Synergistic interactions of begomoviruses with sweet potato chlorotic stunt virus (genus *Crinivirus*) in sweetpotato (*Ipomoea batatas* L.)

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SUMMARY

Three hundred ninety four sweetpotato accessions from Latin America and East Africa were screened by PCR for presence of begomoviruses and forty six were found positive. All were symptomless in sweetpotato and generated leaf-curling and/or chlorosis in *Ipomoea setosa*. The five most divergent isolates, based on complete genome sequences, were used to study interactions with sweet potato chlorotic stunt virus (SPCSV), known to cause synergistic diseases with other viruses. Co-infections led to increased titres of begomoviruses and decreased titres of SPCSV in all cases, although the extent of the changes varied notably between begomovirus isolates. Symptoms of leaf curling only developed temporary in combination with isolate StV1 and coincided with presence of highest begomovirus concentrations in the plant. Small interfering RNA (siRNA) sequence analysis revealed co-infection of SPCSV with isolate StV1 lead to relatively increased siRNA targeting of central part of the SPCSV genome and a reduction in targeting of the genomic ends, but no changes to targeting of StV1 as compared to single infection of either virus. These changes were not observed in the interaction between SPCSV and the RNA virus sweet potato feathery mottle virus (genus *Potyvirus*), implying specific effects of begomoviruses on RNA silencing of SPCSV in dually infected plants. Infection in RNase3 expressing transgenic plants showed this protein was sufficient to mediate this synergistic interaction with DNA viruses, similar to RNA viruses, but exposed distinct effects on RNA silencing when RNase3 is expressed from its native virus, or constitutively from a transgene, despite a similar pathogenic outcome.

INTRODUCTION

Sweetpotato (*Ipomoea batatas* (L.) Lam) is a perennial plant and the sixth most important crop which the world depends on for their food security (FAO, 2013). Because sweetpotato is vegetatively propagated by taking cuttings from a previous crop (directly or from sprouted tubers), build-up of virus infections over generations is a major constraint and contributes to severe losses and cultivar decline (Clark *et al.*, 2012). More than 30 viruses have been reported to infect sweetpotato and half of them are recently described DNA viruses belonging to the families *Geminiviridae* and *Caulimoviridae* (Clark *et al*., 2012). Geminiviruses (family *Geminiviridae*) are plant viruses that have a circular, single-stranded DNA genome encapsidated within twinned isometric particles (Fauquet & Stanley, 2003). They are grouped into four genera based on insect vector, host range, and genome organization (Fauquet & Stanley, 2003). Members of the genus *Begomovirus* are transmitted by whiteflies, have single or bipartite component genomes, and infect dicotyledonous plants. Within the genus *Begomovirus* the sweetpotato infecting viruses are monopartite but are distinct from other monopartite begomoviruses, forming a phylogenetically unique lineage, and are referred to as sweepoviruses as a group (Fauquet & Stanley, 2003, Wasswa *et al.*, 2011, Esterhuizen *et al.*, 2012, Albuquerque *et al.*, 2012). Sweepovirus taxonomy is complex and the currently sequenced sweepoviruses have been suggested to correspond to up to 17 different species based on nt sequence identities (Albuquerque *et al*., 2012). However the occurrence of frequent recombinants and the lack of any complementing biological differences between suggested species currently render these classifications of little practical use and in this paper we will refer to them simply as sweepoviruses.

Despite the lack of obvious symptoms associated with sweepovirus infections, yield reduction of between 10-80% have been reported in infected sweetpotato plants (Clark & Hoy, 2006, Ling *et al.*, 2010). Since they can occur at relatively high incidences in crops they may thus be responsible for considerable crop loss on a global scale.

Studies with several other sweetpotato infecting viruses have shown that most of them can cause severe synergistic disease complexes when co infected with sweet potato chlorotic stunt virus (SPCSV; species: *Sweet potato chlorotic stunt virus,* Genus *Crinivirus,* family *Closteroviridae*) leading to increased yield losses. These include RNA viruses of the genera *Potyvirus, Ipomovirus, Carlavirus* and *Cucumovirus* as well as otherwise symptomless DNA viruses of the genera *Cavemovirus* and *Solendovirus* (Karyeija *et al.*, 2000, Cuellar *et al.*, 2011b, Mukasa *et al.*, 2006, Untiveros *et al.*, 2007). The best studied of these synergistic diseases is the one between SPCSV and sweet potato feathery mottle virus (SPFMV; species: *Sweet potato feathery mottle virus,* genus *Potyvirus,* family *Potyviridae*) and has been shown to be mediated by the SPCSV encoded RNase3 protein, which can also mediate synergistic disease with two other unrelated RNA viruses (Cuellar *et al.,* 2009). RNase3 is a double-stranded RNA (dsRNA)-specific class 1 RNA endoribonuclease III that can digest long as well as short dsRNA and functions as an RNA silencing suppressor (RSS)(Cuellar *et al*., 2009, Weinheimer *et al.*, 2014). RNase3 catalytic activity is required for its RSS activity (Cuellar *et al*., 2009), implicating RNA cleavage in the process of RSS suppression as well as synergistic disease induction. Whereas the exact mechanism of RNase3 action has not yet been elucidated it is clear that it is able to mediate increased susceptibility of sweetpotato to a wide range of viruses (Cuellar *et al*., 2009).

Synergism between SPCSV and sweepoviruses has however not yet been reported. Wasswa *et al*. (2011) reported that a Ugandan sweepovirus isolate was not obviously synergized by co-infection with SPCSV, however our own observations with our reference isolate StV1 seemed to indicate this is not the case for all strains. Therefore, in the current study we screened a selection of sweetpotato germplasm for presence and variability of sweepoviruses and selected the six most diverse isolates for complete sequencing and co-infection experiments with SPCSV to determine if synergism with SPCSV is a general phenomenon for sweepoviruses as it is with other viruses. siRNA profiles in single and mixed infections were also determined with reference isolate StV1 and compared to those of the well-studied interaction between SPCSV and SPFMV.

RESULTS

Virus detection and host symptoms

During standard virus indexing of germplasm material at CIP generally between 10- 20% of samples are found infected with begomoviruses (data not shown). We took advantage of one of the batches of Latin American sweetpotato accessions (329 genotypes; Table S1) going through virus indexing to characterize the amplified begomovirus nucleotide (nt) sequences identified in 39 accessions. In addition we screened by PCR a collection of 65 (symptomless; Table S1) sweetpotato plants collected in East Africa, of which seven were found positive for begomoviruses. Symptomatology was recorded among the sweetpotato accessions from the Americas in the indicator plant *Ipomoea setosa*. No large variation in symptoms was observed among plants infected with begomovirus, but

they could be broadly characterized into three categories: typical upwards leaf curling, chlorosis, or both were observed in indicator plants with all isolates that were positive to sweepoviruses by PCR (Table 1); in several cases plants were co-infected by other viruses, nevertheless, the infected sweetpotato plants themselves were all symptomless. None of the samples used in this work were found co-infected with SPCSV.

Sweepovirus sequence variability and characterization

The sequences of the PCR fragment obtained using the universal sweepovirus primers SPG1 and SPG2 (Li *et al.*, 2004) of forty six isolates identified in this study were determined and compared to those available in the GenBank by PASC and phylogenetic analysis. Phylogenetic analysis using our sequences and others available in the genebank showed that the isolates sequenced in this study spanned the variability found in sweepoviruses except for the cluster corresponding to *Ipomoea yellow vein virus* and *Sweet potato leaf curl canary virus* (data not shown).

The complete genomes of the most divergent sweepovirus based on the analysis of partial sequences described above, and found free of any other virus detectable by index grafting to *I.setosa*, were selected for complete genome sequencing together with our reference isolate. These were isolates StV1 (Saint Vincent), Mex31 (Mexico), Cub5 (Cuba), Jam12 (Jamaica), Per6 and Per10 (Peru). These isolates were then also used in double infection studies with SPCSV-m2-47 as described below. Complete genome comparison confirmed that all six viruses were quite different from each other with <89% nt identity over their genome except for Jam12 and Cub5 (91.7%) and StV1 and Per10 (93.1%). Isolates StV1,

Per-6 and Per-10 are most similar to the ICTV approved species *Sweet potato leaf curl virus* (93, 93 and 98% identity to type isolate respectively), whereas Jam-12 and Cub-5 are most similar to *Sweet potato leaf curl Georgia virus* (91 and 95% similarity to type isolated respectively) and Mex-31 is most similar to *Sweet potato leaf curl South Carolina virus* (93% identity to type isolate). Alignment and phylogenetic analysis using 108 complete sweepovirus genome sequences available from the genebank confirmed the sequenced isolates were positioned well distributed among the known sweepovirus variability (Fig. 1). All sequences were deposited in the GenBank database (see Table 1 for Genbank accession numbers)

Synergistic interaction of sweepoviruses with SPCSV in sweetpotato

Sweepovirus isolates StV1, Mex31, Cub5, Jam12, Per6 and Per10 were used as inoculum for synergistic interaction studies in sweetpotato cultivar 'Huachano'. Co-infection with SPCSV led to symptoms of upward leaf curling transiently around 3-4 weeks post inoculation only in case of the isolate StV1. This was repeated when plants were cut back and symptoms occurred again in the re-growth after about 3-4 weeks. None of the other five isolates caused any visible symptoms at any time-point. No increase in the severity of SPCSV symptoms was observed (i.e. purpling/yellowing of older leaves or stunting) in combination with any of the sweepovirus isolates. Signal intensity analysis of DNA dot-blot hybridization (Fig. S1) from plants infected with the six sweepovirus isolates showed a significantly higher accumulation of the viruses in plants co-infected with SPCSV (Fig. 2) in all cases except isolate Cub5 for which the difference was not significant ($p<0.05$). The extent and time-point of manifestation of this difference varied considerably between

the different isolates, but could be generally divided into three categories. Hybridization signals of isolates StV1 and Mex31 were significantly different from the remaining isolates and showed the overall highest virus titres and a distinct titre development: sweepovirus titres initially increased much faster in mixed infected plants than singly infected plants during the first three weeks, reaching a peak between 4 to 5 weeks post inoculation and another peak at 10 weeks post inoculation. On the other hand, isolates Per6 and Per10 co-infected with SPCSV showed an increase in titres during the first 6 weeks (primary infection) as compared to singly infected plants and after cutting back at 6 weeks the re-grown sweetpotato plants (secondary infection) reached a peak in titers after one week, followed by a reduction. Finally isolate Cub5 and Jam12, although significantly different from each other, showed much lower titres and smaller differences between single and mixed infected plants during primary infection, whereas difference in secondary infection were more pronounced, especially in Jam12. In general the severity of symptoms caused in the indicator plant *I.setosa* correlated positively the titres determined in sweetpotato, with StV1 and Mex31 showing the most rapidly developing and severe symptoms in *I.setosa* and Cub5 the mildest symptoms. Isolate StV1 showed the strongest signals of all isolates tested and except for the first week showed the biggest differences in titer between singly and doubly infected plants throughout the experiment. It was also the only isolate to induce symptoms typical of begomovirus infection, upward leaf curling (Fig. 3a), although this only happened transiently during the 3rd and 4th week after infection and again 4 weeks after cutting back the plants. This happened in all plants co-infected with SPCSV and coincided with the time of maximum virus accumulation in the plants (Fig. 2).

Whereas the dot-blot indicated increased titres of sweepoviruses in dual infections, qRT-PCR analysis of the SPCSV Hsp70 gene region (RNA2) in the same plants, indicated that average SPCSV RNA titres were significantly reduced ($p<0.01$) compared to those in SPCSV single infection in all combinations (Fig. 2, Table S2) and this was confirmed by TAS-ELISA tests (detecting the coat protein) with the isolate StV1 where a decrease in SPCSV antigen was observed (data not shown).

To test if the observed increases in sweepovirus titres could be mediated by RNase3 alone as has been shown for RNA viruses, transgenic plants expressing RNase3 (Cuellar *et al*., 2009) were inoculated with StV1. The infected plants developed typical leaf-curl symptoms in the same temporary fashion as seen for non-transgenic plants co-inoculated with SPCSV and StV1 and a similar increase in virus titres (Fig. 3). Similarly, infection with two unrelated DNA viruses, sweet potato collusive virus (SPCV; species: *Sweet potato collusive virus*, genus: *Cavemovirus*, family: *Caulimoviridae*) and sweet potato vein clearing virus (SPVCV; species: *Sweet potato vein clearing virus*, genus: *Solendovirus*, family: *Caulimoviridae*) also reproduced symptoms similar to those caused by co-infection with SPCSV and resulted in increased viral titres (Fig. S2)

Analysis of virus-derived siRNA from single and double-infected, and RNase3 transgenic sweetpotato plants

Raw sRNA sequence reads of the seven different samples analysed in this study are available at https://research.cip.cgiar.org/confluence/display/cpx/CIP.sweetpotato.2014. Comparison of siRNA sequences determined from uninfected, StV1, SPCSV, and SPCSV and StV1 infected plants showed notable changes in the relative amounts siRNAs

corresponding to each virus as well as the regions to which they mapped, particularly in the case of SPCSV (Fig. 4-5 & S5). The relative number of siRNA reads corresponding to SPCSV increased by more than threefold from 7,500/million reads to 23,708/million reads, with most of the increase corresponding to RNA1 (Fig. 4). Relative amounts of begomovirus specific siRNA did not change, beyond the variation found between individual samples of the same treatment (+/- 0.1%), in double infected plants as compared to singly infected plants (1.1 fold; from 82032 to 90275/million reads). StV1 specific siRNAs also mapped to similar positions in the genome, and no obvious differences could be observed (Fig. 5a). This was in stark contrast to the mapping of reads to the SPCSV genome, where a dramatic change could be observed in the relative amounts and positions to which the siRNAs mapped on the genome (Fig. 5b). This was characterized by a several fold increase in siRNAs corresponding to SPCSV (Fig. 4), a reduction in 21nt siRNA (reduced from 37% to 15.8% of all siRNAs corresponding to SPCSV) and corresponding increase in 22 and 23nt siRNA (increased from 39.6% to 47.1% and 12.8% to 26.5% respectively, of all siRNAs corresponding to SPCSV), as well as a near disappearance of siRNAs matching to the 5' regions of SPCSV (Fig. 5b) in plants co-infected with isolate StV1 as relative to single SPCSV infection. To determine if the effect of change in siRNA mapping to SPCSV was specific to the synergism between SPCSV and StV1 or a general response found in synergistic interactions with other viruses, we sequenced siRNAs from plants infected with SPCSV and SPFMV. No reduction in the mapping of siRNAs to the 5' region was observed in these plants (Fig. 5b), nor was there a change in the total amount of siRNAs corresponding to SPCSV (Fig. 4).

When comparing distribution of StV1 specific siRNAs between StV1 and SPCSV co-infected plants and StV1 infected RNase3-expressing plants on the other hand a surprising difference could be noted, in that a several-fold reduction in 21 and 22 nt siRNAs could be observed in RNase3 expressing plants as compared to SPCSV co-infected plants and also StV1 singly infected plants (Fig. 5b).

DISCUSSION

Until the beginning of this century, most surveys of sweetpotato viruses did not mention begomoviruses (Valverde *et al.*, 2007). Nevertheless closely related begomoviruses have now been reported from all over the world including North America (Lotrakul *et al.*, 2002, Lotrakul *et al.*, 1998, Lotrakul, 2003), the Mediterranean (Lozano *et al.*, 2009, Cohen *et al.*, 1997, Banks *et al.*, 1999, Briddon *et al.*, 2006), Asia (Luan *et al.*, 2007, Onuki *et al.*, 2000, Bi & Zhang, 2012), South America (Fuentes & Salazar, 2003, Rodríguez-Pardina *et al.*, 2012, Albuquerque et al., 2012, Paprotka *et al.*, 2010) and Africa (Miano *et al.*, 2006, Wasswa *et al.*, 2011). Our report corroborates the common occurrence of sweepoviruses and reveals clear synergistic interactions of sweetpotato begomoviruses with SPCSV, another widely distributed virus and mediator of several synergistic interactions (Cuellar et al., 2011b, Karyeija et al., 2000, Untiveros et al., 2007, Mukasa et al., 2006).

Few reports exist on synergistic interactions between RNA and DNA viruses. We previously showed that SPCSV can interact with members of the *Caulimoviridae* family of reverse transcribing viruses (Cuellar *et al*., 2011b), and here we show that this is also the case for sweepoviruses. However, our data also show that the extent of this synergism varies considerably between different sweepovirus isolates and that, in the majority of cases, it was not associated with clear symptoms. This observation may explain why such interactions have not been noticed before. Still, the fact that 5 out of 6 diverse viruses tested showed clear and significant increase in titers in co-infection with SPCSV indicates that this may be a common phenomenon among sweepoviruses. As has been shown for other synergistic interactions with SPCSV (Cuellar et al., 2011b, Mukasa et al., 2006, Cuellar *et al.*, 2008) we also found that an increase in titre of the synergized virus is associated with a corresponding decrease in titre of SPCSV compared to single infection (Fig. 2). It is not yet clear why SPCSV titres are reduced in synergistic interactions, but it may simply be a result of competition for limited resources of the two co-infecting viruses in infected cells, where the association is favoring one over the other. Nevertheless, when we analyzed siRNA targeting of SPCSV in single as compared to dual infection with sweepovirus isolate StV1 we were able to detect a striking difference in the relative amount and distribution of siRNA reads. Total siRNAs corresponding to SPCSV increased several fold (Fig. 4), and 22 and 23nt siRNA increased relatively as compared to 21nt siRNA , whereas siRNAs matching to the 5' regions of SPCSV nearly disappeared (Fig. 5b) in plants co-infected with isolate StV1 relative to single SPCSV infection. This effect was apparently specific to the interaction of SPCSV with StV1, as

similar changes were not observed in the interaction between SPCSV and SPFMV (Fig. 4 and 5b).

The specific changes in distribution and relative quantity of SPCSV specific siRNAs as a result of co-infection with StV1 suggests a modified and increased targeting of SPCSV by the plants RNA silencing system. A plausible explanation is that this is the result of interference by StV1 with the hosts RNA silencing machinery. Although this may seem counterintuitive, it has become clear in recent years that different branches of the RNA silencing system in plants compete with each other for access to cellular machinery (Jauvion *et al.,* 2012). RSS suppression by sweepoviruses is more likely to affect pathways, inhibiting replication of DNA viruses in the nucleus, and this may not necessarily benefit, or even be detrimental to, replicating RNA viruses in the cytoplasm. Two RNA viruses with similar replication strategies on the other hand are less likely to compromise each-others silencing suppression. This alone may explain why similar changes are not seen in the interaction between SPCSV and SPFMV as compared to the interaction between SPCSV and SvT1. Also, although the tissue tropism of sweepoviruses has not yet been determined, many begomoviruses are phloem limited, similar to SPCSV, and if this is the case also for sweepoviruses the effects the two viruses may have on each other's replication may be expected to be more evident than in the case where tissue tropism is distinct such as SPFMV and SPCSV (Karyeija *et al*., 2000).

Although RNA silencing suppressors (RSS) have not yet been reported for sweepoviruses, a number of studies have reported up to three RSS proteins encoded by single and

multipartite begomoviruses, including the (homologs of) V2, C2 and C4 proteins (Amin *et al.*, 2011, Chellappan *et al.*, 2005, Vanitharani *et al.*, 2004, Buchmann *et al.*, 2009, Zrachya *et al.*, 2007, Zhang *et al.*, 2011). These RSS have been found to suppress silencing both at the transcriptional and post-transcriptional level, but not all homologous proteins from different viruses have RSS activity or necessarily function in similar ways (Raja *et al.*, 2010, Amin et al., 2011). Thus although it can be expected that sweepoviruses encode RSS proteins, it is not possible to deduce which they will be or how they will function based on knowledge from other begomoviruses. On the other hand we can use our observations regarding relative changes in siRNA distributions to speculate which components of the RNA silencing machinery might be affected. The phenotype of reduced siRNA targeting of the 5' region and increased targeting towards the 3' of SPCSV is reminiscent to that found in CMV infected *RDR1* defective Arabidopsis (Wang *et al.*, 2010). This may indicate that StV1 interferes with RDR1 function. RDR proteins could be expected to fulfill an important role in antiviral silencing against geminiviruses, since they do not normally produce dsRNA. Nevertheless one must be careful to extrapolate conclusions from the one specific model system to sweetpotato because marked differences have been found between different geminivirus host combinations (Akbergenov *et al.*, 2006, Rodríguez-Negrete *et al.*, 2009, Miozzi *et al.*, 2013).

Previously we demonstrate that RSS encoded by SPCSV (RNase3) is responsible for the enhanced accumulation of co-infecting RNA viruses in synergistic interactions mediated by SPCSV (Cuellar *et al*., 2009). Although the exact mechanism of RNase3 function is

not fully elucidated its dsRNase activity is essential for silencing suppression as well as enhanced accumulation of viruses in transgenic plants (Cuellar et al., 2009, Kreuze *et al.*, 2005). RNase3 has little substrate specificity in vitro, processing both long and short dsRNA including siRNAs and pre-miRNAs (Cuellar et al., 2009, Kreuze et al., 2005, Weinheimer et al., 2014). Nevertheless, its target in plants must be specific, since transgenic plants are phenotypically completely normal except for their extreme susceptibility to viruses (Cuellar *et al*., 2009). We hypothesize that the same mechanism is involved in SPCSV synergisms with RNA and DNA viruses. Indeed infection of RNase3 transgenic sweetpotato plants with StV1 (Fig. 3) as well as SPCV and SPVCV (Fig. S2) provoked characteristic symptoms as seen in plants co-infected with SPCSV. Surprisingly however siRNA distribution patterns of StV1 were perceptibly different in plants constitutively expressing RNase3 as compared to those from plants co-infected with SPCSV, in that 21 and 22 nt siRNAs were strongly reduced in RNase3 plants. This suggests that despite the biologically similar outcomes of enhanced StV1 viral titres and symptom induction, clear differences occur in how RNA silencing is affected in either situation. We offer two possible explanations for this discrepancy: i) constitutive overexpression of RNase3 in all plant cells results in a distinct effect of RNase3 on the silencing pathway as compared to phloem specific expression, or ii) RNase3 function is modulated by other SPCSV encoded proteins to limit its effect to certain sites in the silencing pathway. It is intriguing that in spite of its constitutive expression in all plant cells, RNase3 will not cause visible collateral effects on sweetpotato. Future analysis of siRNA sequences in RNase3 plants infected with different types of viruses may shed

more light on the exact target and mechanism of RNase3 provoked susceptibility to viruses.

Although we did not analyze the potential effect on yield of the different virus combinations in the current study, the strong increase in sweepovirus titres found in some interactions suggests that yield impacts could be expected and this should be a priority for future studies. Indeed, other studies have already shown significant impacts of sweepovirus infection on yield of sweetpotato, despite being largely symptomless (Ling et al., 2010, Clark & Hoy, 2006). In addition, increases in sweepovirus titres in plant tissues could lead to an increased rate of transmission of the virus by its vector contributing to more rapid virus spread.

EXPERIMENTAL PROCEDURES

Virus isolates

The 39 begomovirus isolates described in this study (Table 1 & S1) were identified in sweetpotato accessions from the Central and South America (Mexico, Guatemala, Cuba, Jamaica, Nicaragua, Dominican Republic, Saint Vincent and the Grenadines, Colombia, Ecuador, Peru, Argentina, Paraguay, Panama and Puerto Rico), after indexing by grafting onto the indicator plant *Ipomoea setosa* followed by PCR (see below), during routine virus indexing performed at CIP (329 accessions). These accessions were either collected by CIP or acquired from other collections between 1986 and 1994. Accessions collected by CIP were established under an insect proof screenhouse before being transferred to in-

vitro, where they were maintained as part of CIPs global sweetpotato collection. Samples acquired from other sources were obtained as in-vitro, vine cuttings or roots and in the latter cases established and introduced to in-vitro as described for the CIP collected materials. An additional 7 virus isolates were identified by PCR screening from 65 sweetpotato genotypes collected from different regions of East Africa (Uganda, Kenya, Tanzania) and originally maintained under field conditions for breeding purposes and subsequently transferred to in vitro for transfer to CIPs sweetpotato collection at CIP-Lima. Metadata of the accessions in which sweepoviruses were identified are provided in supplementary Table S1. For synergism experiments SPCSV isolate m2-47, lacking the p22 gene (Cuellar *et al.*, 2011a, Cuellar *et al*., 2008) and maintained in *I. setosa* was used. The SPCV and SPVCV isolates used are described in Cuellar *et al*. (2011b).

DNA amplification, cloning and sequence analyses

The Saint Vincent and the Grenadines isolate (StV1) was isolated from sweetpotato accession CIP400025. The accession has been tested for 10 viruses by ELISA, and grafting onto *I. setosa*. To amplify begomovirus specific fragments from different sweetpotato accessions (Table 1) a simple and quick method of DNA extraction using sodium hydroxide was used to prepare template DNA for PCR (Wang *et al.,* 1993). Shoots were collected from *in vitro* plantlets and homogenized in 0.5M NaOH buffer in ratio of 1/5 (tissue: Buffer). The samples were centrifuged at 12000 g for 10 min to spin down the debris. After a spin down samples were diluted 100 times with Tris-HCl 100mM (pH8) and 1 ul of leaf extract was used directly for PCR in a 25ul reaction using the 2X phusion polymerase readymade master mix (Finnzymes, Finland) and

sweepovirus specific primers SPG1: 5′-CCC CKG TGC GWR AAT CCA T-3′ and SPG2: 5′-ATC CVA AYW TYC AGG GAG CTA A-3′ (Li *et al*., 2004), designed to amplify a 901 bp region encompassing partial AC1 and AC2 ORFs.

For cloning of the selected begomovirus genomes total DNA was extracted using a modified CTAB protocol (see below) followed by separation of small molecular weight DNA using a plasmid isolation protocol (Alkaline lysis) and the Wizard miniprep kit (PROMEGA, USA). The quality and amount of DNA was checked by agarose gel electrophoresis and by espectrophotometry using a nanodrop analyzer (ND-1000, Thermo Fisher Scientific, USA), respectively. In the case of isolates Per10 and Jam12, 5 ug of small molecular weight DNA was used for amplification of circular DNA using Phi29 polymerase (New England Biolabs, USA) reaction with a 5X excess of random hexamer primers according to the manufacturer's instructions. The amplified DNA was then linearized using *Sma*I for Per10 and *Stu*I for Jam12, resulting in the expected 2.7kb fragment. Isolate Per6, Mex31, Cub5 and StV1 were amplified by inverse PCR using a set of degenerate primers designed based on previously amplified and sequenced region (Bego-F: 5' CTG RCC TCC TCT AGC AGA TCK CC -3'; Bego-R: 5'- GAR CCT GCK CCT GGA TTG CAG AGR -3') resulting in the expected 2.3kb fragment.

The PCR and digested Phi29 amplified products were separated by agarose gel electrophoresis and then excised and purified using a gel extraction kit (Promega-WI-Madison). The fragments were cloned into pGEM-T easy vector (Promega). Transformation of *E.coli* DH5α was done by heat shock at 42^{o}C for 90seconds. Using blue white screening putative transformants were screened and confirmed by restriction analysis using Eco*RI* enzyme prior to sequencing. The samples were then prepared for

sequencing (Macrogen, Korea) using SP6 and T7 primers and a set of specifically designed internal primers.

Sequence alignments and phylogenetic analysis were performed using MEGA5.1 (Tamura *et al.*, 2011). Alignments were performed using Muscle and phylogenetic trees were generated after calculating the best fitting model: Maximum-Likelihood method with the General time-reversible model using Gamma distributed rates (with 5 discrete gamma categories) with invariant sites.

Synergistic interaction of sweepoviruses with SPCSV in sweetpotato

Sweetpotato cultivar 'Huachano' (accession CIP420065) obtained from the germplasm collection of the International Potato Center (CIP) was used as a rootstock for graft inoculation of isolates StV1, Per-6, Per-10, Jam-12, Cub-5 and Mex-31 with or without SPCSV. SPCSV isolate m2-47 (Untiveros *et al*., 2007) was maintained in sweetpotato cv. Huachano through cuttings, and all sweepovirus isolates were maintained in *I. setosa* plants by serial grafting. Nodes from the middle part of virus-infected source plants were used as scions to graft inoculate sweetpotato. Four weeks old cuttings of sweetpotato cv "Huachano" were side grafted in the middle of the plant with SPCSV or healthy sweetpotato scions. Two weeks later, two nodes above the initial graft, plants were grafted with healthy or sweepovirus infected *I.setosa* scions, thus generating plants infected with SPCSV alone, SPCSV plus sweepovirus, sweepovirus alone, and mock inoculated. Three plants per treatment were inoculated and formation of graft union confirmed. Plants were cut back below the graft unions 6 weeks after the last graft inoculation and left to re-grow for 4 more weeks. Development of symptoms was

recorded every week after inoculation and total nucleic acid was extracted (see below for methods) from 10 mm leaf disks from a combination of three leaves collected from the apex, middle and bottom part of each plant at 1 to 10 weeks post inoculation for dot-blot detection of begomovirus and quantitative real-time RT-PCR detection of SPCSV. Leaf samples were stored at –20°C. Triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) for SPCSV were carried out as described previously (Karyeija *et al*., 2000), 4-weeks after cutting back infected plants, only on plants with single SPCSV infection and mixed infection of SPCSV and isolate StV1. In a separate experiment three replicates of the RNase3 transgenic sweetpotato 'Huachano' event R3 (Cuellar *et al*., 2009) and three non-transgenic events were also infected with StV1 and tested by DNA dot blot together with non-transgenic plants infected with SPCSV and StV1 four weeks after cutting back infected plants.

Dot-Blot hybridization and signal quantification

For detection by Dot-blot hybridization, total DNA was purified using the CTAB method (Doyle & Doyle, 1987). Frozen leaf tissues (250 mg) were processed immediately by grinding in 2 ml of CTAB buffer (2% CTAB 100 mM Tris-HCl, pH8.0, 20mM EDTA, 1.4M NaCl, 1.0% Na sulphite and 2.0% PVP-40) using polypropylene sack. The homogenate was centrifuged at 10,000 × g for 10 min and the supernatant (750 µl) was transferred to a 1.5-ml microcentrifuge tube and mixed with an equal volume of chloroform:isoamyl alcohol (24:1). The mixture was centrifuged at 12,000 × g for 10 min and the aqueous phase (500 µl) was transferred to a new 1.5-ml microcentrifuge tube before mixing in 550 µl of isopropanol. The mixture was incubated on ice for 10 min and

centrifuged at 12,000 × g for 10 min at 4°C. The pellet was washed with 70% ethanol, centrifuged at 12,000 × g for 5 min, air dried and dissolved in 100 µl of NFW. 5 ug of total DNA in a total volume of 200 ul were used for hybridization. Standards of 125, 50 and 25 ng of plasmid DNA containing the region corresponding to the probe used were also added in duplicate to each membrane tested, to normalize and quantify results. Dioxygenin-labeled probes encompassing the Rep gene region (AC1) of StV1 were synthesized by PCR using primers SPG1 and SPG2 (Li *et al*., 2004), Taq polymerase (Promega) and Dioxygenin-labeled deoxynucleotides (Roche, UK). Total DNA from infected plants were transferred to a nylon membrane (Hybond-N; Amersham Biosciences AB) using Bio-Dot SF Cell (BIO-RAD), cross-linked by UV-irradiation (50mJ) in a cross-linking oven (Stratagene), prehybridized for 90 min at 65°C in 0.02% SDS, 5X SSC (750 mM NaCl, 75mM sodium citrate), 50% formamide, 2% (w v^{-1}) N-lauroylsarcosine and then hybridized in the same solution at 65°C for the 16h after adding the DIG-labeled probe. After hybridization, membranes were washed twice in 2X SSC and 1% SDS at room temperature for 15 min, incubated for 30 min with anti-DIG antibodies conjugated with alkaline phosphatase, and washed twice with maleate buffer with 0.3% Tween-20. The reaction was developed using CSPD chemiluminescent substrate (Roche) and Omat-S film (Kodak). Signal intensities of the hybridized spots was measured from developed films using the Gel Doc equipment in conjunction with the Quantity One software (Biorad) under white light. Signal intensity was determined using volume circle tool, ensuring circles were all the same size and covered each spot exactly, with global background subtraction and avoiding overexposed pixels. The concentration of viral DNA inside each circle was then determined using a regression curve based on

the volumes of the plasmid standards within each membrane using the Volume Analysis Report and Volume Regression Curve within Quantity One. The estimated viral concentrations (in ng) were then used for statistical analysis using the SAS statistical package. Membranes were stripped and hybridized using rDNA specific probes (amplified using primers Ribosomal F: 5'- ACA GCA GAA CGA CCA GAG AAC GC -3', and Ribosomal R: 5'- GCA CGC TAG GTA CGA CCA CCA CT -3') to confirm equal loading of DNA between samples. First a Repeated Measures Analysis of Variance was performed revealing highly significant (<0.0001) probability of interactions between timepoint, isolate and treatment. Subsequently an analysis of variance was performed for each time point to determine the effect of treatments and isolates at each timepoint. A full analysis of variance considering time and isolate as factors was also performed to determine the global effect of single vs. dual infection for each isolate.

Real-time quantitative RT-PCR for detection of SPCSV

The same samples described for dot-blot hybridizations above were used to extract RNA using the CTAB method described previously, but modified to precipitate total RNA by adding an equal volume of 4M LiCl rather than isopropanol, and overnight incubation at 4°C followed by centrifugation at 10,000 × g for 20 min. The pellet was washed with 70% ethanol as described above and re-dissolved in 100 µl of NFW.

A TaqMan real-time RT-PCR assay was then used for detecting SPCSV. One-step real-time RT-PCR assays were performed using the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) in a 25-µl final reaction volume containing 2U of MMLV, 300 nM of each primer, 100 nM of the probe, and 2 µl of template RNA. The

following thermal cycling conditions were used: 42°C for 30 min (cDNA synthesis), 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Primers and probe for SPCSV were SPCSV-Uni-E-F(5'-CGG AGT TTA TTC CCA CYT GTY T-3') and SPCSV-Uni-E-R (5'-GGG CAG CCY CAC CAA-3') and probe SPCSV-Uni-E-P (5'-[FAM]-TCT GTC ACG GCT ACA GGC GAC GTG-[TAMRA]-3'), corresponding to the Hsp70h region on RNA2. Cytochrome oxidase (COX) was used as internal reference gene using the primers COX-F (5'-CGT CGC ATT CCA GAT TAT CCA-3'), COX-R (5'-CAA CTA CGG ATA TAT AAG AGC CAA AAC TG-3' and probe COX-P: 5'-[VIC]-TGC TTA CGC TGG ATG GAA TGC CCT-[TAMRA]-3'.

Results were analyzed with MxPro QPCR Software and Statistical differences between single and mixed infections were determined for each timepoint/virus combination as well as each virus combination over all time-points using the Relative Expression Software Tool (REST) v2.0.12 (QIAGEN GmbH, Germany).

siRNA sequencing and analysis

Total RNAs were extracted from three leaves each of healthy, SPCSV infected, StV1 infected, SPCSV+StV1 infected, SPFMV infected and SPCSV+SPFMV infected 'Huachano' plants, as well as StV1 infected RNase3 transgenic 'Huachano' plants (Cuellar *et al*., 2009) at several months after inoculations using TRIZOL reagent. siRNAs were purified from 4% agarose gel and sent for library preparation and Illumina sequencing (Provider: Fasteris Life Sciences SA, Plan-les-Ouates, Switzerland).

Reads were mapped to the corresponding genomes using the MAQ software, and results were visualized using a custom script (Fuentes *et al.,* 2012) and Microsoft Excel (bar-charts).

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SUPPORTING INFORMATION LEGENDS

Table S1. Origin of plants, collection date, genotype name and germplasm accession numbers of plants screened for sweepoviruses in this study.

Table S2. Relative expression of SPCSV in mixed infection with 6 sweepovirus isolates as compared to single infection over all time-points based on REST analysis of qRT-PCR results.

Figure S1. a) DNA dot-blot results of sweepoviruses used to generate graphs in figure 2. b) Results of same blots with ribosomal DNA probe demonstrating equal loading

Figure S2. Symptoms of pararetroviruses (SPVCV and SPCV) in transgenic sweetpotato cultivar 'Huachano' expressing the RNase3 gene of Sweet potato chlorotic stunt virus (SPCSV). a) and c) = Non transgenic 'Huachano' infected with SPVCV and SPCV, respectively. b) and d) = transgenic 'Huachano' plants expressing RNase3 and infected with SPVCV and SPCV, respectively. e) = Dot-Blot detection of SPVCV in transgenic vs. non-transgenic sweetpotato 'Huachano' plants. N.I.= Not infected plant. F = Detection of SPCV by NCM-ELISA 1-2 = Not infected 'Huachano' plant, 3-4 = SPCV, 5-6=SPCV in RNase3 transgenic plants, 7-8 = SPCV in co-infection with SPCSV.

Figure S3. Distribution of total small RNA size classes corresponding to SvT1 (white bars), SPCSV (black bars), SPFMV (dark grey bars) and other sequences (light grey bars) in a) wild-type plants singly and dually infected with StV1 and SPCSV or RNase3

transgenic plants infected with StV1, b) wild-type plants singly and dually infected with SPFMV and SPCSV, or c) non-infected wild-type plants. Vertical axis shows total number of small RNA reads, and horizontal axis indicates size class of siRNA, and the exact numbers are tabulated below the graph including the sums and grand total.

TABLES

Table 1. Sweepovirus isolates used in this study

FIGURE LEGENDS

Figure 1. Maximum likelihood phylogenetic tree based on complete genome sequences of 123 isolates. Sequences are indicated with their accession numbers and country of origin, sequences determined in this study are in bold and highlighted. Country codes: CHN China, PRI Puerto Rico, JPN Japan, KOR Republic of Korea, ESP Spain, USA United States of America, BRA Brazil, IND India, UGA Uganda, ITA Italy, Mex Mexico, Jam Jamaica, Per Peru , Cub Cuba, StV Saint Vincent and the Grenadines.

Figure 2. Sweepovirus titres and relative SPCSV titres from single and dually infected plants with different sweepovirus isolates. For each sweepovirus isolate the titres of sweepovirus, determined by signal intensity analysis of DNA dot-blots, are shown in the top graph in single (solid line) and mixed (dotted line) infection with SPCSV m2-47. The bottom graph indicates the titers of SPCSV (grey line) in dual infection with sweepovirus isolates relative to single SPCSV infection as determined by quantitative real-time PCR. Horizontal axis indicates weeks post inoculation of sweepovirus and the dotted vertical line through the graphs indicates the time point at which plants were cut back and left to re-grow. Error bars indicate range of minimum and maximum values.

Figure 3. Symptoms and increased sweepovirus titres in SPCSV co-infected and RNase3 expressing transgenic sweetpotato plants 10 weeks after inoculation. a) Phenotype of typical leaves from uninfected non-transgenic, uninfected RNase3 transgenic, and SPCSV infected non-transgenic sweetpotato cultivar Huachano respectively (top), or

StV1 infected non-transgenic, StV1 infected RNase3 transgenic, and SPCSV and StV1 dual infected non-transgenic plants respectively of the same cultivar. b) DNA dot blot of uninfected and StV1 infected non-transgenic, StV1 infected RNase3 transgenic, and SPCSV and StV1 dual infected non-transgenic plants respectively.

Figure 4. Distribution of siRNA size classes corresponding to sweepovirus (white bars), SPCSV (black bars) and SPFMV (grey bars) in single and dually infected plants. Vertical axis shows number of siRNA reads per million total reads, and horizontal axis indicates size class of siRNA. Numbers above 21 and 22 nt siRNAs in the right bottom graph indicate the number of siRNAs corresponding to SPFMV, which exceed the scale of the graph. Analysis of the total number of all small RNA reads are provided in supplementary Fig. S3.

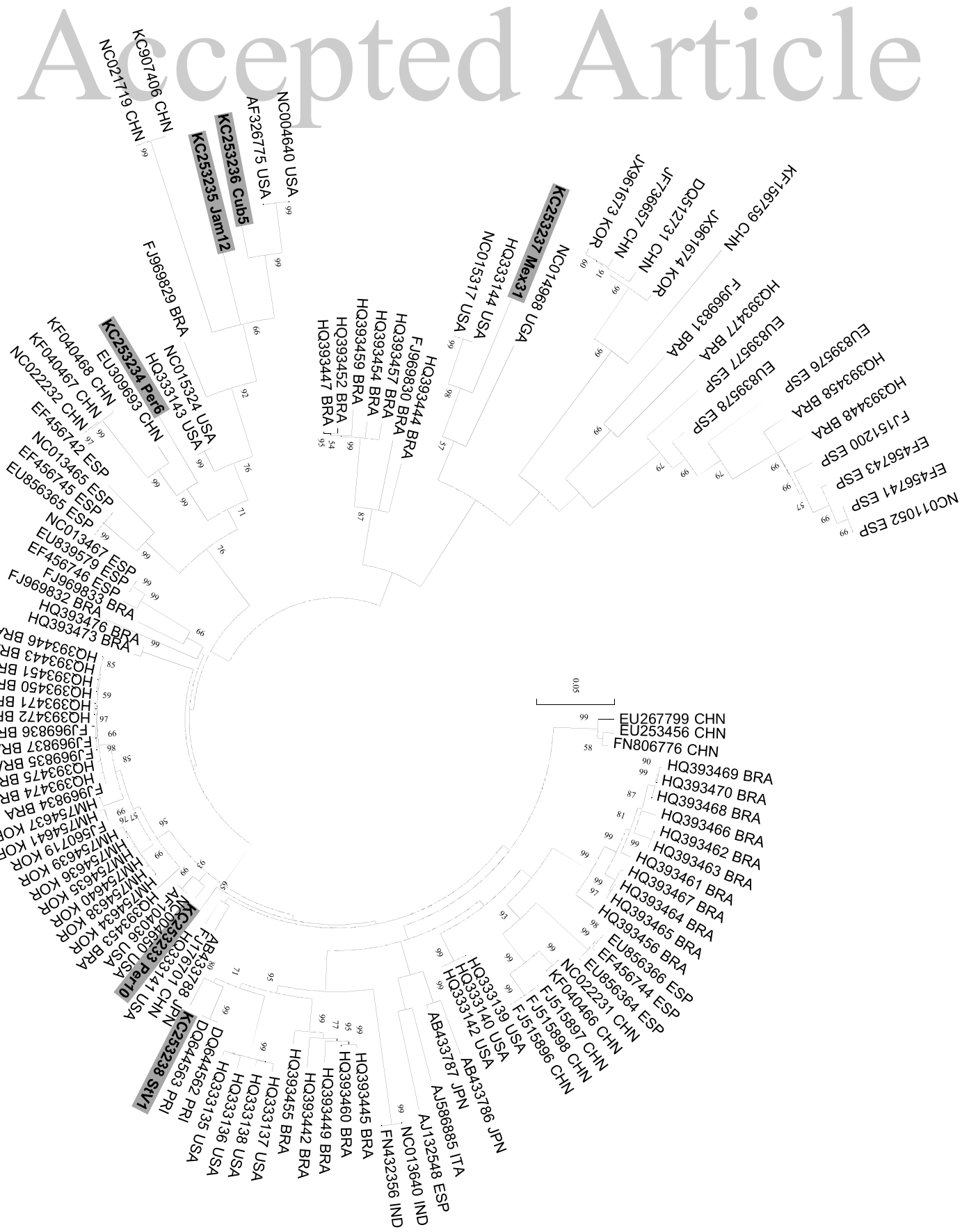
Figure 5. Coverage of viral genomes by different siRNA size classes a) coverage of sweepovirus StV1 genome in single (top graph) or dual (middle graph) infection with SPCSV or in RNase3 transgenic plants (bottom graph). b) Coverage of SPCSV genome by different siRNA size classes in single (top graph) or dual infection with StV1 (middle graph) or SPFMV (bottom graph). Vertical axis shows number of siRNA reads per million total reads, and horizontal axis indicates nucleotide position on the genome. Red green, black and blue lines indictate 21, 22, 23 and 24 nt RNAs respectively. A schematic representation of the virus genome is provided on top with named box arrows representing the locations of the respective encoded genes.

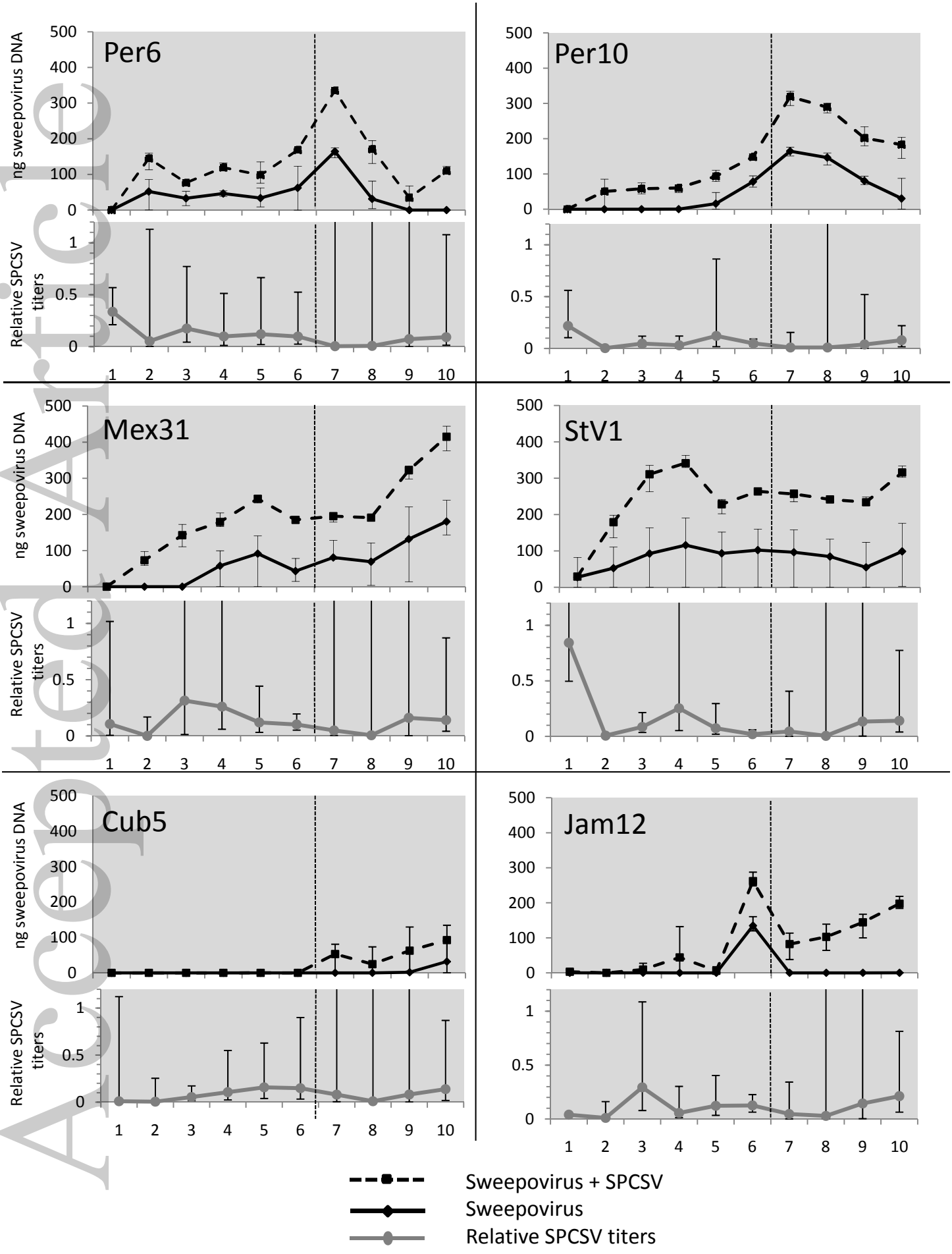
Table 1. Sweepovirus isolates used in this study

Isolate name	Symptoms in *I.setosa*[1]	Co-infected with	Country of origin	sequence accession number
Arg33	C,RU		Argentina	KC253260
Arg34	C,RU,D		Argentina	KC253261
Arg35	R,C,RD,RU,D,Vc	SPFMV	Argentina	KC253262
Arg36	C,RU,D		Argentina	KC253263
Arg37	RU		Argentina	KC253264
Arg38	R,C,Cp,RU,D,Vc,IVC	SPFMV	Argentina	KC253265
Col22	RU,Vc,Np,Ln		Colombia	KC253252
Col9	C,RU		Colombia	KC253242
Cub5*	C		Cuba	KC253236
Cub42	C,IVC		Cuba	KC288164
Dom13	C,RU,IVC		Dominican Republic	KC253244
Dom2	C,RU		Dominican Republic	KC253239
Ecu8	C,Cp,RU,Vc	SPFMV, SPVG	Ecuador	KC253241
Gtm16	C,RU		Guatamala	KC253247
Gtm17	C,RU	SPVCV	Guatamala	KC253248
Gtm19	C,RU,D,IVC		Guatamala	KC253250
Gtm20	C,RU		Guatamala	KC253251
Jam12*	C,RU		Jamaica	KC253235
Jam23	C,RU	SPVCV	Jamaica	KC253253
Jam24	C		Jamaica	KC253254
Mex31*	C,RU,D		Mexico	KC253237
Mex32	C,RU,D		Mexico	KC253259
Mex39	C,RU,D,Np	SPCV	Mexico	KC288161
Nic25	C,RU,D		Nicaragua	KC253255
Pam18	C,RU,D,IVC		Panama	KC253249
Pan14	C,RU,D		Panama	KC253245
Pan15	RD,RU,D,Ld		Panama	KC253246
Per10*	C,RU		Peru	KC253233
Per6*	C,RU		Peru	KC253234
Per7	C,RU		Peru	KC253240
Pri21	C,RU,D,IVC		Puerto Rico	KC288165
Pry11	C		Paraguay	KC253243
Pry26	Cp,RU,D,Vc	SPFMV, SPVG	Paraguay	KC253256
Pry27	C,RU		Paraguay	KC253257
Pry29	RU		Paraguay	KC253258
Pry30	C,RU		Paraguay	KC288166
Pry40	C,RU		Paraguay	KC288162
StV1*	RD,RU,D,Ld		St. Vincent and the Grenadines	KC253238
StV41	D, IVC		St. Vincent and the Grenadines	KC288163
Tza13	nt		Tanzania	KC288167
Tza16	nt	SPMMV	Tanzania	KC288169
Uga15	nt		Uganda	KC288168
Uga19	nt		Uganda	KC288170
Uga29	nt	SPFMV	Uganda	KC288171
Uga34	nt		Uganda	KC288172
Uga37	nt		Uganda	KC288173

* Genomes were fully sequenced and isolates used in synergism studies

[1] Symptoms on leaves, C: Chlorosis; CP: Chlorotic point; D: Dwarfing; IVC: interveinal chlorosis; Ld: Leaf deformation; Ln: leaf necrosis; Np; Necrotic point; R: rugosity; RD: Roll down; RU: Roll up; Vc: vein clearing.; nt: not tested





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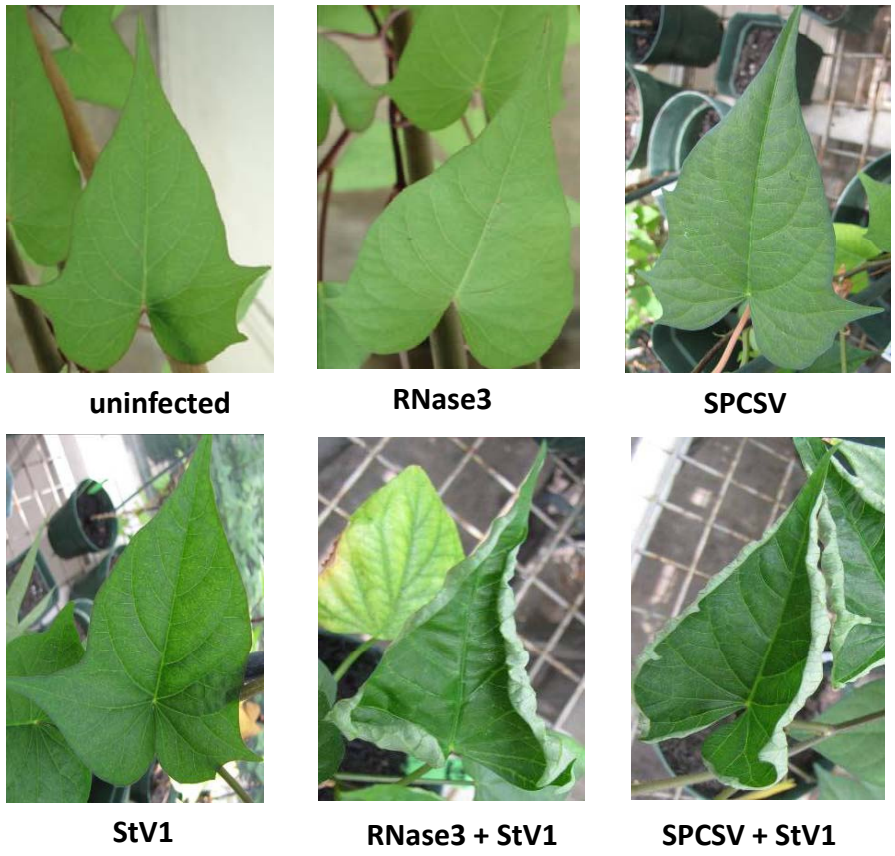

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