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Short communication

Tritimovirus P1 functions as a suppressor of RNA silencing and an enhancer of disease symptoms

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ABSTRACT

Wheat streak mosaic virus (WSMV) is an eriophyid mite-transmitted virus of the genus *Tritimovirus*, family *Potyviriidae*. Complete deletion of helper component-proteinase (HC-Pro) has no effect on WSMV virulence or disease synergism, suggesting that a different viral protein suppresses RNA silencing. RNA silencing suppression assays using *Nicotiana benthamiana* 16C plants expressing GFP were conducted with each WSMV protein; only P1 suppressed RNA silencing. Accumulation of GFP siRNAs was markedly reduced in leaves infiltrated with WSMV P1 at both 3 and 6 days post infiltration relative to WSMV HC-Pro and the empty vector control. On the other hand, helper component-proteinase (HC-Pro) of two species in the mite-transmitted genus *Rymovirus*, family *Potyviriidae* was demonstrated to be a suppressor of RNA silencing. Symptom enhancement assays were conducted by inoculating *Potato virus X* (PVX) onto transgenic *N. benthamiana*. Symptoms produced by PVX were more severe on transgenic plants expressing WSMV P1 or potyvirus HC-Pro compared to transgenic plants expressing GFP or WSMV HC-Pro.

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Plants utilize RNA silencing as a defense against virus infection; in turn, plant viruses encode virulence factors that suppress RNA silencing (for review see Baulcombe, 2004; Ding, 2010; Ding et al., 2004; Voinnet, 2005; Vance and Vaucheret, 2001). Helper component-proteinase (HC-Pro) encoded by viruses of the genus *Potyvirus* (family *Potyviriidae*) is a multifunctional protein that mediates vector transmission by aphids (Ammar et al., 1994; Blanc et al., 1998), polypeptide processing via proteolytic cleavage of its carboxy-terminus (Carrington and Herndon, 1992; Carrington et al., 1989), and suppression of RNA silencing (Anandalakshmi et al., 1998; Carrington et al., 2001; Kasschau and Carrington, 1998). RNA silencing appears to be the underlying mechanism of certain pathogen-derived transgenic resistance strategies (Baulcombe, 1996; Lindbo and Dougherty, 1992; Lindbo et al., 1993; Van den Boogart et al., 1998). In contrast, suppression of RNA silencing may

contribute to disease synergism in which symptom severity is exacerbated upon double infection of a host by two unrelated viruses (Pruss et al., 1997; Vance et al., 1995).

Among divergent genera of the family *Potyviriidae*, six (*Potyvirus*, *Tritimovirus*, *Rymovirus*, *Ipomovirus*, *Poacevirus*, and *Brambyvirus*) encode HC-Pro homologues. Whereas gene function of *Potyvirus* spp. has been the subject of intensive investigation, much less is known for other genera of the family. Species of the genera *Tritimovirus* (Rabenstein et al., 2002; Stenger et al., 1998), *Rymovirus* (French and Stenger, 2005), and *Poacevirus* (Seifers et al., 2009; Tatineni et al., 2009) are vectored by eriophyid mites, share a gene organization in common with *Potyvirus* spp., but retain only limited sequence conservation. Species of the genus *Ipomovirus* are whitefly-transmitted (Lecoq et al., 2000) but differ in gene complement: *Sweet potato mild mottle virus* (SPMMV) encodes HC-Pro (Colinet et al., 1998) but SPMMV P1 has been shown to suppress RNA silencing by binding to argonaute and inhibiting RNA-induced silencing complex activity (Giner et al., 2010); whereas *Cucumber vein yellowing virus* (CVYV) has a second divergent copy of P1 (P1b) in place of HC-Pro that serves as a suppressor of RNA silencing mediated by binding 21 nucleotide (nt) sRNAs (Lakatos et al., 2006; Valli et al., 2006, 2011). *Brambyvirus* contains a single virus species infecting *Rubus* (Susaimuthu et al., 2008) but no vector has been identified and knowledge of gene function is limited to sequence annotation.

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Fig. 1. Evaluation of *Wheat streak mosaic virus* isolate Sidney81 (WSMV Sidney81) proteins for suppression of RNA silencing. Diagrams of constructs cloned into the pPZP212 binary vector and transformed into *Agrobacterium tumefaciens* are presented in Panel A (LB = left border; P35S = 35S promoter; TL = Tobacco etch virus [TEV] leader sequence; T35S = 35S transcription terminator; RB = right border). Constructs were designed to express various viral proteins (test ORF) or green fluorescent protein (GFP). Panel B depicts RNA silencing suppression assays conducted in infiltrated leaves of *Nicotiana benthamiana* 16C plants transgenic for GFP. Leaves infiltrated with *A. tumefaciens* were detached after six days and illuminated with UV light. Northern blot (5 µg total RNA) showing accumulation of GFP mRNA in *N. benthamiana* 16C plants used in RNA silencing suppression assays is shown in Panel C. Ethidium bromide stained 28S rRNAs are shown as loading controls.

Fig. 2. RNA silencing suppression assays for proteins (P1/HC-Pro, P1, or HC-Pro) encoded by tritimoviruses, rymoviruses, and potyviruses. Panel A depicts RNA silencing suppression assays conducted in infiltrated leaves of *Nicotiana benthamiana* 16C plants transgenic for GFP. Leaves infiltrated with *A. tumefaciens* were detached after six days and illuminated with UV light. Northern blot (5 µg total RNA) showing accumulation of GFP mRNA in *N. benthamiana* 16C plants used in RNA silencing suppression assays is shown in Panel B. Ethidium bromide stained 28S rRNAs are shown as loading controls.

Fig. 3. Tritimovirus P1 and rymovirus HC-Pro suppressors of post-transcriptional gene silencing reduce accumulation of small interfering (si) RNAs. Presented are northern blots (1.5 μ g low molecular weight RNA) of GFP siRNAs at 3 and 6 days post-infiltration of *Nicotiana benthamiana* 16C plants with *A. tumefaciens* expressing GFP and *A. tumefaciens* expressing tritovirus (WSMV), rymovirus (AgMV), or potyvirus (TuMV) proteins. Ethidium bromide stained gel of low molecular weight (LMW) RNAs shown as loading controls.

Wheat streak mosaic virus (WSMV) is the type species of the genus *Tritovirus* (Stenger et al., 1998) and is perhaps the best characterized species of the *Potyviridae* not belonging to the genus *Potyvirus*. WSMV HC-Pro shares two functions in common with potyvirus HC-Pro: mediation of vector transmission and cysteine proteinase activity (Stenger et al., 2005a,b, 2006a; Young et al., 2007). Mutational analyses of WSMV HC-Pro suggested a role in replication and systemic movement (Stenger et al., 2006a,b), phenotypes expected for mutants of an RNA silencing suppressor (Cronin et al., 1995; Kasschau et al., 1997). Surprisingly, complete deletion of the HC-Pro coding region had no effect on WSMV virulence (Stenger et al., 2005a,b) or disease synergism in double infections with *Maize chlorotic mottle virus* (MCMV) (Stenger et al., 2007). These results indicated that replication and movement defects associated with point mutations or partial deletions of WSMV HC-Pro (not affecting polyprotein processing) were due to mutant interference and that WSMV HC-Pro was unlikely to function as a suppressor of RNA silencing. Here, we address this issue by evaluating tritovirus proteins for RNA silencing suppression activity. For comparison, select rymovirus proteins also were evaluated for RNA silencing suppression activity. Finally, we demonstrate that the WSMV P1 protein, identified as a suppressor of RNA silencing, is sufficient for enhancement of disease symptoms of an unrelated virus.

RNA silencing suppression assays were conducted essentially as described by Qu et al. (2003) using the *Nicotiana benthamiana* GFP transgenic line 16C developed by Brigneti et al. (1998). Briefly, leaves were co-infiltrated with *Agrobacterium tumefaciens* harboring expression cassettes for GFP (to induce RNA silencing) and with *A. tumefaciens* expressing various open reading frames (ORFs) to be evaluated for RNA silencing suppression activity (Fig. 1A). ORFs to be tested were amplified by PCR from previously cloned cDNAs of an infectious clone of WSMV Sidney81 (Choi et al., 1999), the divergent WSMV isolate El Batán3 (Choi et al., 2001), the tritovirus *Oat necrotic mottle virus* (ONMV) (Stenger and French, 2004), and the rymoviruses *Agropyron mosaic virus* (AgMV) and *Hordeum mosaic virus* (HoMV) (French and Stenger, 2005). Constructs containing the P1/HC-Pro coding region of the potyviruses *Tobacco etch virus* (TEV) and *Turnip mosaic virus* (TuMV) were

provided by J.C. Carrington; individual coding regions of potyvirus P1 or HC-Pro were amplified by PCR from the constructs containing both coding regions. Test ORFs were inserted into pRTL2 (Carrington and Freed, 1990), then transferred to the *A. tumefaciens* binary vector pPZP212 (Hajdukiewicz et al., 1994). Coding sequences including P1 utilized the native translational initiation codon; other test ORFs were modified to include an engineered initiation codon. Termination of translation for most test ORFs was directed by engineered stop codons located immediately 3' of coding sequences while the WSMV Sidney81 coat protein (CP) was terminated by the native polyprotein stop codon.

Nine WSMV Sidney81 proteins were tested for RNA silencing suppression activity (Fig. 1B). Six days post-infiltration, leaves were detached and GFP expression detected by fluorescence upon exposure to UV light. Negative controls consisted of *N. benthamiana* 16C plants infiltrated only with *A. tumefaciens* expressing GFP or buffer (mock). For comparison, detached leaves of non-transformed *N. benthamiana* also were imaged. Suppression of RNA silencing was observed (based on GFP fluorescence) only with constructs expressing WSMV-Sidney81 P1 (either alone or with HC-Pro) and the positive control (TEV P1/HC-Pro). Suppression of RNA silencing was not observed with WSMV Sidney81 proteins HC-Pro, P3, 6K1, CI, 6K2, NIa, NIb, or CP tested individually. Total RNA samples were extracted from leaves 5 days post-infiltration and evaluated for accumulation of GFP mRNA by Northern blot hybridization (Fig. 1C). GFP mRNA was detected in samples from leaves co-infiltrated with constructs shown (Fig. 1B) to suppress RNA silencing based on GFP fluorescence.

In addition to proteins generated by proteolytic cleavage of the polyprotein, potyviruses express an additional protein (PIPO) via minus 1 translational frameshifting in the P3 coding region (Chung et al., 2008). PIPO is likely expressed by WSMV as the ORF is conserved among divergent strains (Choi et al., 2001) and a debilitating mutation in WSMV Sidney81 (synonymous with respect to P3) that alters the putative PIPO frameshift site (Choi et al., 2005). Therefore, one additional test ORF consisting of WSMV P3 bearing a minus 1 frameshift mutation was constructed. Co-infiltration leaves with *A. tumefaciens* expressing this mutant (N-terminal P3-PIPO) with *A. tumefaciens* harboring GFP indicated that N-terminal P3-PIPO did not suppress RNA silencing (data not shown).

To determine whether RNA silencing suppression by P1 is a trait conserved among mite-transmitted members of the family *Potyviridae*, *A. tumefaciens* expressing P1/HC-Pro, P1 or HC-Pro of two additional tritoviruses (WSMV El Batán3 and ONMV) and two rymoviruses (AgMV and HoMV) were co-infiltrated with *A. tumefaciens* bearing GFP (Fig. 2A). Corresponding constructs derived from the potyviruses TEV (Fig. 2A) and TuMV (data not shown) also were evaluated for RNA silencing suppression. All constructs bearing both P1 and HC-Pro suppressed RNA silencing. However, when P1 and HC-Pro were tested individually, tritovirus P1 but not HC-Pro suppressed RNA silencing. In contrast, HC-Pro derived from rymoviruses or potyviruses suppressed RNA silencing whereas P1 did not. Results of Northern blot assays for GFP mRNA (Fig. 2B) were consistent with that of GFP expression as measured by fluorescence (Fig. 2A).

RNA silencing is mediated through production of small interfering (si) RNAs homologous to the target mRNA. Depending on which step in the RNA silencing pathway is blocked, siRNA accumulation may be reduced. Accumulation of GFP siRNAs in plants co-infiltrated with *A. tumefaciens* bearing GFP and *A. tumefaciens* expressing select viral coding regions was determined by Northern blots of samples of low molecular weight RNAs enriched from total RNA extracted from plants at 3 and 6 days post-infiltration (Fig. 3). WSMV P1, AgMV HC-Pro, and TuMV HC-Pro reduced accumulation of GFP siRNAs, particularly the 21 nt size class of siRNA, at both

Fig. 4. Disease symptom enhancement assays. (A) Images of healthy (right) and *Potato virus X* infected *Nicotiana benthamiana* (left) plants transgenic for *Wheat streak mosaic virus* (WSMV) HC-Pro, WSMV P1, *Turnip mosaic virus* (TuMV) HC-Pro, or GFP. Note increased severity of symptoms for plants expressing post-transcriptional gene silencing suppressors (WSMV P1 and TuMV HC-Pro). (B) Northern blot analysis of total RNA extracted from upper non-inoculated leaves of PVX inoculated transgenic plants at 6 dpi hybridized with a probe specific for PVX (upper panel) and ethidium bromide-stained agarose gel showing 28S ribosomal RNA as RNA loading controls.

time points relative to WSMV HC-Pro, WSMV P3, AgMV P1, or the empty vector control.

Symptom enhancement assays were conducted by inoculating *Potato virus X* (PVX) onto *N. benthamiana* transgenic plants expressing WSMV P1, WSMV HC-Pro, TuMV HC-Pro, or GFP. Transformed plants were produced at the University of Nebraska-Lincoln Plant Transformation Core Facility using the same constructs as those used in RNA silencing suppression assays (Fig. 1). These were introduced into *A. tumefaciens* strain C58C1 and used to transform leaf disks of *N. benthamiana* as described (Horsch et al., 1985) and the presence of each transgene was confirmed by PCR with primers specific for each test ORF. Seven independent transgenic lines were evaluated for each construct with identical results for each test ORF. Leaves of transgenic plants expressing WSMV HC-Pro and GFP were normal in appearance while those of plants expressing WSMV P1 and TuMV HC-Pro exhibited a slight downward rolling and cupping of leaf margins (Fig. 4A). PVX infection of plants transformed with TuMV HC-Pro or WSMV Sidney81 P1 resulted in enhancement of symptoms compared to mild symptoms resulting from PVX infection of plants transformed with WSMV HC-Pro or GFP (Fig. 4A). PVX genomic RNA accumulated to higher levels in PVX-infected leaves of TuMV HC-Pro or WSMV Sidney81 P1 transgenic plants compared to PVX RNA accumulation in PVX-infected leaves of *N. benthamiana* plants transformed with WSMV HC-Pro or GFP (Fig. 4B). Enhancement of symptoms on WSMV P1 transgenic plants infected with PVX is consistent with RNA silencing suppression activity of WSMV P1 and provides a plausible explanation for disease synergism in maize co-infected with a WSMV HC-Pro complete deletion mutant and MCMV (Stenger et al., 2007).

Collectively, the results indicate that tritrovirus P1 but not HC-Pro mediates suppression of RNA silencing. WSMV P1 has serine proteinase activity (Choi et al., 2002) similar to that of potyviruses and serine proteinase motifs are present in P1 proteins of other divergent genera, suggesting this trait arose early in the evolution of the family *Potyviridae*. The same is true for HC-Pro cysteine proteinase activity/motif conservation. In contrast, evolution of RNA silencing suppression in the family *Potyviridae* appears more complex, following at least three distinct paths: P1 for tritroviruses, P1 or P1b for ipomoviruses, and HC-Pro for potyviruses and rymoviruses. Valli et al. (2007) previously noted the structural divergence of P1 proteins of tritroviruses from those of potyviruses and suggested that there may be functional divergence as well. Interestingly, rymoviruses and potyviruses share a most recent common ancestor exclusive of other genera of the family (French and Stenger, 2005). Thus, it is possible that suppression of RNA silencing by HC-Pro represents a more recent character, relative to RNA silencing suppression by P1.

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