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Stable expression of Rice dwarf virus Pns10 suppresses the post-transcriptional gene silencing in transgenic *Nicotiana benthamiana* plants

P. ZHOU, B. REN, X.M. ZHANG, Y. WANG, C.H. WEI, Y. LI*

Peking-Yale Joint Center for Plant Molecular Genetics and Agrobiotechnology, National Laboratory of Protein Engineering and Plant Genetic Engineering, College of Life Science, The National Plant Gene Research Center, Peking University, Beijing 100871, China

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Summary. – RNA silencing is a conserved mechanism that defends against viral infection and retrotransposon activity for protection of the genome. Segment 10 (S10) of Rice dwarf virus (RDV) encodes Pns10 protein, a viral suppressor of RNAi that suppresses the host RNA silencing machinery. In this study, we obtained stable transgenic RDV-S10 *Nicotiana benthamiana* plants expressing Pns10. Suppression of post-transcriptional gene silencing (PTGS) by Pns10 supported the conclusion that this protein exhibited the RNA silencing suppressor activity. In particular, the transgenic plants stably expressing a viral suppressor of RNAi (VSR) provide a model system for investigating the mechanism of RNA silencing.

Keywords: RNA silencing; VSR; Rice dwarf virus; Pns10; transgenic plant

Introduction

RNA silencing is a conserved mechanism in fungi, yeasts, invertebrates, plants, and vertebrates. RNA silencing protects against viral infections, regulates endogenous gene expression, and protects the genome against inappropriate retrotransposon activity, as well as repetitive sequences (Carthew and Sontheimer, 2009; Ding and Voinnet, 2007; Kalmykova *et al.*, 2005; Tomari and Zamore, 2005; Zambon *et al.*, 2006). RNA silencing is triggered by dsRNA that can be derived from diverse sources, such as abnormal RNA, virus RNA, transgene RNA, transposons, and microRNA primary transcripts (Fire *et al.*, 1998; Geley and Muller, 2004; Hamilton and Baulcombe, 1999; Hannon, 2002). The trigger dsRNAs are processed into 21–24 nts short interference RNAs (siRNAs) by the RNase III enzymes Dicer and Drosha (Tuschl *et al.*, 1999; Xie *et al.*, 2003; Zamore *et al.*, 2000). The strands with the less stable 5'-ends (Khvorova *et al.*, 2003) are then preferentially recruited *via* their 5'-ends by different Argonaute proteins (Mi *et al.*, 2008) into RNA-induced silencing complexes (RISCs). The RISC guides the complex to the target mRNA by Watson-Crick base pairing. After the base-pairing, the targeted mRNA was degraded and processed by the complex of the RNase H enzyme, Argonaute (slicer) resulting in RNA silencing (Hutvagner and Simard, 2008; Vaucheret, 2008). RNA silencing can occur also post-transcriptionally and is called PTGS. It was discovered in transgenic *Petunia* plants that lost expression of both transgenes and homologous endogenous genes (Napoli *et al.*, 1990).

RNA silencing has evolved as a common mechanism to defend against viral infection in plants. To suppress the host RNA silencing, viruses often encode various proteins termed VSRs (Ding and Voinnet, 2007; Li and Ding, 2006). In recent years, numerous VSRs have been identified in many plant viruses and some animal viruses (Li and Ding, 2006; Qu and Morris, 2005; Voinnet, 2005) including non-structural protein Pns10 encoded by RDV (Cao *et al.*, 2005).

Rice dwarf virus (the genus *Phytoreovirus*, the family *Reoviridae*) (Zheng *et al.*, 2000) is composed of 12 genome segments of linear dsRNA. The genome of RDV is a natural

*Corresponding author. E-mail: liyi@pku.edu.cn; fax: +86-10-62756903.

Abbreviations: dpi = days post infiltration; GFP = green fluorescent protein; PTGS = post-transcriptional gene silencing; RDV = Rice dwarf virus; RISC = RNA-induced silencing complex; segment 10 = S10; siRNA = short interference RNA; TEV= Tobacco etch virus; VSR = viral suppressor of RNAi

inducer of RNA silencing and therefore, VSRs encoded by RDV are of particular interest. The non-structural protein Pns10 encoded by the S10 of RDV was identified as a VSR using transient expression assay (Cao *et al.*, 2005).

In this study, we generated transgenic *N. benthamiana* plants that stably expressed Pns10. To investigate the VSR activity of Pns10 in stable transgenic system we transiently expressed green fluorescent protein (GFP) gene in wild-type and stable Pns10 transgenic plants by agro-infiltration. Our results showed that Pns10 can suppress PTGS in the stable transgenic expression system and this result support the conclusion of Pns10 as a VSR from the transient expression. In addition, these transgenic plants provide a model system to investigate in detail the mechanism of RNA silencing suppression and to characterize Pns10 as a VSR.

Materials and Methods

Plants. Wild-type *N. benthamiana* plants and transgenic RDV-S10 *N. benthamiana* plants were grown in a greenhouse with 16 hrs supplemental lighting at a constant temperature of 25/20°C (day/night).

Agrobacterium tumefaciens strains and plasmids. Preparation of the plasmid pE3S10 and *Agrobacterium tumefaciens* strain AGL1: pE3S10 was described previously (Cao *et al.*, 2005). This strain was used to transform *N. benthamiana* plants and obtain the transgenic RDV-S10 plants. To construct a plasmid pCambia1301-GFP, the GFP gene fragment under control of the 35S promoter with a terminator was released from pRTL2-GFP (Itaya *et al.*, 1997), digested with *Hind* III, and inserted into the same restriction sites of pCambia1301 (http://www.cambia.org/daisy/cambia/585.html). The T-DNA region of the pCambia1301-GFP is shown in Fig. 2a. pCambia1301-GFP was electroporated into *A. tumefaciens* AGL-1 according to the manufacturer's instructions (Model ECM 630; BTX) to obtain the strain AGL1:pCambia1301-GFP.

Transformation of N. benthamiana. A. tumefaciens-mediated transformation was modified and used for generating transgenic plants (Horsch, 1985). The MSb medium (full-strength MS salts (Murashige and Skoog, 1962), full-strength B5 vitamins (Gamborg *et al.*, 1968), 87.6 mmol/l sucrose, 0.56 mmol/l inositol, 3 mmol/l MES, 0.54 μmol/l 1-naphthylacetic acid, 4.4 μmol/l 6-benzylaminopurine, 1.2% agar, pH5.8) was used for co-cultivation and selection (containing 0.5 mmol/l cefotaxime and 0.6 mmol/l kanamycin mono sulphate).

Southern blotting hybridization. Genomic DNA was extracted from the fresh tissues using cetyl-trimethyl-ammonium bromide (CTAB) method (Murray and Thompson, 1980). 15 μg of genomic DNA was digested with *Eco*RI and separated on a 1% agarose gel and transferred onto Hybond-N+ membrane (Amersham Biosciences) with 20×SSC. As a probe, the RDV-S10 gene was labeled with digoxigenin (DIG) using the PCR DIG Probe Synthesis kit (Roche). Hybridization and detection were performed as described previously (Men *et al.*, 2003).

Northern blotting hybridization. Total RNA was extracted from leaves with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (10 μg) was separated on a 1.2% formaldehyde-denatured agarose gel and transferred onto Hybond-N+ membrane (Amersham Biosciences) with 20×SSC. The membrane was UV-crosslinked at 1500 KJ and hybridized with DIG-labeled probes corresponding to the full-length ORF of RDV-S10 or GFP, overnight in DIG high SDS hybridization buffer at 50°C (Cao *et al.*, 2005). The washing and detection of blots were performed as described in Southern blotting hybridization.

For northern blotting analysis of siRNAs, enrichment of low-molecular-weight RNAs was performed as described previously (Cao *et al.*, 2005). Low-molecular-weight RNA sample (10 μg) were separated on a 15% polyacrylamide-urea gel and transferred onto Hybond-N+ membrane (Amersham Biosciences) with 20×SSC. The membrane was hybridized with DIG-labeled probes corresponding to the full-length ORF of GFP. The hybridization and detection of siRNAs were performed as described previously (Goto *et al.*, 2003).

Western blotting hybridization. For western blotting analysis of RDV-Pns10 protein in the transgenic plants tissues, we used the procedure described previously (Guo and Ding, 2002; Hagiwara *et al.*, 2003). Polyclonal antiserum specific for Pns10 was prepared in rabbits (Laboratory Animals Center, Chinese Academy of Sciences) and applied at a dilution of 1:5,000.

Agro-infiltration and GFP imaging. The suspension of *A. tumefaciens* strain AGL1:pCambia1301-GFP was infiltrated into the axial airspaces of 6–8-week-old *N. benthamiana* plants according to a described method (Hamilton *et al.*, 2002; Johansen and Carrington, 2001). 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.

Semi-quantitative image analysis of hybridization signals. The free image analysis software ImageJ (http://rsb.info.nih.gov/ij/download.html) was used for semi-quantitative image analysis according to the manual (http://rsb.info.nih.gov/nih-image/). Northern blotting images were inverted and then the area and mean gray values of the hybridization bands and ethidium bromide-stained control RNAs were computed by the ImageJ software. The uncalibrated optical density values were obtained using the following function: uncalibrated OD = log10 [255/(255–Gray Value)]. The total quantities were calculated by the function: total quantity = uncalibrated OD* area. The GFP transcript levels and siRNA levels of RDV-S10 transgenic lines were normalized to the ethidium bromide-stained control RNAs signal and expressed as the ratio to the corresponding value of the wild-type (RA).

Results

Generation of stable transgenic RDV-S10 N. benthamiana plants

We utilized a simple, rapid, and efficient system for obtaining stable transgenic *N. benthamiana* plants using *A. tumefaciens*-mediated transformation and kanamycin as the selection marker. After inoculation, co-cultivation, selection, and rooting we obtained more than 15 transgenic *N. benthamiana* lines that showed resistance to kanamycin.

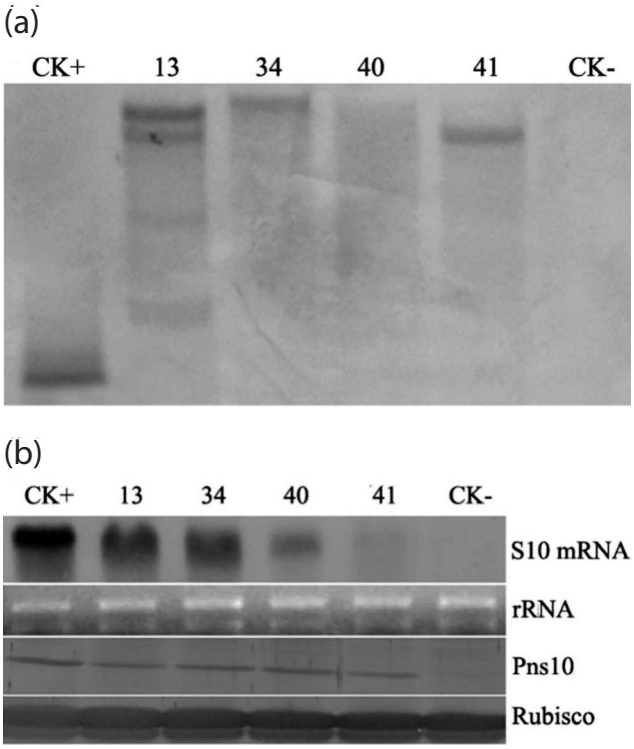

Fig. 1

Southern blotting (a), Northern and western blotting (b) analyses of transgenic *N. benthamiana* plants

Transgenic lines are represented by Nos.13, 34, 40, and 41. CK+, CK- represent positive and negative control, respectively.

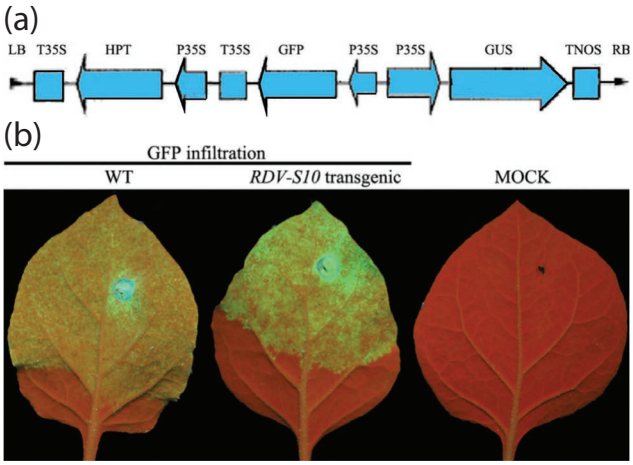

Fig. 2

Pns10 suppression activity of local RNA silencing triggered by GFP in transgenic *N. benthamiana* plants

(a) T-DNA regions of pCambia1301-GFP; LB – left border; T35S – terminator of CaMV 35S; HPT – coding region of hygromycin phosphotransferase gene; P35S – CaMV 35S promoter; GFP – GFP full coding region; TNOS – terminator of nopaline synthase gene; GUS – ß-glucuronidase reporter gene full coding region; RB – right border. (b) GFP fluorescence images of wild-type (WT), RDV-S10 transgenic, and mock-transfected plants.

These transgenic lines were analyzed to confirm presence of Pns10 gene expression.

Molecular analysis of transgenic RDV-S10 plants

Transgenic lines exhibiting resistance to kanamycin signed as No. 13, 34, 40, and 41, were selected randomly for Southern blotting hybridization. The plasmid pE3S10 digested with *Eco*RI was used as a positive control and DNA from a non-transgenic plant was used as a negative control. RDV-S10 has no *Eco*RI sites and therefore, digestion of genomic DNA of transformants with *Eco*RI generated a unique fragment for each integrated copy. Thus, the Southern blotting results could provide an estimation of the transgene copy number in genome of the transformant. The Southern blotting results indicated that all tested transgenic lines showed the positive hybridization bands with RDV-S10 gene probes in agreement with the positive control and no hybridization signal was detected in genomic DNA from the negative control plants (Fig. 1a). These results indicated that the RDV-S10 gene was incorporated into the genome of 4 transgenic lines. The patterns of the hybridization signals were different from plant to plant indicating that independent transformation events and random integration had occurred in the transformation process. The copy number of integrated genes ranged from a single copy to four copies indicating a multiple insertion.

Northern blotting with an RDV-S10 gene probe and western blotting analysis with polyclonal antiserum against Pns10 were used for determination of RDV-S10 gene expression (Fig. 1b). All 4 transgenic lines contained a transcript corresponding to the appropriate size of the RDV-S10 coding sequence and expressed Pns10 protein. Total mRNA and total proteins extracted from the leaves infiltrated with the strain AGL1: pE3S10 at 3 day post infiltration (dpi) were used as the positive control in northern and western blotting analysis. Total RNAs and proteins extracted from non-transgenic plant were used as the negative control and rRNAs stained with ethidium bromide together with rubisco stained with Coomassie brilliant blue were used as the loading controls.

Pns10 suppresses RNA silencing triggered by sense GFP RNA

We selected transgenic line 34 for further study, since it contained only one copy of RDV-S10 in its genome and expressed Pns10 at a higher level. Transient bioassays showed

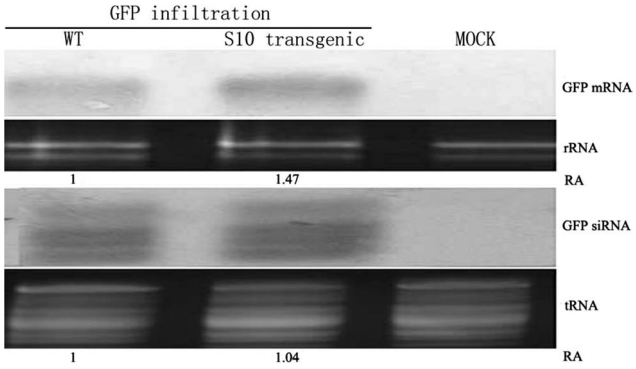

Fig. 3

Northern blotting analysis of the steady-state levels of GFP mRNAs and siRNAs in wild-type (WT), transgenic RDV-S10 and mock-transfected *N. benthamiana* plants

Relative quantity of GFP mRNA/rRNA and GFP siRNA/tRNA. RA – normalized ratio.

that Pns10 suppressed local GFP RNA silencing triggered by sense RNA (Cao *et al.*, 2005). however, we used the transgenic plants expressing Pns10 to study the function of Pns10 and to compare the difference between the transient expression and stable expression. We infiltrated wild-type and transgenic RDV-S10 *N. benthamiana* plants (line 34) with *A. tumefaciens* strains AGL1: pCambia1301-GFP. Afterward, we monitored GFP fluorescence under long wavelength UV light. A wild-type *N. benthamiana* plant without infiltration served as a mock-transfected control.

As expected, the green fluorescence intensity of all leaves infiltrated with GFP reached the highest level at 2–3 dpi. Thereafter, the level of fluorescence declined in the wild-type plants (Johansen and Carrington, 2001; Voinnet and Baulcombe, 1997), but remained high in the transgenic RDV-S10 plants at 5 dpi (Fig. 2b). In fact, GFP was still present in the transgenic RDV-S10 plants at 7 dpi, what is the same outcome as demonstrated in the transient system (data not shown). This result proved that Pns10 suppressed the GFP fluorescence decline.

Subsequently, mRNAs and siRNAs were extracted from the infiltrated leaves and hybridized with the DIG-labeled GFP gene probe. rRNAs and tRNAs stained with ethidium bromide were used as the loading controls.

The mRNA blotting hybridization revealed that the steady-state levels of GFP mRNA in transgenic leaves were 1.47 times higher as in wild-type leaves (Fig. 3). Therefore, the expression of Pns10 in transgenic plants contributed to the stabilization of GFP mRNAs, what resulted in the elevated GFP fluorescence.

The presence of siRNAs is a hallmark of RNA silencing (Hamilton *et al.*, 2002; Hamilton and Baulcombe, 1999). therefore, we also examined the accumulation of GFP-specific siRNAs in the infiltrated leaves to confirm that the observed mRNA decline was a consequence of RNA silencing. As expected, northern blot analysis showed that the wild-type *N. benthamiana* plants exhibited abundant amount of small RNAs. However, in the transgenic plants, where the degradation of GFP mRNA was suppressed by expression of Pns10, equal amounts of GFP siRNAs were detected (Fig. 3). This result suggested that RDV Pns10 suppressed RNA silencing triggered by the sense RNA, but did not downregulate the siRNA accumulation.

Discussion

The transient expression and stable expression assays are the main methods used to identify plant VSRs. The transient expression assay is rapid and easy (Guo and Ding, 2002; Johansen and Carrington, 2001; Voinnet *et al.*, 2000). Two *A. tumefaciens* strains expressing a candidate suppressor and GFP reporter gene, respectively, are co-infiltrated into the leaves of *N. benthamiana*. When the candidate suppressor has RNA silencing suppressor activity, the infiltrated patch would remain bright green under UV right at 5 dpi. When the candidate suppressor did not have the suppressor activity, the patch would turn red due to silencing of GFP expression. To date, many VSRs have been identified by this method including some VSRs encoded by animal viruses (Iwamoto *et al.*, 2005; Li *et al.*, 2002).

The stable expression assay has several merits in comparison to the transient expression assay. It can identify certain suppressors that suppress systemic silencing, but not local silencing, such as the coat protein of Citrus tristeza virus (Lu *et al.*, 2004). RNA silencing can regulate endogenous gene expression and then control the organism's development. On the other hand, VSRs disturb the normal RNA silencing pathway and affect the organism's development (Chapman *et al.*, 2004). Therefore, the stable expression of VSRs in transgenic plants is an effective tool to study the mechanism of RNA silencing and VSRs. The crossing of VSR transgenic plants and well-characterized transgenic lines expressing reporter genes such as GFP or beta-glucuronidase (GUS), can be used to study transgene-induced RNA silencing (Kasschau and Carrington, 1998). The stable expression assay is also well suited for the investigation of the suppression of systemic silencing using grafting (Guo and Ding, 2002; Mallory *et al.*, 2001, 2003).

In this work, we generated transgenic RDV-S10 *N. benthamiana* plants by the leaf disc method and showed that Pns10 was expressed in transgenic plants by molecular analysis. This study provided a useful transgenic material for further research about the function of Pns10.

Non-transgenic as well as transgenic *N. benthamiana* plants were infiltrated with *A. tumefaciens* expressing GFP

as the PTGS inducer. The patches on transgenic plants remained bright green, while those on the wild-type plants turned red at 5 dpi. These results demonstrated that Pns10 displayed PTGS suppressor activity in a stable transgenic system similar to that in the transient system. Pns10 could suppress the RNA silencing triggered by sense-GFP and enhance the accumulation of GFP mRNAs.

Our results using transgenic lines differed slightly from the results obtained using the *A. tumefaciens*-mediated transient expression assay. The accumulation of siRNAs was eliminated, when the GFP and RDV-S10 were co-infiltrated into leaves, whereas equal levels of siRNAs were observed in transgenic RDV-S10 plants and wild-type plants. RNA silencing is a complicated phenomenon involving many cell factors, such as Dicers, RNA-dependent RNA polymerases, and Argonautes. VSRs might be involved in more than one pathway for suppression of the host RNA silencing. For example, HC-Pro protein encoded by Tobacco etch virus affects the host Dicer activity, RISC formation, even affects the sequestering of siRNAs (Lakatos *et al.*, 2006). Earlier reports also indicated that different methods of studying RNA silencing can provide different results for the accumulation of siRNA. For example, HC-Pro prevents or largely eliminates siRNA accumulation in transgenic plants (Llave *et al.*, 2000; Mallory *et al.*, 2001), but not in transient expression or protoplast systems (Johansen and Carrington, 2001; Lakatos *et al.*, 2006; Qi *et al.*, 2004). There is no convincing explanation for this difference, but it may be the basic difference between transient and transgene system (Mallory *et al.*, 2002; Zhang *et al.*, 2008). We also presume that the stable expression of Pns10 in transgenic plants can induce steady and persistent effects on the host RNA silencing system. We believe that the stable transgenic plants will provide a better system for further investigation of Pns10 as a VSR in systemic silencing, transgene-induced RNA silencing, and organism development.

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