Identification of an RNA silencing suppressor encoded by a mastrevirus

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Wheat dwarf virus (WDV) is a DNA virus belonging to the genus Mastrevirus of the family Geminiviridae. In this study, we report that the Rep protein encoded by WDV is a RNA silencing suppressor as determined by co-infiltration assays using transgenic Nicotiana benthamiana line 16c carrying the GFP reporter gene. The Rep protein was shown to inhibit both local and systemic RNA silencing of the GFP gene as well as the spread of systemic GFP RNA silencing signals. Gel mobility shift assays showed that the Rep protein binds 21 nt and 24 nt small interfering RNA (siRNA) duplexes and single-stranded (ss)-siRNA. To our knowledge, this is the first identification of an RNA silencing suppressor encoded by mastreviruses. Furthermore, deletion mutagenesis indicates that both the N- and C-terminal regions of the Rep protein are not critical for silencing suppression and self-interaction, but the N terminus of Rep is necessary for its pathogenicity.

Post-transcriptional gene silencing (PTGS) is a sequence-specific defence mechanism that can target both cellular and viral mRNA for degradation in plants. A common feature of PTGS involves structured or dsRNA that is processed into small interfering RNA (siRNA) molecules of 21–25 nt by an RNase III-like enzyme, named Dicer (Fire et al., 1998). When PTGS is induced at one site the silencing signals move both cell to cell and long distances to trigger systemic silencing of target RNA in distant tissues of plants (Palauqui et al., 1997; Voinnet & Baulcombe, 1999; Himler et al., 2003).

Geminiviruses are plant viruses that have either bipartite or monopartite circular ssDNA genomes. The family Geminiviridae is divided into seven genera, Begomovirus, Mastrevirus, Curtovirus, Topocuvirus, Becurtovirus, Turncurtovirus and Eragrovirus (Adams et al., 2013). Many studies have demonstrated that plants employ PTGS as a natural defence mechanism against geminivirus infection. As a counter-defensive measure, geminiviruses have evolved in different ways resulting in expression of unique proteins that function as suppressors of PTGS (Sharma & Ikegami, 2008). The AC2, AC4, V2, C2, C4 and βC1 proteins associated with several begomoviruses and their associated betasatellite have been found to act as silencing suppressors (Vanitharani et al., 2004; Cui et al., 2005; Gopal et al., 2007; Zrzacha et al., 2007; Amin et al., 2011; Luna et al., 2012; Sharma & Ikegami, 2010; Zhang et al., 2012). Recently, the alphasatellite-encoded Rep protein associated with a begomovirus was also found to have strong silencing suppression activity (Nawaz-ul-Rehman et al., 2010). PTGS suppressors have also been described in the genus Curtovirus (Wang et al., 2005).

Wheat dwarf virus (WDV) belongs to the genus Mastrevirus (Schubert et al., 2007), members of which possess a monopartite genome. For WDV, the single transcription unit produced from the complementary-sense promoter has the potential to encode two proteins, RepA (formerly C1) and Rep (formerly C1:C2), the latter after a splicing event (Schalk et al., 1989). Two ORFs that encode the movement protein (MP or V1) and the coat protein (CP or V2) are on the viral-sense strand.

To investigate if any proteins encoded by WDV can suppress PTGS, a pCHF3 binary expression vector was employed to express individual WDV ORFs in plants. Fragments carrying individual ORFs (i.e. V1, V2, RepA and Rep) were amplified by PCR, using ORF-specific primers, from a recombinant plasmid that contains a full-length infectious clone of WDV-Wz-1 (Dr Joerg Schubert, Federal Centre for Breeding Research on Cultivated Plants, Germany). The resulting PCR products were first cloned into the pMD18-T vector (TaKaRa) and then into the pCHF3 vector. A construct capable of constitutively expressing tomato bushy stunt virus p19 from the cauliflower mosaic virus 35S promoter (Xiong et al., 2009), or empty vector (pCHF3) containing no insert were used as positive and negative controls, respectively.

RNA silencing suppression assays were conducted essentially as described previously (Bucher et al., 2003; Voinnet et al., 2003). Leaves of transgenic Nicotiana benthamiana

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One supplementary figure and one supplementary table are available with the online version of this paper.
Fig. 1. Effect of WDV Rep protein on local and systemic silencing of GFP in transgenic N. benthamiana line 16c in an agro-co-infiltration assay. (a) Suppression of GFP local silencing by Rep. Photographs of plants were taken under long-wavelength UV light at 3 days p.i. (b) Northern blot analysis of GFP mRNA and GFP siRNA extracted from agroinfiltrated patches of 16c leaves shown in (a). Ethidium bromide staining of rRNA were used as loading control of blot analysis of GFP mRNA and the small housekeeping RNA U6 blot served as loading control of blot analysis of GFP siRNA. (c) Suppression of GFP systemic silencing by Rep. Photographs of agroinfiltrated plants taken at 30 days p.i. (d) Northern blot analysis of GFP mRNA and GFP siRNA extracted from the systemic leaves of agroinfiltrated plants shown in (c). (e) Suppression of the GFP silencing signal spread by Rep. The agroinfiltrated plants were examined for systemic GFP silencing and photographed at 25 days p.i. Schematics above photographs illustrate the location of the 35S-Rep (blue) and 35-GFP (purple) inocula. B and T denote the base and tip portions of the leaf, respectively, while Letters L and U denote the lower and upper leaves, respectively. (f) Northern blot assays of GFP mRNA extracted from the systemic leaves of agro-infiltrated plants shown in (e).
line 16c plants carrying GFP were infiltrated at the four-leaf stage with a mixture of Agrobacterium harbouring 35S-GFP and either a test or a control construct. By 3 days post-infiltration (p.i.), strong green fluorescence was only seen in leaves co-infiltrated with 35S-GFP plus either 35S-Rep or 35S-P19 (Fig. 1a, Fig. S1 available in the online Supplementary Material). In contrast, GFP fluorescence in leaves co-infiltrated with 35S-GFP and either the empty vector or 35S-RepUT (an untranslatable Rep mutant, in which the second codon of the Rep gene was replaced with a stop codon) was significantly reduced and was almost undetectable under UV light (Fig. 1a). Northern blot analysis was performed using a [α-32P]dCTP-labelled probe specific for the GFP gene to determine any correlation between the observed GFP fluorescence and GFP mRNA. As shown in Fig. 1(b), GFP mRNA levels were reduced in leaves co-infiltrated with 35S-GFP plus either the empty vector or 35S-RepUT at 3 days p.i. In contrast, GFP mRNA accumulated to higher levels in leaves co-infiltrated with 35S-GFP plus 35S-Rep, although mRNA levels were much lower than in leaves co-infiltrated with 35S-GFP plus 35S-P19. To further determine whether Rep suppresses accumulation of siRNA that guides sequence-specific degradation (Voinnet, 2001; Waterhouse et al., 2001), low-molecular-mass RNA was extracted from the infiltrated zones at 3 days p.i. and hybridized to fragments of the GFP gene end-labelled with [γ-32P]dATP (Fig. 1b). The analysis of siRNA accumulation indicated that 21 nt and 24 nt GFP siRNAs, characteristic of RNA silencing, had accumulated to significantly higher levels at 3 days p.i. in leaves co-infiltrated with 35S-GFP plus either the empty vector or 35S-RepUT. In contrast, GFP-specific siRNA levels were reduced in leaves co-infiltrated with 35S-GFP plus either 35S-P19 or 35S-Rep. Therefore, these results strongly suggest that expression of WDV Rep is capable of suppressing local gene silencing in N. benthamiana plants.

Previous evidence has shown that the C-terminal domain of tomato leaf curl Java virus V2 is likely required to suppress RNA silencing (Sharma & Ikegami, 2010). In order to test whether the truncated N and/or C termini of WDV Rep are able to suppress PTGS, mutants containing a deletion of the first 9 aa at the N terminus (located between aa 1 and 9), or a 30 aa deletion at the C terminus of Rep (located between aa 323 and 352; full length Rep encodes 352 aa), were introduced into the pCHF3 vector to produce the constructs of 35S-RepDN9 and 35S-RepΔC30, respectively. Transient expression of the two mutant constructs together with 35S-GFP resulted in strong green fluorescence at the site of infiltration (Fig. 1a). Consistent with these results, Northern blot analysis of GFP mRNA and GFP-specific siRNA shows that the N-terminal 9 aa and C-terminal 30 aa of the Rep protein are not critical for its local silencing suppression activity (Fig. 1b).

After the onset of RNA silencing, a mobile silencing signal is thought to spread systemically and induce systemic silencing of homologous sequences in upper leaves (Guo & Ding, 2002). To investigate whether Rep can interfere with the long-distance spread of the silencing signal, GFP fluorescence was monitored in upper leaves of 16c plants co-infiltrated with a mixture of Agrobacterium carrying 35S-GFP and either the control, or various Rep-containing constructs. Systemic silencing occurred in 75–85% of plants co-infiltrated with 35S-GFP plus either empty vector or 35S-RepUT by 40 days p.i. (Table S1). In contrast, systemic silencing only occurred in 4–10% of plants co-infiltrated with 35S-GFP plus either 35S-P19 or 35S-Rep, even at 40 days p.i. The remaining plants continued to show strong GFP fluorescence in their upper young leaves (Fig. 1c). Interestingly, at 40 days p.i., less than 17% of plants co-infiltrated with 35S-GFP plus either 35S-RepDN9 or 35S-RepΔC30 developed systemic GFP silencing. Northern blot analysis showed negligible accumulation of GFP mRNA and high accumulation of GFP-specific siRNAs in systemic leaves co-infiltrated with 35S-GFP plus either empty vector or 35S-RepUT. On the other hand, systemic leaves co-infiltrated with 35S-GFP plus either 35S-P19 or 35S-Rep or either of the Rep deletion mutants showed a high accumulation of GFP mRNA and negligible accumulation of GFP-specific siRNAs (Fig. 1d). Taken together, these results provide evidence that WDV Rep functions as a suppressor of systemic silencing and both the N-terminal 9 aa and C-terminal 30 aa of the Rep protein are not critical for this activity.

To characterize how Rep interferes with spread of the systemic silencing signal, we used a previously developed assay (Guo & Ding, 2002). As illustrated in Fig. 1(e) and Table S1, when the tip (T) part of a 16c plant leaf was inoculated with 35S-GFP and the basal (B) of the same leaf was simultaneously inoculated with 35S-Rep, systemic silencing only occurred in 1 of 18 inoculated plants. However, systemic silencing occurred in all plants inoculated with the same Agrobacterium cultures but in the opposite direction. On the other hand, when lower (L) leaves were inoculated with 35S-GFP and upper (U) leaves of the same plant simultaneously inoculated with 35S-Rep, systemic silencing was not observed. Systemic silencing did occur in all plants inoculated with the same Agrobacterium cultures but in the opposite direction. Northern blot analysis showed negligible accumulation of GFP mRNA and high accumulation of GFP specific siRNAs in systemic leaves infiltrated with Rep(T) plus GFP(B) or Rep(L) plus GFP(U). In contrast, high accumulation of GFP mRNA and negligible accumulation of GFP siRNAs in systemic leaves infiltrated with Rep(B) plus GFP(T) or Rep(U) plus GFP(L) were observed (Fig 1f). These results reveal that Rep can suppress systemic RNA silencing by blocking or inactivating the spread of silencing signals.

Previous reports have shown that siRNA binding activity is required for the silencing suppression function of P19, cucumber mosaic virus 2b and rice dwarf phytoavirace virus p10 (Ye et al., 2003; Ren et al., 2010; González et al., 2012). To test whether Rep can bind siRNA, gel mobility shift assays were conducted essentially as described previously (Ren et al., 2010). The intact Rep ORF was...
cloned into the vector pMBP-28. MBP-tagged Rep fusion proteins were purified in their native, non-denatured form using a Ni-NTA affinity column (Qiagen). The purified fusion protein was incubated either with 21 and 24 nt siRNA duplexes or single-stranded (ss)-siRNA with 2 nt 3’ overhangs as probes in RNA binding assays. As shown in Fig. 2, WDV Rep bound both 21 and 24 nt siRNA duplexes and ss-siRNAs forming complexes. It should be noted that binding between Rep and siRNA duplexes appeared to be weaker than that seen between Rep and ss-siRNAs. However, it remains to be determined whether the siRNA binding of WDV Rep is also important for its silencing suppressor activity.

In some cases, virus-encoded RNA silencing suppressors have been reported to have specific roles as avirulence determinants (Chapman et al., 2004). To test whether Rep displays such a function, the WT Rep gene and the Rep deletion mutants (Rep<sup>dN9</sup> and Rep<sup>dC30</sup>) were cloned into the potato virus X (PVX) vector PGR106 to produce PVX-Rep, PVX-Rep<sup>dN9</sup> and PVX-Rep<sup>dC30</sup>, respectively, as well as the untranslatable Rep construct, PVX-Rep<sup>UT</sup>. Agroinfiltration assays showed that <i>N. benthamiana</i> leaves infiltrated with PVX-Rep or PVX-Rep<sup>dC30</sup> produced more severe mosaic and necrotic symptoms in systemic leaves at 9 days p.i. and plants became dwarfed at 15 days p.i. In contrast, plants infected with PVX-Rep<sup>UT</sup> or PVX-Rep<sup>dN9</sup> initially developed veinal chlorosis at 6–9 days p.i. followed by mild chlorotic symptoms on upper leaves, similar to those induced by the PVX vector alone (Fig. 3a). ELISA and Northern blot analysis indicated that the relative titre of PVX and accumulation of PVX RNA were much higher in plants infected with PVX-Rep and PVX-Rep<sup>dC30</sup> as compared with those infected with PVX, PVX-Rep<sup>UT</sup> or PVX-Rep<sup>dN9</sup> at 15 days p.i., while only minimal differences were observable at 9 days p.i. (Fig. 3b, c). These results suggest that WDV Rep protein can function as a pathogenicity determinant in the PVX heterologous expression system and that the N-terminal 9 aa of Rep are necessary for its pathogenicity.

Multimerization is necessary for the betasatellite Y10<sub>B</sub>C1, encoded by tomato yellow leaf curl China virus, to induce symptoms in <i>N. benthamiana</i> (Cheng et al., 2011). Interestingly, oligomerization of WDV Rep <i>in vitro</i> has been described (Castellano et al., 1999). Here, we carried out bimolecular fluorescence complementation (BiFC) to determine whether WDV Rep self-interacts <i>in planta</i>. The Rep ORF was cloned into BiFC transformation vectors to produce p2YN-Rep and p2YC-Rep. Following agroinfiltration, confocal microscopy images showed strong yellow fluorescent protein fluorescence localized mainly to amorphous aggresomes located in the cytoplasm of

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**Fig. 2.** Immunodetection of recombinant Rep and gel mobility shift assay of recombinant Rep binding to siRNA. (a) Western blot analysis of crude (non-purified) and purified recombinant (MBP-tagged) Rep protein using with anti-MBP mouse mAb. The molecular mass of the protein markers are indicated. (b) Binding activity between WT Rep protein and 21 nt ss-siRNA (left) or 24 nt ss-siRNA (right). ss-siRNA probes were incubated with increasing concentrations of recombinant Rep protein. Free (unbound probe) and bound (protein–siRNA complexes) are indicated. (c) Binding activity between WT Rep protein and 21 nt siRNA duplex (left) or 24 nt siRNA duplex (right). siRNA probes were incubated with increasing concentrations of recombinant Rep protein. Free (unbound) and bound (protein–siRNA complexes) are indicated.
epidermal cells at 48 h p.i. No fluorescence was observed in cells co-infiltrated with the two vectors, p2YN and p2YC, alone (Fig. 3d). Consistent with the predominantly cytoplasmic nature of Rep–Rep complexes, confocal microscopy images showed that both RepN9–RepN9 and RepC30–RepC30 complexes accumulated in the...

**Fig. 3.** The relationship between pathogenicity and self-interaction of Rep. (a) Symptoms of *N. benthamiana* plants infected with various potato virus X (PVX) expression constructs at 15 days p.i. (b) ELISA analysis of PVX titre in systemic leaves of plants shown in (a), using an anti-PVX mouse mAb. (c) Northern blot analysis of viral RNA in systemic leaves of plants shown in panel (a), using a probe specific for PVX CP RNA. Ethidium bromide staining of rRNA shows loading control. The position of PVX genomic RNA (gRNA) and subgenomic RNA (sgRNA) are indicated. (d) Visualization of Rep self-interaction in *N. benthamiana* epidermal cells by BiFC assay. YFP, Yellow fluorescent protein.
cytoplasm (Fig. 3d). These findings allow us to conclude that self-interaction and pathogenicity of Rep are not necessarily linked and that the Rep protein may contain different domain(s) necessary for its multimerization and pathogenicity.

The RNA silencing-based defence and counter-defence interplay has important ramifications for virus infection. The results presented in this paper demonstrate that the WDV Rep protein can suppress both local and systemic RNA silencing in *N. benthamiana* as well as the spread of a systemic GFP RNA silencing signal. WDV Rep and RepA genes share identical primary sequences in their N-terminal amino acid residues (Fondong, 2013). Since RepA is not able to suppress PTGS, it is possible that the C-terminal domain of Rep is essential for this silencing suppression activity. To our knowledge, this is the first silencing suppressor identified to date that is encoded by mastreviruses. It is well known that virus-encoded PTGS suppressors act through divergent mechanisms. However, the mechanism by which mastreviruses deal with host RNA silencing is poorly understood. Future efforts will be necessary for elucidation of the mechanisms by which WDV Rep proteins interfere with the gene-silencing pathway.

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**References**


