Molecular studies on virus interactions in sweetpotato

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Sweetpotato (*Ipomoea batatas*) is an important subsistence and famine reserve crop grown in developing countries. The most severe disease (SPVD) and yield losses are caused by a synergistic virus interaction between *Sweet potato chlorotic stunt virus* (SPCSV; *Closteroviridae*) and *Sweet potato feathery mottle virus* (SPFMV; Potyviridae). RNA interference (RNAi) is a conserved eukaryotic mechanism used by plants to counteract viral infections via virus-derived short RNA sequences known as small interfering RNA (siRNA); consequently viruses encode proteins able to block RNAi, known as RNAi suppressor proteins (RSP). Our data show that transformation of an SPFMV-resistant sweetpotato variety with SPCSV-encoded RSP proteins (RNase3 and p22) broke down resistance to SPFMV, leading to high accumulation of SPFMV and severe disease symptoms similar to SPVD. Interestingly RNase3-transgenic sweetpotatoes also accumulated higher titers and displayed enhanced symptoms of unrelated RNA viruses and two previously non-characterized DNA viruses. These viruses have been previously shown to synergize with SPCSV. Although siRNA-binding has been reported as a relatively common mechanism for suppression of RNAi by RSPs, SPCSV-RNase3 RSP function depended on its endonuclease activity. We show that siRNAs and total small RNA isolated from virus-infected sweetpotato plants were cleaved *in vitro* by RNase3, suggesting a novel viral mechanism for suppression of RNAi by cleavage of small RNA. Because some SPCSV isolates causing synergistic interactions do not encode p22, results implicate RNase3 as a sufficient factor for the development of SPVD and suggest a target for engineering virus resistance in sweetpotato.

Keywords: Plant virus, RNA silencing, suppression of RNAi, viral sinergismo.

Sweetpotato (*Ipomoea batatas*) is an important crop used in developing countries as a famine reserve food. More than 20 viruses are known to infect this crop worldwide (Valverde *et al.*, 2007) but many sweetpotato cultivars are highly resistant to single virus infection or are able to recover from initial single-infection during plant growth (Gibson *et al.*, 1998; Karyeija *et al.*, 2000; Untiveros et al., 2007). However, mixed virus infections can develop into severe symptoms and cause significant yield losses (Gibson *et al*, 1998). *Sweet potato chlorotic stunt virus* (SPCSV, *Closteroviridae*) has been identified as a critical component in these synergistic interactions. SPCSV infection renders sweetpotato susceptible to accumulation of several unrelated viruses while SPCSV titres remain little affected (Karyeija *et al.*, 2000; Mukasa *et al.*, 2006; Untiveros *et al.*, 2007). Indeed, the most severe disease affecting sweetpotatoes and thereby food security is caused by a virus complex that can reduce yields by 90% (Gibson *et al.*, 1998). This sweet potato virus disease (SPVD) develops in plants infected with SPCSV and *Sweet potato feathery mottle virus* (SPFMV, *Potyviridae*). SPVD is characterized by severe leaf malformation, chlorosis, and stunting of the plants. The synergistic effect of SPCSV on several unrelated viruses is observed independently of the strain of SPCSV (Untiveros *et al.*, 2007; Cuellar *et al.*, 2008) and indicates a general loss of resistance to viruses in sweetpotato in the presence of SPCSV.

Viruses are inducers and targets of RNA interference (RNAi), a fundamental antiviral defense mechanism in eukaryotic organisms (Haasnoot *et al.*, 2007) and essential for virus resistance and recovery from virus disease in plants (Covey *et al.*, 1997, Ratcliff *et al.*, 1997). RNAi is a cell surveillance system to recognize double-stranded RNA (dsRNA) and specifically eliminate by cleavage RNAs homologous to the inducer RNA (Fire *et al.*, 1998; Hammond *et al.*, 2000). Cleavage of dsRNA is carried out by Dicer, which is a class 3 RNase III endonuclease (Bernstein *et al.*, 2001). Plants encode 4 Dicer-like (DCL) enzymes that recognize and cleave long dsRNA molecules to 21-, 22-, and 24-bp fragments that act as small interfering RNA (siRNA). siRNA are key for the efficiency and specificity of the RNAi response (Hamilton and Baulcombe, 1999; Dunoyer *et al.*, 2007; Ding and Voinnet 2007).

Viruses express a wide range of dedicated RNAi-suppressor proteins (RSP) to interfere with the different steps of the RNAi pathway (Li and Ding 2006). It is therefore conceivable that in mixed viral infections, the presence of several RSPs might help to overcome RNAi, generating a synergism that allows at least one of the co-infecting

viruses to accumulate at higher titers than observed in single virus infections (Anandalakshmi *et al.*, 1998; Pruss *et al.*, 1997). However, so far this has been shown only for the N-proximal part (P1/HC-Pro) of the potyviral polyprotein that is known as the potent and sufficient mediator of synergism in transgenic plants infected with unrelated viruses (Pruss *et al.*, 1997). The central part of HC-Pro that mediates viral synergism is involved in suppression of RNAi (Shi et al., 1997; Kasschau and Carrington 2001). Given that RNase III endonucleases are key enzymes involved in RNAi, the presence of a homologous enzyme encoded by SPCSV prompted us to its characterization.

We report here that SPCSV, the causal agent of several virus synergistic interactions encodes one RSP protein (RNase3) that is sufficient to replicate SPCSV synergistic interactions with heterologous viruses. Our data suggest that SPCSV RNase3 is a novel suppressor of a basic antiviral defense system of sweetpotato. In addition, some SPCSV isolates encode one more RSP protein (Cuellar *et al.*, 2008) functionally different to RNase3, and may therefore have a further role in SPCSV synergistic virus interactions.

Materials and methods

Transgenic sweetpotato lines expressing SPCSV RSP

Pathogen-free in vitro plants of the SPFMV-resistant Peruvian sweet potato landrace 'Huachano' (accession no. CIP420065) were obtained from the germplasm collection of the International Potato Center (CIP). The *RNase3* and the *p22* gene of SPCSV-Ug were placed in the binary vector pKOH200, as described (Karim *et al.*, 2007), and used to transform sweetpotato leaf explants with *Agrobacterium. tumefaciens* strain EHA105. Plants were transformed and regenerated following a somatic embryogenesis protocol (Kreuze *et al.*, 2008). Independent transgenic lines were analyzed for transgene-protein expression and used as rootstocks for grafting different viruses.

Plant inoculation and virus detection

Sweetpotato plants were graft-inoculated with the East African strains of SPFMV-Piu, SPMMV and SPCSV-m2–47 (Untiveros *et al.*, 2007; Kreuze *et al.*, 2008), two recently characterized members of the *Caulimoviridae:* 'Sweetpotato cavemovirus A' (previously known as SPCaLV), 'Sweetpotato cavemovirus B' (previously known as C-9) and an isolate of Sweetpotato leaf curl virus (SPLCV-54) using scions from the virus propagation host, *Ipomoea setosa* in an insect-proof greenhouse at CIP. Viruses were detected from approximately 150 mg of the tissue sampled from the youngest fully opened leaves by double antibody sandwich ELISA (DAS-ELISA) (Cuellar *et al.*, 2008) and NCM-ELISA. Circular DNA viruses (SPLCV-54, 'Sweetpotato cavemovirus A' and 'Sweetpotato cavemovirus B' were amplified using phi29 polymerase (BioLabs) from small molecular weight DNA obtained after passing total plant DNA through a plasmid miniprep kit (Promega). Identification was done by sequence analysis of PCR-amplified regions or full virus genomes. DNA virus detection was carried out by PCR using a direct extraction protocol (Wang *et al.*, 1993).

Western blot analysis

Proteins were isolated from 200 mg sweet potato leaf tissue, separated in a denaturing 12% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Hybond-P) by electroblotting. RNase3 protein was detected with a specific rabbit antiserum raised against SPCSV RNase3, as described (Kreuze *et al.*, 2005). Anti-rabbit monoclonal mouse antibodies conjugated with horseradish peroxidase (Amersham), the Supersignal West Pico chemiluminescent substrate (Pierce Biotechnology), and exposure to X-ray film were used to detect signals by the ECL method according to the manufacturer's instructions (Amersham).

Agroinfiltration assay

The cloning strategy and vector plasmids used in the agroinfiltration assays in this study have been described (Kreuze *et al.*, 2005). Sequences of the *RNase3* genes of SPCSV-Ug, SPCSV-M2–47, and SPCSV-Is have been described and encode the most different RNase3 protein sequences currently known (amino acid sequence identity 80–97%) (Cuellar *et al.*, 2008). In the mutated RNase3 of SPCSV-Ug (designated as RNase3-Ala), 2 substitutions (E37A and D44A) were made in the highly conserved RNase III signature motif required for the dsRNA endonuclease activity of RNase III enzymes. pA-GUS expresses the ß-glucuronidase (GUS) gene with a plant intron to prevent GUS expression in *Agrobacterium*. pBIN35S-GFP expressed the "cycle 3" GFP gene. Constructs were verified by sequencing. Agroinfiltration was done as described using different *A. tumefaciens*

cultures that were combined before infiltration (Kreuze *et al.*, 2005). For co-infiltration treatments that included fewer constructs than others, the missing volume was replaced by the *Agrobacterium* strain expressing GUS. Infiltrations were carried out on the *N. benthamiana* line 16c which constitutively express the jellyfish (*Aequoria victoria*) GFP (Brigneti *et al.*, 1998) in a controlled growth chamber. Infiltrated tissues were monitored daily for GFP fluorescence using a hand-held UV lamp.

## Nucleic acids isolation and northern blot hybridization

For amplification using phi29 total DNA was extracted from 500 mg of infected leaf tissue using the CTAB protocol. For PCR detection of SPLCV-54, a quick DNA extraction from 200 mg of leaf tissue was tested using NaOH (Wang *et al.*, 1993) and PCR using primers targeting the REP gene. Total RNA was isolated from 400 mg fresh leaf material using TRIzol (Invitrogen) following the manufacturer's instructions. Low molecular weight (LMW) RNA was obtained by LiCl precipitation and used to detect siRNA, whereas high molecular weight (HMW) RNA was used to detect *gfp* mRNA accumulation as described (Hamilton and Baulcombe 1999; Kreuze *et al.*, 2005). A probe complementary to *gfp* was prepared and labeled with [alpha-32P]UTP (Amersham) by in vitro transcription of *gfp* cloned into pCR-Blunt (Invitrogen) behind the T7 promoter. After hybridization and washing, membranes were exposed to X-ray film (Kodak) for 4, 16, and 48 h and developed using an X-Omat 1000 automated developer (Kodak).

RNA cleavage assays with RNase3

SPCSV RNase3 and RNase3-Ala proteins were overexpressed and purified from *E. coli* BL21(DE3)-RIL cells. Purification of the 6x-His-tagged proteins was accomplished using Ni-NTA agarose columns according to the manufacturer's instructions (Expressionist, Qiagen). Synthetic siRNA oligonucleotides were labeled by phosphorylation with [alpha-32P]-ATP using T4 polynucleotide kinase (Fermentas). And used as substrates in a reaction mix containing RNase3, RNase3-Ala, or *E. coli* RNase III (New England Biolabs). The reaction products were separated by electrophoresis as above and visualized using a PhosphorImager (Fuji FLA-5010). For testing cleavage of siRNA isolated from sweetpotato plants, LMW-RNA was isolated using TRIzol (Invitrogen) and LiCl precipitation, 5ug was heated to 95 °C for 10 min, and then let to cool for 2 h at room temperature. The LMW-RNA samples were treated with RNase3 and RNase3-Ala in the presence of 10 mM MgCl2 for 2 hours and separated by electrophoresis as above gel stained with ethidium bromide, and visualized using an Epichemi3-Darkroom (UVP Bioimaging System) gel documentation equipment. SPFMV-specific siRNA was detected by hybridization using a radioactive probe. To reveal the proportions of SPFMV-derived double-stranded and singlestranded siRNA in SPFMV-infected sweet potato, small RNAs of 20–30 nucleotides in size were isolated by polyacrylamide gel purification, and sequenced on the Illumina Genome Analyzer at Fasteris Life Sciences SA according to the service provider's recommendations. The resulting sequences 21–24 nucleotides in length (~95% of all sequences) were mapped to the complete sequence of SPFMV-Piu (FJ155666) using the program MAQ (http://maq.sourceforge.net). Sequences were sorted according to size and polarity and the exact number of putative double stranded siRNA could then be analyzed for each size class.

Results and discussion

RNase3 is the second viral protein directly implicated in viral synergism in plants. The first was the P1/HC-Pro polyprotein of an unrelated virus family (*Potyviridae*) (Pruss *et al.*, 1997; Shi *et al.*, 1997) whose ability to mediate synergism suggested interference with RNAi. Suppression of RNAi was subsequently shown for HC-Pro (Kasschau and Carrington 1998; Brigneti *et al.*, 1998; Anandalakshmi *et al.*, 1998) and many other RSPs from a wide range of plant and animal viruses (Li and Ding 2006), but no other RSPs besides P1/HC-Pro were reported as causal agents of synergistic viral diseases. Our data show that RNase3 also mediates synergism with viruses of other taxa, several of them known to synergize with SPCSV (Mukasa *et al.*, 2006; Untiveros *et al.*, 2007). These data suggest that SPCSV encodes a suppressor of a basal antiviral defense system of sweetpotato that normally protects the plants. Transgenic 'Huachano' plants expressing SPCSV RNase3 following inoculation with different viruses accumulated high titers of them and developed more severe symptoms in comparison to the non-transgenic plants (Fig. 1). In particular, disease symptoms and SPFMV titers in RNase3-transgenic plants were similar to non-transgenic plants co-infected with SPCSV and SPFMV. Therefore, RNase3 alone was sufficient to predispose the plant to SPVD, increase titres and induce more severe symptoms of RNA and DNA viruses.
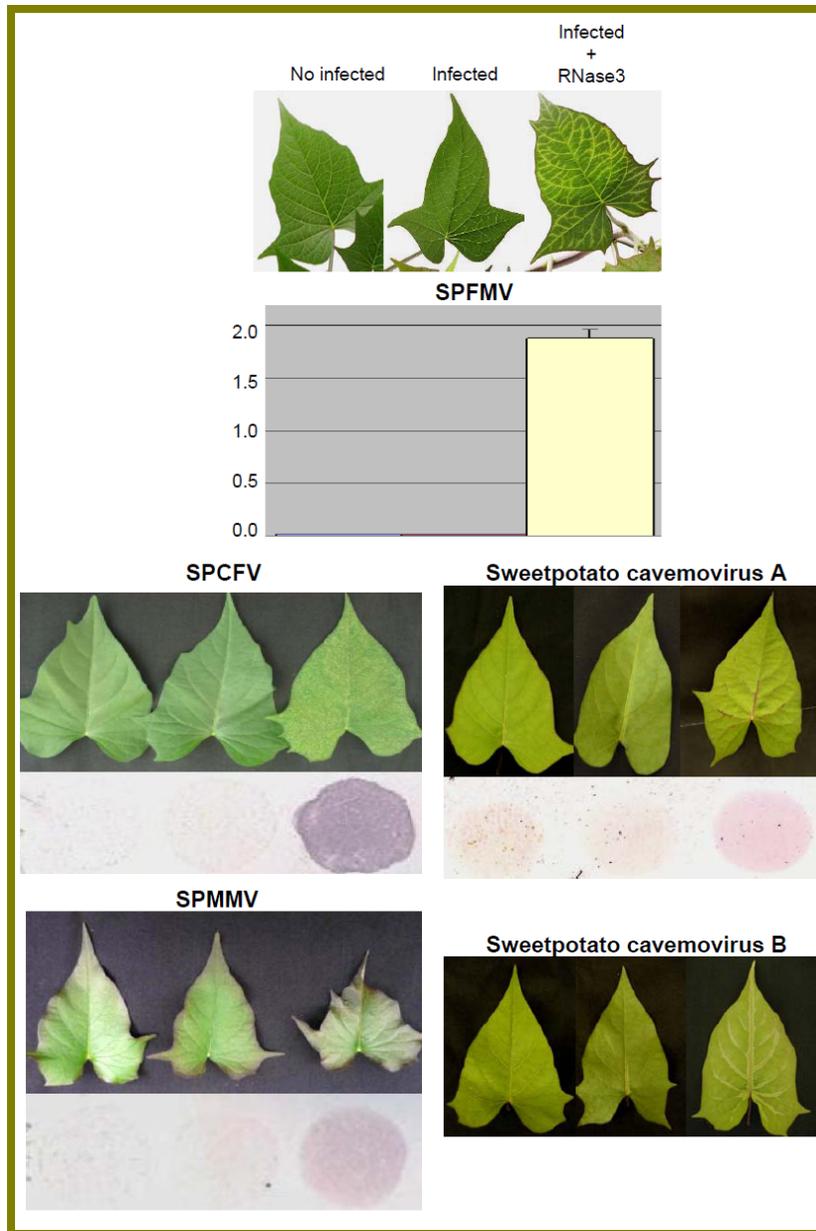
Figure 1. Systemic infection of non-transgenic (NT) and RNase3-transgenic sweet potato cv. 'Huachano' with *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato chlorotic fleck virus* (SPCFV), *Sweet potato mild mottle virus* (SPMMV), 'Sweet potato cavemovirus A' and 'Sweet potato cavemovirus B'. For each virus a 'no infected' (*left*), an 'infected' (*middle*) and an 'infected + RNase3' (*right*) leaf is shown. Viruses are barely detectable in the leaves of the inoculated NT plants (results shown from DAS-ELISA for SPFMV or NCM-ELISA for other viruses) and no symptoms are observed. However, in RNase3-transgenic plants, virus concentrations are elevated and readily detected; in all RNase3-transgenic plants virus symptoms are more severe. We are currently developing an antiserum for 'Sweet potato cavemovirus B' detection.

RNase3 is one of two reported RSP found in SPCSV, the other is p22 (Kreuze *et al.*, 2005). However, recent studies have revealed that many SPCSV isolates lack the *p22* gene but still synergize with SPFMV and with other unrelated RNA viruses (Untiveros *et al.*, 2007; Cuellar *et al.*, 2008), indicating that p22 is dispensable for synergy between SPCSV and other viruses but not ruling out a role for p22 in other virus interactions.

Viral RSPs may bind siRNA (Ye *et al.*, 2003), affect their methylation status and stability (Vogler *et al.*, 2007), and interfere with formation of the effector complexes required for RNAi (Ding and Voinnet 2007; Li and Ding 2006). These modes of action are known or suggested for RSPs encoded by many viral taxa (Li and Ding 2006), including those related to the sweetpotato viruses that cause synergistic diseases in co-infection with SPCSV (Mukasa *et al.*, 2006, Untiveros *et al.*, 2007). However, the viral RNase3 enzyme of SPCSV is the first viral RSP found to destroy siRNAs. This was confirmed by agroinfiltration in leaves of transgenic *N. benthamiana* 16c expressing GFP (Brigneti *et al.*, 1998) were the induced RNAi of GFP mRNA is suppresses by the expression of RNase3. In addition, two point mutations introduced into the endonuclease signature motif of RNase3, known to abolish its RNase III activity also affected its RSP activity (Fig. 2). Therefore, RNase3 endonuclease activity is required for its RSP function.
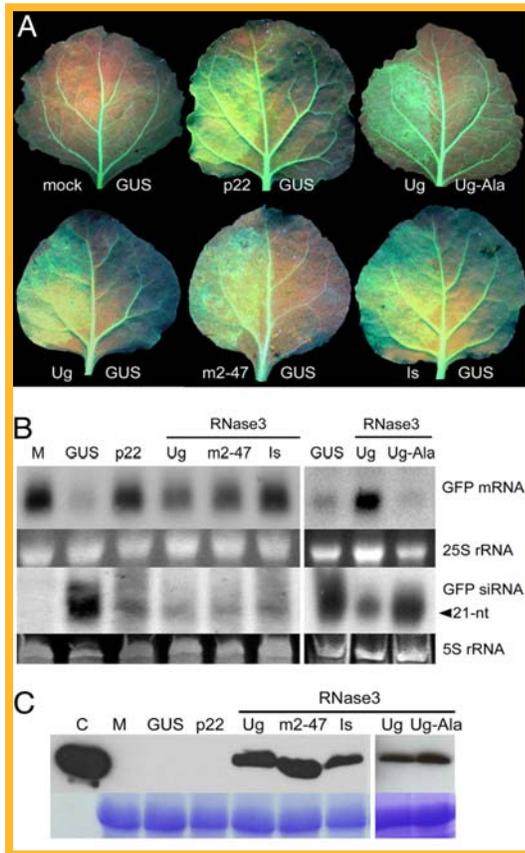
Figure 2. RNase3 from SPCSV suppresses RNA silencing. (*A*) *N. benthamiana* leaves were mock-infiltrated with buffer or with an *A. tumefaciens* strain expressing *gfp* and a strain expressing *p22* of a Ugandan isolate of SPCSV (Ug) or *RNase3* from isolates Ug, M2–47 (Peru) or Is (Israel). GUS indicates that leaves were infiltrated with a negative control. Ug-Ala indicates the RNase3-Ala mutant defective for endonuclease activity. The transgenic *N. benthamiana* plants (line 16c) constitutively express *gfp* (green fluorescence in veins of the leaf at the upper left corner and the right sides of the leaves). Leaves were photographed and analyzed 3 days post-infiltration. (*B*) Northern blot of *gfp* mRNA and siRNA in the leaf tissues illustrated in *A*. M=16c line mock-infiltrated with buffer. *Upper* shows the accumulation of *gfp* mRNA in the respective infiltrated regions. *Lower* shows accumulation of *gfp*-derived siRNA. Ethidium bromide-stained gels of rRNA were used as loading controls. (*C*) Western blot of the RNase3 protein in the infiltrated tissues. C= purified recombinant RNase3 protein. *(Lower)* Coomassie blue–stained gel.

Following this observation we tested whether RNase3 could target and modify the double-stranded siRNA essential for RNAi (Ding and Voinnet 2007). We observed that synthetic siRNA were all cleaved to products of ~14 bp by RNase3 (Fig. 3A), which are inefficient triggers of RNAi (Elbashir *et al* 2001; Paddison *et al.*, 2002; Yang *et al.*, 2002). RNase3 (but no RNase3-Ala) was also able to cleave 21–25 nt siRNA isolated from SPFMV-affected sweetpotato plants however detection with an SPFMV-specific probe revealed that the amounts of virus-specific siRNAs following treatment with RNase3 were only slightly less than with RNase3-Ala. These data indicated that RNase3 can act on the double-stranded forms of host-derived siRNA and/or miRNA, and that SPFMV derived double-stranded siRNA might be in a minority in the pool of total SPFMV-derived siRNA (Fig 3B).
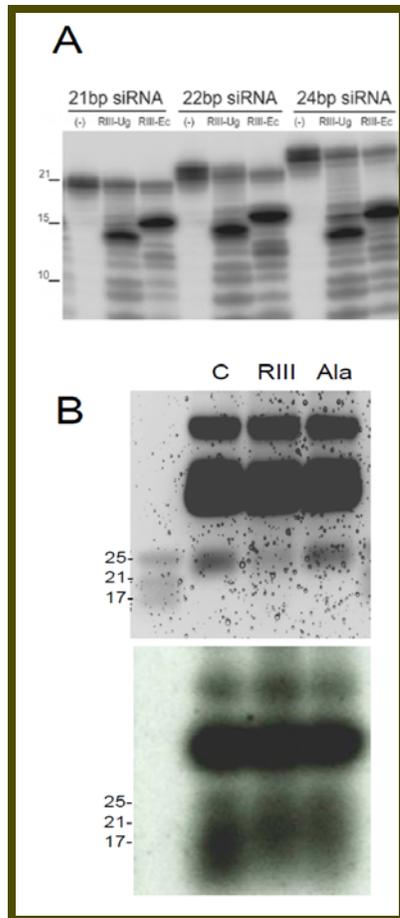
Figure 3. RNase3 of SPCSV cleaves synthetic siRNAs. ^{32}P-labeled double-stranded siRNA of the indicated sizes were incubated for 1 h with purified recombinant RNase3 proteins of SPCSV (RIII-Ug) or *E. coli* (RIII-Ec). Numbers at the left of the figure indicate the sizes in nucleotides. Samples were analyzed by electrophoresis in a TBE-UREA. (*B*) RNase3 [RIII (wt)] cleaved siRNAs isolated from an SPFMV-infected sweetpotato plant as revealed by comparison to the same amount of siRNA that was not treated (C) or was treated with mutant RNase3-Ala (Ala). The small RNAs were analyzed by 4% Agarose gel electrophoresis and stained with ethidium bromide (B-*Up*). Subsequently, RNA was transferred to Hybond NX membrane by capillary blotting and detected by Northern blot hybridization using an SPFMV-specific radioactive RNA probe (B-*Bottom*)

The latter was confirmed by calculating that only 3.95% of the total siRNA derived from SPFMV may form double-stranded siRNA. In addition, these results suggest that RNase3 may target a specific dsRNA host component in a manner which other viruses are unable to do and which releases a key obstacle that prevents other viruses from accumulating in higher titers. It is possible that after reaching a certain level thanks to the effect of SPCSV or RNase3 these co-infecting viruses can suppress silencing with its own RSS proteins. Identification of the specific target of RNase3 remains as an interesting topic for further study.

The results of this study provide a mechanistic understanding of synergism that is addressed to an important disease of a subsistence crop in developing countries. Identification of a viral class 1 RNase III enzyme as a key factor behind severe virus diseases and yield losses suggests possibilities for disease control. This is important because extensive screening of sweetpotato germplasm for sources of resistance, and conventional approaches of engineered, pathogen-derived resistance used in sweetpotato varieties have rendered little progress possible toward durable resistance to, for example, SPVD (Kreuze *et al.*, 2008). Our preliminary results indicate that an additional RSP encoded in some African isolates of SPCSV may also have an effect on virus accumulation (Fig. 4). The possibility that p22 may modify the outcome of SPCSV synergistic virus interactions in a different way as RNase3 remains to be studied.
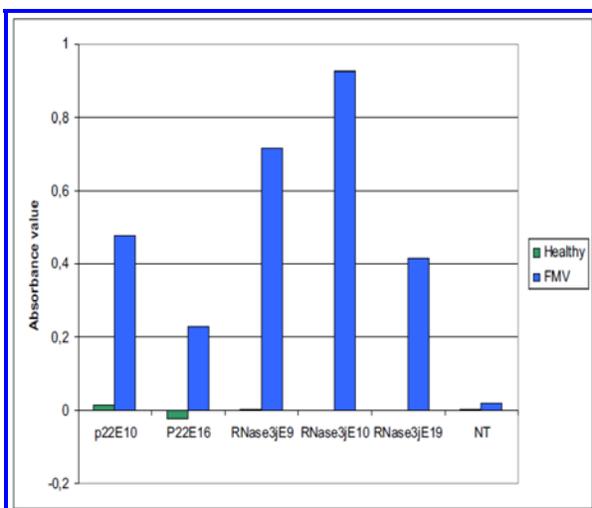
Figure 4. Relative amounts of *Sweet potato feathery mottle virus* (FMV) coat protein antigen in the systemically infected leaves of p22-transgenic (lines E10 and E16), RNase3-transgenic (lines E9, E10, E19) and non-transgenic (NT) sweet potato cv. 'Huachano' 3 weeks post-inoculation as detected by double antibody sandwich ELISA. Only RNase3-transgenic plants developed the severe symptoms of sweet potato virus disease (SPVD) following infection with SPFMV. The p22-transgenic and NT plants remained symptomless or expressed mild mottling 3 weeks post-inoculation

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