). To quantify the expression of *TAS3*-related ta-siRNAs in

transgenic rice plants expressing P6 (6myc-P6 and 3HA-P6), we conducted reverse transcription-polymerase chain reaction (RT-PCR) (data not shown), Western blot analyses, and Northern blot analyses. The results showed that P6 transgenic rice plants and controls had similar levels of *Oryza sativa TAS3* 5'D6(+) ta-siRNA (Supplementary Fig. S1). Collectively, our data suggest that transgenic rice plants expressing P6 do not influence the function of OsRDR6 in the biogenesis of ta-siRNAs.

Table 1. Suppression efficiency of systemic silencing in *Nicotiana benthamiana* line 16c^a

Construct	Infiltrated plants (n)	Systemic silencing plants (n)	Suppression efficiency (%)
GFP	16	15	6.25
GFP+PΔ6	22	18	18.1
GFP+P6	21	3	85.7
GFP+P19	15	1	93.3

^a *Agrobacterium* strains carrying indicated constructs were agroinfiltrated to *N. benthamiana* line 16c and the green fluorescence was monitored under a long-wave UV lamp to detect the suppression efficiency of systemic silencing at 16 days postinfiltration (dpi). n = number of plants.

Fig. 2. Transient expression assay in leaves of *Nicotiana benthamiana* line 16c and suppression effect of *Rice yellow stunt rhabdovirus* P6 on systemic silencing caused by sense GFP (plasmid pCambia1302, which expresses the green fluorescent protein) RNA. **A**, Leaves were infiltrated with suspensions of *Agrobacterium tumefaciens* carrying different constructs as indicated above the figure. Leaves were photographed at 6 days postinfiltration (dpi). **B**, Northern blot analysis of accumulation of GFP mRNA and GFP siRNAs in leaves infiltrated with *Agrobacterium* strains carrying indicated constructs. **C**, Western blot analysis of 3HA-P6 protein expressed in *N. benthamiana* line 16c leaves infiltrated with *Agrobacterium* strains carrying GFP and 3HA-P6 using specific monoclonal antibodies against the hemagglutinin (HA) tag. Coomassie Brilliant Blue staining of the large subunit of RUBISCO served as a loading control. **D**, Plants of *N. benthamiana* line 16c agroinfiltrated with *Agrobacterium* strains carrying indicated constructs were photographed with a yellow filter under a long-wave UV lamp at 16 dpi. **E**, Northern blotting of GFP mRNA and GFP siRNAs extracted from newly emerged leaves of infiltrated plant at 16 dpi. Methylene blue staining and U6 hybridization served as loading controls for mRNA and siRNA, respectively. Scale bar = 1 cm.

P6 did not suppress dsRNA-induced silencing in rice protoplasts.

The rice protoplast system was used to study the suppression activity of P6 in rice cells. An inverted-repeat construct,

dseGF, similar to dsGF but corresponding to the 5' part of the eGFP gene (eGF) was created. We cotransformed plasmids of eGFP, dseGF with P6, PΔ6, or P19 (pX-DR-3HA-P6, pX-DR-3HA-PΔ6, and pX-DR-3HA-P19, respectively) into

Fig. 3. Effect of P6 protein on primary and secondary siRNA accumulation. **A**, *Nicotiana benthamiana* plants were agroinfiltrated with agrobacteria containing GFP construct (plasmid pCambia1302 which expresses the green fluorescent protein), dsGF, and buffer (mock), PΔ6, P6, or P19. Photographs were taken at 6 days postinfiltration (dpi). Scale bar = 1 cm. **B**, RNAs were extracted at 3 and 6 dpi, respectively, and were analyzed for the presence of GFP mRNA and siRNA. Small RNAs were hybridized with specific probes for the GF or P parts of GFP. Methylene blue staining and U6 hybridization served as loading controls for mRNA and siRNA, respectively. Expression of P6 was confirmed by Western blotting, using monoclonal hemagglutinin (HA) antibodies. Coomassie Brilliant Blue staining of the large subunit of RUBISCO served as a protein loading control.

Fig. 4. Interaction between P6 and RDR6 in vivo and intracellular localization of P6-eCFP and eYFP-AtRDR6. **A**, 3HA-P6 was coexpressed with 6myc-AtRDR6, 6myc-AtSDE3, 6myc-AtSGS3, or 6myc-OsRDR6 in *Nicotiana benthamiana*. Total soluble proteins were extracted (Input) and immunoprecipitated (IP) with anti-c-myc M2 affinity gel. Western blot was carried out using monoclonal myc or hemagglutinin (HA) antibodies to detect myc-tagged proteins (top panels) or coimmunoprecipitated HA-tagged P6 protein (bottom panels), respectively. **B**, *Agrobacterium* cells were co-infiltrated into leaves of *N. benthamiana*. Co-localization of P6-eCFP and eYFP-AtRDR6 was observed mainly in the nucleus. Infiltration with P6-eCFP or eYFP-AtRDR6 was used as controls. All images were taken at the same magnification. Scale bar = 10 μm.

the protoplasts at a weight ratio of 1:4:4. By 10 h post-transformation, the RNAs were isolated and were analyzed by RNA gel blotting.

As shown in Figure 5A, codelivery of eGFP and silencing inducer dsGFP caused a drastic reduction in eGFP mRNA. This silencing effect was prevented in the presence of P19 but not in the presence of P6 or PΔ6. These results in rice protoplasts were in accordance with the results we obtained in *N. benthamiana* leaves (Fig. 3B). Five independent experiments produced essentially the same results.

The expression level of eGF primary siRNAs and eP secondary siRNAs were also analyzed, using RNA gel blots. The results showed that the presence of P6 could slightly decrease the eGF primary siRNA expression but PΔ6 or P19 had no effects. As shown in the middle panel of Figure 5B, the expression of eP secondary siRNAs was undetectable. The expression of suppressors fused with DsRed and tagged with HA was confirmed by Western blot (Fig. 5C).

Transgenic rice plants expressing P6 showed enhanced sensitivity to RSV.

The above data suggested that P6 is a part of the counter-RNA silencing system in RYSV. By inference, the P6 trans-

genic plants may be more sensitive to virus infection than untransformed plants. Six independent P6 transgenic rice lines and untransformed wild-type rice plants were challenged with RSV, and the infected plants were harvested at 3 and 9 days postinoculation (six plants of each transgenic line and control plants). Total RNAs of inoculated plants (mixture of six plants) were isolated and levels of NSvc3 RNA (encoding the RSV CP protein) were quantified by quantitative RT-PCR. The results showed that there were larger quantities of the RSV RNA in most transgenic lines compared with the control plants (Fig. 6). These results suggest that the presence of the RYSV P6 protein facilitates the viral RNA accumulation via its RNA silencing suppressor activity.

DISCUSSION

RNA silencing is a defense strategy evolved by plants and other eukaryotic organisms against invading microbes such as viruses. As a counterdefense, viruses have evolved silencing suppressors that target multiple steps of the RNA silencing pathway of their hosts (Burguán and Havelda 2011; Wu et al. 2010). Many VSR have been identified from RNA and DNA viruses with diverse functions (Bisaro 2006; Qu and Morris 2005).

P6 is a structural protein of RYSV with unknown functions. Transgenic rice plants expressing P6 did not show any significant change in morphology and were more susceptible to virus infection than untransformed plants. Expression of RYSV P6 by PVX enhances the virulence of the heterologous virus PVX in *N. benthamiana*, *N. tabacum* SR1, and *N. glutinosa* (data not shown). Symptoms in the leaves of the above plants infiltrated with PVX-P6, e.g., chlorotic spots, stunting, and leaf yellowing, were also observed in RYSV-infected rice (Hibino 1996). Interestingly, this phenotype resembles the symptoms of PVX infection in RDR6-knockdown plants (Schwach et al. 2005). Taken together, our data imply that RYSV P6 possesses VSR functions in vivo. Specifically, our data suggest that RYSV P6 interferes with the function of the host plant RDR6 protein.

Fig. 5. Expression of *Rice yellow stunt rhabdovirus* P6 did not suppress enhanced green fluorescent protein (eGFP) silencing induced by double-stranded (ds)RNA in rice protoplasts. Plasmids encoding 35S-eGFP, 35S-dsGFP, and viral suppressors were introduced into rice protoplasts. After incubation in the dark for 10 h, RNAs were extracted and were analyzed for the presence of **A**, eGFP mRNA and **B**, primary and secondary siRNA. mRNAs and secondary siRNAs were hybridized with specific probes for the enhanced green fluorescence (eGF) part of eGFP. Methylene blue-stained rRNA and U6 hybridization served as loading controls for mRNA and siRNA, respectively. **C**, Western blot analysis of P6, PΔ6, and P19 with monoclonal antibodies against hemagglutinin (HA). Arrowheads (◄) indicate transiently expressed proteins fused with DsRed and HA tags.

Fig. 6. *Rice stripe virus* (RSV) RNA expression level in the P6 transgenic rice lines (Myc-P6 and HA-P6) after RSV infection. Levels of the RSV NSvc3 transcripts were analyzed by quantitative reverse transcription-polymerase chain reaction at 3 and 9 days postinoculation. The NSvc3 expression levels were normalized using the signal from the rice *actin* gene. The accumulation level of RSV NSvc3 in the wild-type rice plants was set to 1.0. Three biological repeats are shown (solid circles). Horizontal bars represent the average values.

The RNA silencing signals are amplified with the help of host proteins such as RDR6, SGS3, SDE3, and others in a process known as transitivity (Himber et al. 2003). The results from this study showed that the P6 protein could inhibit systemic RNA silencing by restricting the transitivity of the silencing signals triggered by dsRNA but had no effect on local RNA silencing. Coimmunoprecipitation experiments showed specific interaction of P6 with RDR6 but not with SGS3 or SDE3. However, no evidence for a direct or indirect interaction between P6 and RDR6 within the immunoprecipitated complex is known. P6 was colocalized with RDR6 in the nuclei. This suggests that P6 might inhibit amplification of the silencing signals by interfering with the activity of RDR6. Since RDR6 is indispensable for RNA silencing signal movement, we propose that P6-RDR6 interaction may limit the accumulation of secondary siRNAs, thereby inhibiting non-cell autonomous silencing. The levels of *TAS3* ta-siRNAs in P6 transgenic rice plants were similar to those in control rice plants, probably because the expression level of P6 in transgenic plants was too low to affect the function of RDR6 in producing ta-siRNAs. The possibility that other RYSV proteins function with P6 to regulate the biogenesis of secondary siRNAs during viral infection cannot be ruled out. It was reported that RDR6 colocalized with SGS3 in cytoplasmic granules (Kumakura et al. 2009). It may also be interesting to investigate the role of SGS3 in the P6-RDR6 partnership in future studies.

Unlike the TBSV silencing suppressor P19 that functions by binding 21- to 25-nt siRNAs (Silhavy et al. 2002) and the poliovirus protein P0, which inhibits the formation of secondary siRNAs and the local silencing by degrading the AGO1 protein (Baumberger et al. 2007; Bortolamiol et al. 2007), P6 neither interferes with local silencing nor binds 21-nt siRNA (Supplementary Fig. S2). PVX-P6 infected plants showed more severe symptoms and increased PVX genomic RNA levels compared with the control plants. Based on our study, we propose that P6 acts as a pathogenicity determinant, at least partially as a result of its counter-RNA silencing effects.

In the rice protoplast-based transient RNA silencing system, P6 could not interfere with RNA silencing triggered by dsRNA. Since this transient assay is conducted in single cells, it is not a good choice to study systemic silencing (Qi et al. 2004). We also did not detect secondary siRNAs in this system, probably because the end point analysis was performed 10 h post-transformation. Transgenic rice plants expressing P6 were more susceptible to RSV infection, resulting in higher levels of viral RNA accumulation than in infected control plants. We observed at least twofold higher levels of RSV RNA accumulated in HA-P6 transgenic rice lines than in Myc-P6 lines. Whether different fusion tags affect the partner protein differentially in its solubility or stability remains to be studied.

The role of RYSV P6 in the virus-insect vector interaction is still unknown. The P6 protein can be detected in viruliferous vector leafhoppers (Huang et al. 2003). Even though RNA interference directs innate immunity against pathogens in insect organisms (Wang et al. 2006), there is no RdRP homolog identified in any insect genomes until now (Gordon and Waterhouse 2007; Zong et al. 2009). It will be interesting to investigate the roles of RYSV P6 in the insect vector cells.

MATERIALS AND METHODS

Plasmid construction.

The plasmid pBin61-P19 was provided by D. C. Baulcombe (Department of Plant Sciences, University of Cambridge, Cambridge, U.K.). For construction of dsGF, a GF fragment (corresponding to the 5' 1 to 392 nt sequence of mgfp5) was amplified from pCambia1302 using two pairs of primers:

GF-F1 5'-AAACTCGAGCCCGGGAGAAGAAGCTTTTCAC TG-3'; GF-R1 5'-AAAAAAGCTTCCTTAAGCTCGATCCTG-3'; GF-F2 5'-AAAACTAGTGGAGAAGAAGCTTTTCACTG-3'; GF-R2 5'-AAAAGAATTCCTTAAGCTCGATCCTG-3'. Restriction sites (underlined) were added to these primers as follows: *Xho*I and *Hind*III sites with the first primer pair, *Spe*I and *Eco*RI sites with the second primer pair. The PCR-generated fragments, *Xho*I-GF-*Hind*III and *Eco*RI-FG-*Spe*I encoding sense and antisense mgfp5 5' proximal RNAs, respectively, were inserted into the plasmid pSK-intron (Guo et al. 2003) at the 5' and 3' arms of the intron between appropriate restriction sites to generate pSK-dsGF. After digestion with *Sma*I and *Sac*I, the fragment containing the sense GF, intron, and antisense FG sequence was purified and was ligated to pBI121 (Jefferson 1987; purchased from Clontech, Palo Alto, CA, U.S.A.) digested with the same enzymes. The resulting plasmid was denoted pBI-dsGF.

Similarly, dseGF, which produces a dsRNA consisting of the strand eGF (corresponding to nt 34 to 373 of the eGFP 5' terminal sequence) and its complementary strand eFG, was constructed by amplifying an eGF fragment (corresponding to nt 34 to 373 of the eGFP 5' terminal sequence) from pBI221-eGFP, using two pairs of primers: eGF-F1 5'-AAACTC GAGCCCGGGGTGGTGGCCATCCTGGTTCGA-3'; eGF-R1 5'-AAAAAAGCTTCGATGCGGTTCCAGGGGTGT-3'; eGF-F2 5'-AAAACTAGTGTGGTGGCCATCCTGGTTCGA-3'; eGF-R2 5'-AAAAGAATTCGATGCGGTTCCAGGGGTGT-3'. The PCR-generated fragments, *Xho*I-eGF-*Hind*III and *Eco*RI-eFG-*Spe*I were inserted into the plasmid pSK-intron to generate pSK-dseGF. After digestion with *Sma*I and *Sac*I, the fragment containing the sense eGF, intron and antisense eFG DNA sequences was purified and ligated to pBI221 (Jefferson 1987; purchased from Clontech) to obtain pBI-dseGF.

The binary vector pBA002, consisting of the CaMV 35S promoter, the nopaline synthase terminator, and a 6×myc tag or a 3×HA tag attached, was used for *Agrobacterium* infiltration experiments (Zhang et al. 2005). The ORF6 encoding the P6 protein was amplified by PCR, using the following two primers: P6-F1 5'-AAAAACCCGGGATGTCCAGCCAACAAG AAC-3' (*Sma*I site underlined) and P6-R1 5'-AAAAGAGCT CTTAATCTGGCCAGCCGTTGCA-3' (*Sac*I site underlined). A nontranslatable mutant DNA fragment of P6, which had a single nucleotide substitution (G to T) that converted the sixth codon (GAA) of ORF6 to a stop codon (TAA), was amplified by PCR using two primers: PΔ6-F 5'-AAAACCCGGGATGT CCAGCCAACAATAAAC-3' (*Sma*I site underlined) and PΔ6-R1 as stated above. The purified PCR products were double digested with *Sma*I and *Sac*I and were inserted into pBA002-6myc or pBA002-3HA to generate pBA002-6myc-P6 or pBA002-3HA-P6, pBA002-6myc-PΔ6 or pBA002-3HA-PΔ6. Then the 3HA-P6, 3HA-PΔ6 fragments were amplified from pBA002-3HA-P6 and pBA002-3HA-PΔ6, with the primers as follows: t-3HA-F 5'-tATGTATCCTTATGATGTTCCAGA-3', P6-R2 5'-TTAATCTGGCCAGCCGTTGCA-3'. After purification, the DNA fragments were ligated to the vector pX-DR (Chen et al. 2009), digested with *Xcm*I enzyme, to obtain pX-DR-3HA-P6, pX-DR-3HA-PΔ6. pX-DR-3HA-P19 was constructed using a similar method.

The RDR6, SGS3, and SDE3 genes of *Arabidopsis* were amplified from RT products of mRNA extracted from *Arabidopsis thaliana* (Col-0) plant leaves using the following primers: AtRDR6-F 5'-AAAACCCGGGTTATGGGGTCA GAGGGAAAT-3', AtRDR6-R 5'-AAAATTAATTAATTAGA GACGCTGAGCAAG-3', AtSGS3-F 5'-AAAACCCGGGTTA TGAGTTCTAGGGCTGGT-3', AtSGS3-R 5'-AAAAGAGCT CTTATCAATCATCTTCATTGTGAAG-3'; AtSDE3-F 5'-AA AACCCGGGTTATGAGTGTGAGTGGATA-3'; AtSDE3-R

5'-AAAATTAATTAATTACTTCCACCCATCAGACCAC TC-3'. *SmaI* sites (underlined) were added to the forward primers, and *PacI* or *SacI* sites (underlined) were added to the reverse primers. The PCR products of AtRDR6, AtSGS3, and AtSDE3 were double digested with *SmaI* and *PacI* or *SacI* and were ligated to pBA002-6myc previously restricted with the same enzymes, to produce pBA002-6myc-AtRDR6, pBA002-6myc-AtSGS3, and pBA002-6myc-AtSDE3, respectively. *OsRDR6*, also known as rice *SHOOTLESS2* (*SHL2*) (Nagasaki et al. 2007), was amplified from cDNA of *Oryza sativa* seedlings with the primers OsRDR6-F 5'-AAAATTAATTAACA TGCGATCGCCGCGCGGGCGGGCGG-3' (*PacI* site underlined) and OsRDR6-R 5'-AAAAGAGCTCTCATATCCTCTC GGAGATGTATGCTGCAAG-3' (*SacI* site underlined). The PCR product was double digested with *PacI* and *SacI* and ligated to pBA002-6myc previously restricted with the same enzymes to obtain pBA002-6myc-OsRDR6.

To prepare a CaMV 35S promoter-driven construct expressing P6-eCFP fusion protein, the P6 gene was amplified with primers P6-F3 5'-AAAAGGATCCGCATGTCCAGCCAACA AG-3' and P6-R3 5'-AAAACCATGGTATCTGGCCAGCCGT TGCA-3'. A *BamHI* site was introduced into the forward primer and an *NcoI* site into the reverse primer (underlined). The purified PCR product was digested with *BamHI* and *NcoI* and was ligated to pCambia1300-eCFP previously restricted with the same enzymes, to obtain pCambia1300-P6-eCFP. The eYFP gene was amplified with primer eYFP-F 5'-AAAA TCTAGATGGTGAGCAAGGGCGAG-3' and eYFP-R 5'-AA AACCCGGGGCTTGTACAGCTCGTCCATGC-3'. An *XbaI* site was introduced into the forward primer and a *SmaI* site was introduced into the reverse primer (underlined). The purified PCR product was digested with *XbaI* and *SmaI* and was ligated to pBA002-6myc-AtRDR6 previously restricted with the same enzymes, to obtain pBA002-eYFP-AtRDR6.

The full-length cDNA clone of PVX, pP2C2S, was provided by D. C. Baulcombe. The full-length cDNA of PVX was introduced into pBI121 vector in two steps. First, the 5' end (nt 1 to 550) of the PVX genome was amplified by PCR, using a forward primer corresponding to the 5' end of the PVX genome (5'-ggactagtGAAACTAAACCATACAC-3') incorporating a *SpeI* site (underlined, additional nucleotides in lowercase) and a reverse primer (5'-TCGGATCCAAGAAGTGCAG-3'), corresponding to nucleotides from positions 550 to 533 nt of the PVX genome and containing a *BamHI* site (underlined). The resulting PCR product was double digested with *SpeI* and *BamHI* and was inserted between the *XbaI* and *BamHI* sites of pBI121 to produce the plasmid pPVX550. Then, the pP2C2S was double digested with *BamHI* and *SacI*, and the remaining part of the PVX genome was inserted into the *BamHI* and *SacI* sites of pPVX550 to yield pBI121-PVX. ORF6 and the non-translatable mutant (discussed above) were obtained by PCR amplification, using pSK-P6 as template. An *EcoRV* and a *SalI* site were introduced in the forward and the reverse primers, respectively. The PCR products were then digested with *EcoRV* and *SalI* and were ligated into pP2C2S between the *EcoRV* and *SalI* sites. The resulting plasmids were double digested with *BamHI* and *SacI* and were ligated into the *BamHI* and *SacI* sites of pPVX550, to yield pBI121-PVX-P6 and pBI121-PVX-PA6.

The binary vector pCambia1300-221, a derivative of pCambia1300, was used for rice transformation. After double digestion with *HindIII* and *SacI*, the fragment of pBA002-6myc-P6 or pBA002-3HA-P6 containing the CaMV 35S promoter and 6myc-P6 or 3HA-P6 was ligated with pCambia1300-221 digested with the same enzymes to yield pCambia1300-6myc-P6 or pCambia1300-3HA-P6, respectively.

Plant materials, agroinfiltration, GFP imaging, and rice transformation.

N. benthamiana, *N. tabacum* SR1, GFP transgenic *N. benthamiana* (line 16c), and *Oryza sativa* spp. *japonica* cv. Nipponbare plants were kept under incubator conditions with 25°C 16 h of light and 8 h of dark. The *Agrobacterium* infiltration method has been described previously (Brigneti et al. 1998). GFP fluorescence was observed using a long-wave UV lamp (Black Ray model B 100AP; UV products, Upland, CA, U.S.A.) and was photographed with a Nikon 9500 digital camera equipped with a Kenko yellow lens (Tokyo).

Agrobacterium-mediated transformation of rice was performed as described previously with some modifications (Hiei et al. 1994). Briefly, we used actively growing calli induced from the scutella of mature seeds and cocultivated for 20 min with *Agrobacterium tumefaciens* EHA105 harboring the gene of interest in cell suspension containing 100 mM acetosyringone. After incubation at 28°C in the dark for 48 h on solid cocultivation medium, the calli were transferred twice to the selection medium containing 250 mg of carbenicillin per liter, each, for 2 weeks. Then the calli were plated on the regeneration medium to obtain transgenic plants.

Rice protoplast preparation and transformation.

Young rice seedlings 10- to 14-days postgermination were harvested; the leaf and stem tissues were cut into approximately 0.5-mm strips, using a sharp razor, and were soaked in 0.6 M mannitol. The soaked diced tissues were collected, transferred to the enzyme solution (0.6 M mannitol, 10 mM MES at pH 5.7, 1.5% cellulase RS, 0.75% macerozyme R-10, 1 mM CaCl₂, 0.1% bovine serum albumin, 5 mM β-mercaptoethanol, and 50 μg of carbenicillin per milliliter), digested at 28°C for 4 h in the dark under gentle shaking (50 rpm), and filtered through 35-μm nylon mesh. The protoplasts were collected by centrifuging at 150 × g for 5 min from the filtrate. After washing twice with W5 solution (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, 2 mM MES at pH 5.7), the protoplasts were resuspended in MMG solution (0.6 M mannitol, 15 mM MgCl₂, 4 mM MES at pH 5.7) at a concentration of 1 × 10⁶ cells/ml for polyethylene glycol (PEG)-mediated transformation.

Plasmid DNAs were mixed with 300 μl of resuspended protoplasts (3 × 10⁵ cells), 330 μl PEG solution (0.6 M mannitol, 100 mM CaCl₂, 40% (wt/vol) PEG 3350). For cotransfection, 2 μg of pBI221-eGFP, 8 μg of pBI-dseGF, and 8 μg of suppressor expression plasmids were used. The mixture was gently shaken and incubated at room temperature for 15 min. Following the incubation, 1.0 ml of W5 solution was added and mixed gently by inverting the tube, and the protoplasts were pelleted by centrifuging at 150 × g for 3 min. After being washed once more with W5 solution, the protoplasts were resuspended in 1.5 ml of W5 solution and were cultured in the dark at 28°C for 10 h.

Northern blot analysis.

Total RNAs were extracted from plant tissues using TRIzol (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. To detect PVX gRNA and GFP mRNA, 1 to 5 μg of RNA samples were separated on 1.2% agarose gels containing 1.0% formaldehyde and were transferred to Amersham Hybond N+ membranes (GE Healthcare, Buckinghamshire, U.K.). To detect siRNAs, 10 to 50 μg of total RNA samples were separated on 7 M urea and 15% polyacrylamide gels and were transferred to Amersham Hybond N+ membranes. For ta-siRNA hybridization, 80 μg of total RNA was separated and transferred in the same way as for siRNA detection. The probes for PVX gRNA, GFP mRNA, and siRNAs were ³²P-la-

beled using the Amersham Rediprime II DNA labeling system (GE Healthcare). Specifically, the probe to detect PVX gRNA corresponded to the PVX coat protein gene. Locked nucleic acid (LNA)-modified oligonucleotide complementary to *Oryza sativa* TAS3 ta-siRNA (5'-gtgggtcttacaAgGtCaAgaa-3', LNA nucleotides are shown in uppercase) was purchased from Exiqon A/S (Skelstedet 16, Vedbaek, Denmark) and labeled with ³²P on the 5'-end (Liu et al. 2007).

Coimmunoprecipitation and Western blot experiments.

Four-week-old *N. benthamiana* plants were infiltrated with *Agrobacterium tumefaciens* carrying pBA002-3HA-P6 together with *Agrobacterium tumefaciens* carrying pBA002-6myc-AtRDR6, pBA002-6myc-AtSDE3, pBA002-6myc-AtSGS3, pBA002-6myc-OsRDR6, or pBA002-6myc as a negative control. After 2 days, total proteins were extracted from 2 g of leaf tissue with 4 ml of extraction buffer (100 mM NaCl, 10 mM MgCl₂, 50 mM Tris-HCl at pH 7.5, 0.2% Triton X-100, 5 mM dithiothreitol) containing EDTA-free protease inhibitor cocktail (Roche Diagnostic, Indianapolis, IN, U.S.A.). After two centrifugations (each 15 min at 20,000 × g), the supernatants were immobilized on anti-c-myc M2 affinity gel (Sigma, St. Louis) at 4°C overnight. All washing steps were repeated four times in the same extraction buffer. Finally, the beads were taken up in polyacrylamide gel electrophoresis loading buffer and were heated at 100°C for 5 min. After centrifugation, the proteins were analyzed by Western blot with monoclonal antibodies against myc and HA tags.

Subcellular localization experiments and confocal microscopy.

Four-week-old *N. benthamiana* plants were co-infiltrated with the *Agrobacterium tumefaciens* harboring the plasmid pBA002-eYFP-AtRDR6 and that harboring pCambia1300-P6-eCFP. Plants were maintained for 2 days at 25°C (16 h of light and 8 h of dark) in an incubator. YFP and CFP were observed under a Zeiss LSM 5 EXCITER confocal laser scanning microscope (Carl Zeiss Microimaging GmbH, Oberkochen, Germany). YFP was excited at 514 nm and fluorescence emission was collected through a 530-nm long-pass emission filter (LP530). CFP was excited at 433 nm and fluorescence emission was collected through a 470 to 500 nm band-pass emission filter (BP470-500).

Virus challenge assay.

RSV, which belongs to the genus *Tenuivirus*, was used to challenge the P6 transgenic rice plants. Small brown planthoppers (*Laodelphax striatellus*), the insect vector of RSV, were cultured as previously described (Zhang et al. 2010). Transgenic and control rice seedlings at the two-leaf stage were exposed to viruliferous *L. striatellus* in an inoculation chamber for 48 h, at an insect to plant ratio of 5:1. After the insects were completely removed from the plants, the rice seedlings were continually grown in an incubator. Plants were harvested at 3 and 9 dpi, respectively. The whole plants (except the root) were cut and frozen in liquid nitrogen for isolating RNA. About 1 µg of total RNA was reverse-transcribed by SuperScript III reverse transcriptase (Invitrogen), using random hexamers as primers, according to the manufacturer's instructions. To quantify the relative viral RNA transcription level, the 2^{-ΔΔCt} method was applied with a Bio-Rad CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, U.S.A.) and the Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan). The primers rsv-rt-F GATGCGTTGTCTTACCTGACTGC and rsv-rt-R CA CTATCCCATACCTCGACACCA were used for measuring RSV CP gene expression, and Os-actinF GACTCTGGTGAT GGTGTCAGC and Os-actinR GGCTGGAAGAGGACCTCA

GG were used for assaying the level of *Osactin11* expression as an internal standard.

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