Distinct cavemoviruses interact synergistically with sweet potato chlorotic stunt virus (genus *Crinivirus*) in cultivated sweet potato

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Two serologically unrelated sweet potato viruses causing symptoms of vein clearing in the indicator plant *Ipomoea setosa* were isolated and their genomes have been sequenced. They are associated with symptomless infections in sweet potato but distinct vein-clearing symptoms and higher virus titres were observed when these viruses co-infected with sweet potato chlorotic stunt virus (SPCSV), a virus that is distributed worldwide and is a mediator of severe virus diseases in this crop. Molecular characterization and phylogenetic analysis revealed an overall nucleotide identity of 47.6 % and an arrangement of the movement protein and coat protein domains characteristic of members of the genus *Cavemovirus*, in the family *Caulimoviridae*. We detected both cavemoviruses in cultivated sweet potato from East Africa, Central America and the Caribbean islands, but not in samples from South America. One of the viruses characterized showed a similar genome organization as, and formed a phylogenetic sublineage with, tobacco vein clearing virus (TVCV), giving further support to the previously suggested separation of TVCV, and related viral sequences, into a new caulimovirid genus. Given their geographical distribution and previous reports of similar but yet unidentified viruses, sweet potato cavemoviruses may co-occur with SPCSV more often than previously thought and they could therefore contribute to the extensive yield losses and cultivar decline caused by mixed viral infections in sweet potato.

INTRODUCTION

Sweet potato [*Ipomoea batatas* (L.) Lam.] is one of the most important subsistence crops in developing countries and the third most important root crop after potato (*Solanum tuberosum* L.) and cassava (*Manihot esculenta* Crantz) (FAOSTAT, 2008). More than 20 viruses are known to infect sweet potato and severe viral diseases affecting this crop have been reported (Valverde *et al.*, 2007; Loebenstein *et al.*, 2009). Often these viral diseases are caused by mixed virus infections involving the crinivirus sweet potato chlorotic stunt virus (SPCSV), a member of the family *Closteroviridae*. The most common and severe of these is

called sweet potato virus disease and is caused by co-infection of SPCSV and sweet potato feathery mottle virus (SPFMV), in the genus *Potyvirus*, and the family *Potyviridae* (Gibson *et al.*, 1998a, b; Mukasa *et al.*, 2006; Untiveros *et al.*, 2007; Aritua *et al.*, 2007).

In plants, RNA silencing is involved in virus resistance and recovery from virus disease (Covey *et al.*, 1997; Ratcliff *et al.*, 1997). On the other hand, viruses express a wide range of proteins that interfere with different steps of RNA silencing (Li & Ding, 2006). In mixed viral infections, the presence of these viral proteins might help to overcome RNA silencing, generating a synergism that allows at least one of the co-infecting viruses to accumulate at higher titres than observed in single virus infections (Pruss *et al.*, 1997; Anandalakshmi *et al.*, 1998). This has been shown for P1/HC-Pro of the potyviral genomes (Shi *et al.*, 1997), and we have recently reported that this is the case also with the RNase3

protein of SPCSV, which has RNA silencing suppression activity and is sufficient to mediate several synergistic interactions between SPCSV and unrelated RNA viruses (Cuellar et al., 2009). Synergistic interactions of SPCSV with DNA viruses have, however, not yet been reported.

Members of the family Caulimoviridae, or plant pararetroviruses, have a circular dsDNA genome ranging between 7.7 and 8.1 kbp encoding between four and six ORFs and they replicate by reverse transcription (Fauquet et al., 2005). Based on sequence conservation and genome organization, members of the family Caulimoviridae are divided into six genera: the bacilliform Badnavirus and Tungrovirus and the isometric Caulimovirus, Soymovirus, Petuvirus and Cavemovirus. Cauliflower mosaic virus (CaMV; Franck et al., 1980) is the type species of the family Caulimoviridae (Fauquet et al., 2005; Bousalem et al., 2008). CaMV transcribes its genome into two major transcripts: (i) the 35S RNA which serves as a template for the reverse transcription step of viral DNA replication, and (ii) the 19S RNA, which encodes P6 (62 kDa) a multifunctional protein with reported roles in virulence, host specificity, translational transactivation and RNA-silencing suppression (Haas et al., 2002; Kobayashi & Hohn, 2003; Love et al., 2007).

To date only a single caulimovirid, sweet potato caulimo-like virus (SPCV) has been reported (Atkey & Brunt, 1987; De Souza & Cuellar, 2010), but despite its widespread occurrence (Atkey & Brunt, 1987; Wambugu, 1991; Aritua et al., 2007), SPCV is not officially recognized by the International Committee on Taxonomy of Viruses (Fauquet et al., 2005). In addition to the complete sequence of SPCV, we report here the isolation and complete genome characterization of a second caulimovirid showing similar but not identical characteristics to SPCV. We designate it as sweet potato vein clearing virus (SPVCV) because of the symptoms associated with it in Ipomoea setosa. Both viruses group together with cassava vein mosaic virus (CsVMV) and tobacco vein clearing virus (TVCV), which are the only members of the genus Cavemovirus. They occur in sweet potato from East Africa and Central America, but not in South America. Interestingly, isolates of SPCV form geographically distinct subgroups, something that is not observed for SPVCV isolates. Comparative sequence and genome organization analyses of CsVMV, TVCV, SPCV and SPVCV suggest that genus Cavemovirus may be further divided into two distinct subgroups. Most interestingly, we show that both these cavemoviruses can be synergized by the crinivirus SPCSV in sweet potato. This is important given that SPCSV is distributed worldwide and can mediate severe diseases in sweet potato. This is also the first report of an RNA virus mediating a synergistic interaction on DNA viruses, indicating SPCSV affects a basal antiviral defence mechanism in plants.

RESULTS

In single infection, SPVCV and SPCV are associated with vein-clearing symptoms in the indicator plant *I. setosa* but are symptomless in sweet potato

We were unable to transmit SPCV or SPVCV using the insect vector *Myzus persicae* or by mechanical inoculation of indicator plants. No symptoms could be observed in any of the inoculated plants during the 8 weeks that the assays lasted. On the other hand, both viruses were readily transmitted by grafting to sweet potato and *I. setosa* plants with 100 % efficiency. *I. setosa* infected with SPCV or SPVCV displayed vein-clearing symptoms around 4–6 weeks after graft infection (Fig. 1a). In most cases, symptoms continued to develop into necrosis of veins and eventually necrosis of the whole leaf. SPCV could be detected by ELISA on nitrocellulose membranes (NCM-ELISA) from the indicator plant *I. setosa* 3–5 weeks after graft infection along with the development of symptoms (Fig. 1b). Virus titres increased over time and older leaves showed higher titres of the virus compared with young top leaves (data not shown). We observed variability in the levels of detection of SPCV among graft-infected plants by NCM-ELISA, while PCR tests (see below) showed that all grafted plants were infected even before the symptoms were apparent. Detection of SPVCV by PCR showed that all grafted plants displaying vein-clearing symptoms were positive for PCR (data not shown). Both viruses caused symptomless infections when they were graft transmitted to sweet potato (cultivar 'Huachano'), but were readily detected by PCR around 4–5 weeks after graft infection; SPCV levels were undetectable by NCM-ELISA in single infection.

Severe symptoms and higher titres of SPVCV and SPCV are detected in sweet potato upon co-infection with SPCSV

Although SPCV has been detected in different parts of the world there are no reports of its co-occurrence with the worldwide-distributed crinivirus SPCSV which has been shown to synergize several unrelated viruses in sweet potato (Untiveros et al., 2007). To study a possible interaction between cavemoviruses and SPCSV, we carried out co-infection studies in sweet potato cultivar 'Huachano' and SPCSV isolate m2-47 which has been previously shown to synergistically interact with heterologous RNA viruses in sweet potato (Untiveros et al., 2007). As originally observed for SPCV (Atkey & Brunt, 1987), no symptoms could be observed, and serological detection was impossible in sweet potato plants infected with either cavemovirus under our conditions (Fig. 1b). In double infection with SPCSV, however, detection of SPCV or SPVCV in sweet potato was possible and correlated with the appearance of vein-clearing symptoms. On the other hand, triple antibody sandwich ELISA (TAS-ELISA) tests
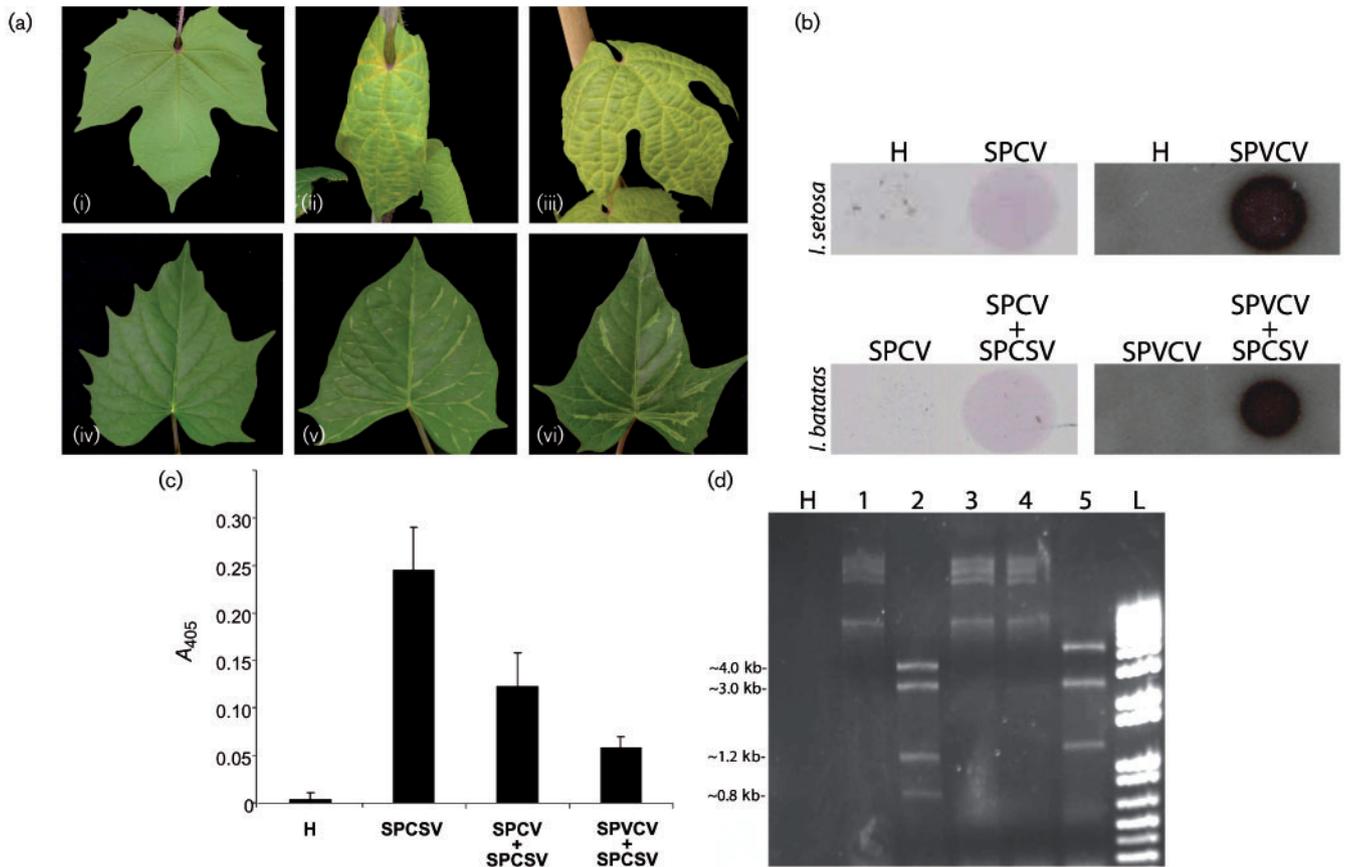
Fig. 1. (a) In *I. setosa* both cavemoviruses cause vein-clearing symptoms in single infection (ii and iii; i: healthy plant). Clear vein-clearing symptoms are also observed in sweet potato (*I. batatas*) but only in co-infection with the crinivirus SPCSV (v and vi, respectively; iv: single infection with SPVCV). (b) In *I. setosa* (top) cavemoviruses are detected in single infection by NCM-ELISA (SPCV, left) or dot-blot hybridization (SPVCV, right). However, both cavemoviruses are below the level of detection in single-infected *I. batatas* (bottom) but are readily detected after co-inoculation with SPCSV. H, non-infected plants. (c) TAS-ELISA detection of SPCSV levels in single- and double-infected sweet potato plants. (d) RFLP analysis of SPVCV viral DNA isolated from *I. setosa*. Different restriction enzymes (lane 1, *Pst*I; lane 2, *Hin*dIII; lane 3, *Bst*ZI; lane 4, *Not*I; lane 5, *Eco*RI) were used. The approximate size of *Hin*dIII restriction fragments is indicated on the left. L, 1 kbp Plus DNA ladder.

revealed that SPCSV titres did not increase, but were lower in double-infected plants compared with the levels in single infection (Fig. 1c). In co-infected plants symptoms became severe and resembled those observed in *I. setosa* when single-infected by SPCV or SPVCV (Fig. 1a); vein clearing first appeared along the main vein and later spread through secondary veins; sometimes purpling of the veins could be observed in SPCV-infected plants (data not shown). These results indicate that SPVCV and SPCV are synergized by SPCSV in sweet potato plants in a similar manner as has been described for RNA viruses (Untiveros *et al.*, 2007).

SPVCV and SPCV are distinct members of the genus *Cavemovirus* in the family *Caulimoviridae*

A band of approximately 9 kbp corresponding to the linearized genomic DNA and additional slower and faster migrating bands were obtained from preparations of

SPVCV when visualized in agarose gels after electrophoresis (data not shown). Similar profiles have been observed for SPCV (De Souza & Cuellar, 2010) and other caulimovirid preparations (Donson & Hull, 1983; Covey *et al.*, 1998). The amount of DNA obtained by this method was ~2 μg per 400 mg fresh *I. setosa* leaf tissue. This amount was enough for digestion with different restriction enzymes and molecular cloning of the fragments for sequencing. The restriction profile for SPVCV with *Hin*dIII was distinct from the profile described for SPCV (De Souza & Cuellar, 2010; Fig. 1d). Assembly of all obtained sequences revealed an 8837 bp circular genome for SPVCV and 7723 bp for SPCV (Fig. 2). These sizes are comparable to other known genomes in the family *Caulimoviridae* which are in the range of 7.2–8.1 kbp (Fauquet *et al.*, 2005). Overall nucleotide sequence identity between both viruses was 47.6 % at nucleotide level and the highest overall similarity with other genomes in the family

Caulimoviridae was 63.1 % between SPCV and CsVMV (GenBank accession no. NC001648) (de Kochko *et al.*, 1998), and 45.8 % for SPVCV and TVCV (GenBank accession no. NC003378) (Lockhart *et al.*, 2000). Pairwise sequence comparison (PASC) (Bao *et al.*, 2008) produced similar results (data not shown). Comparative sequence analysis detected several functional domains shared by all members of the family *Caulimoviridae* (Fig. 3). Phylogenetic analysis of complete nucleotide sequence alignments of representative members of the family *Caulimoviridae* (Table 1) placed SPCV and SPVCV in a strongly supported phylogenetic lineage within the genus *Cavemovirus*, and further into two sublineages comprising SPCV and CsVMV or TVCV and SPVCV (Fig. 4). Phylogenetic analysis using the replicase sequence produced similar trees (data not shown) as previously reported for members of the family *Caulimoviridae* (Bousalem *et al.*, 2008; Geering *et al.*, 2010).

The A + T composition of SPVCV and SPCV was 69.9 and 74.3 %, respectively, which corresponds to the range found for the genus *Cavemovirus* and is considerably higher than those found in other members of the family *Caulimoviridae* (60–64.6 %; Fauquet *et al.*, 2005). A region complementary to the 3′-terminal end of the host tRNAmet is recognized by the viral reverse transcriptase as the transcription initiation site and is characteristic of pararetroviral genomes (Fauquet *et al.*, 2005; Fig. 2). The first nucleotide in this region has been defined as the first base of the genomic sequence (Fauquet *et al.*, 2005) and corresponds to the sequence 5′-**TGGTATCAGAGC**ATA**GTT**-3′ and 5′-**TGGTATCAG-AGC**TAGTCC-3′ (letters in bold are complementary to the sequence at the 3′ end of tRNA met) for SPCV and SPVCV,

respectively (Fig. 2). RNAfold predicted a region in both SPVCV and SPCV with potential to form a large stem–loop structure, characteristic of the pre-genomic RNA 5′ leader sequences found in caulimovirid members (Fütterer *et al.*, 1988; Fig. 2). The interaction of the leader region with the zinc finger domain of the coat protein (CP) might have a role in CaMV infectivity and packaging of the virion (Guerra-Peraza *et al.*, 2000). In addition, a putative TATA box and AS1 elements upstream of this leader sequence suggest the presence of a promoter region at a similar position as the 35S promoter of CaMV (Fig. 3).

In SPCV and SPVCV, as in all cavemoviruses described so far, the domains corresponding to CP and movement protein (MP) are in inverted order with respect to homologous domains in members of the family *Caulimoviridae* (Fauquet *et al.*, 2005; Fig. 2). SPCV encodes both these domains as a fusion protein (MP/CP) of a predicted 1264 aa (CP: 785 aa, MP: 433 aa) similar to CsVMV. In contrast SPVCV, like its closest relative TVCV, encodes both domains in separate ORFs of 471 aa (CP) and 389 aa (MP), respectively (Lockhart *et al.*, 2000; Fig. 2). In addition, both viruses have predicted ORFs encoding an aspartic protease/reverse transcriptase, RNase H polyprotein (replicase) and a putative inclusion body protein (IBP) found at the same genomic position and showing marginal sequence similarity to the P6 protein of CaMV (Kobayashi & Hohn, 2003; Love *et al.*, 2007; Martinière *et al.*, 2009). Additional short ORFs were found in the same genomic region in SPVCV and TVCV (Fig. 2). These were designated ORFs a–d and they shared an amino acid similarity (identity) of 34.8 % (17.4 %) for ORF a, 27.4 %
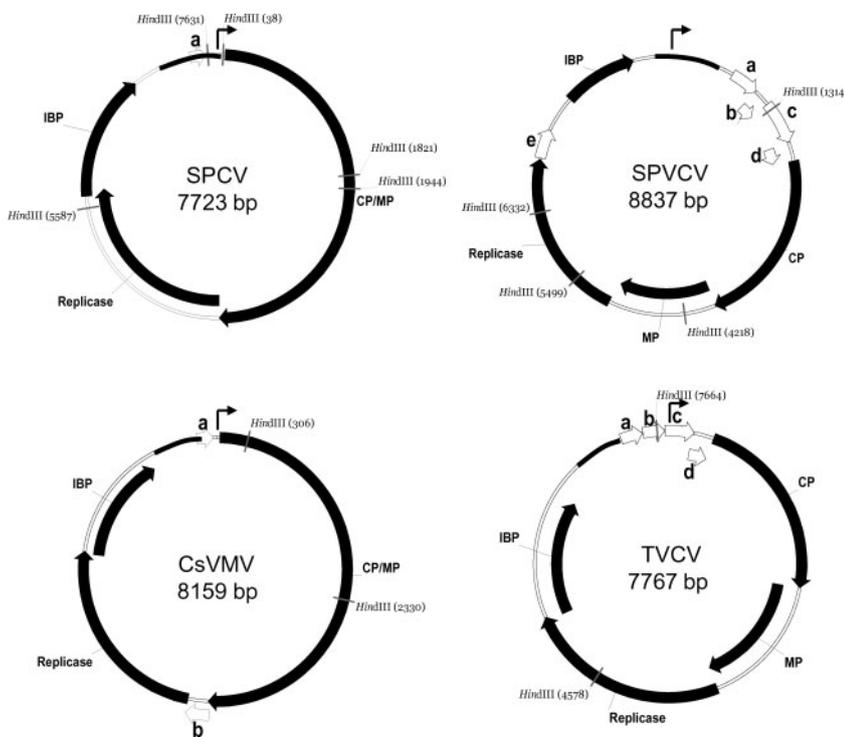
Fig. 2. Genome organization of cavemoviruses. Line arrows indicate the position of the first nucleotide of the genome (tRNAmet sequence). Black block arrows indicate ORFs found in all caulimoviruses while white block arrows (a–e) indicate ORFs for which no function could be assigned on the basis of sequence similarity. The black solid line indicates the relative position of the 5′ leader sequence (hairpin) in each genome. *Hind*III restriction sites are indicated for comparison. Virus isolates are as indicated in Table 1. CP, Coat protein; IBP, inclusion body protein; MP, movement protein.

TATA box

			Zinc finger domain	
CaMV:	TCCTC**TATATAA**GGAAG	(nts 7400-7416)	DC**R**C**W**I**C**NIEG**H**YANE**C**P	(ORF4 aa 411-428)
FMV:	CCCTC**TATATAA**GAAGG	(nts 6891-7907)	KC**R**C**W**I**C**TEEG**H**YANE**C**P	(ORF4 aa 558-575)
CERV:	CTGGC**TATATAA**AGGGA	(nts 7046-7062)	TC**R**C**W**V**C**NIEG**H**YANE**C**P	(ORF4 aa 417-434)
MMV:	TTCCT**TATATAA**GGAAC	(nts 7043-7059)	KC**R**C**W**I**C**SEEG**H**YANE**C**P	(ORF4 aa 427-444)
SbCMV:	TACTT**TATTAAA**AGTGG	(nts 6042-6058)	KC**Q**C**W**L**C**HEEG**H**YANE**C**P	(ORF4 aa 380-397)
PCSV:	TTGCC**TATAAAT**AAGTT	(nts 6045-6061)	TC**R**C**W**I**C**QEEG**H**YANE**C**P	(ORF4 aa 389-406)
CsVMV:	TTTCC**TATATAA**GGAAC	(nts 7566-7582)	AC**K**C**Y**N**C**GEEG**H**ISPN**C**K	(ORF1 aa 738-755)
TVCV:	CATAC**TATATAT**CCTAA	(nts 7364-7380)	EC**T**C**Y**N**C**GLG**H**IAKD**C**K	(ORF1 aa 505-522)
SPCV:	TCGAA**TATATAA**AAGTT	(nts 7169-7213)	PC**T**C**Y**N**C**GEIG**H**ISPN**C**K	(ORF1 aa 732-749)
SPVCV:	TCGCC**TATATAA**ACCGA	(nts 8733-8749)	DC**K**C**Y**N**C**GLG**H**YANE**C**R	(ORF1 aa 593-610)
PVCV:	TTGCC**TATATAA**GGTAA	(nts 6873-6889)	PR**S**C**F**T**C**GKIG**H**FSRN**C**P	(ORF3 aa 1109-1126)
RTBV:	TCCAG**TATATAA**GGAGC	(nts 7368-7384)	KC**R**C**Y**I**C**QDEN**H**LANR**C**P	(ORF3 aa 771-788)
ComYMV:	TTTCC**TTATTTAA**GCAC	(nts 7317-7333)	KC**K**C**Y**I**C**GQEG**H**YANQ**C**R	(ORF3 aa 878-895)
BSV:	AGTTC**TTATAAA**TGGTC	(nts 139-156)	DC**K**C**F**A**C**GDVG**H**FASE**C**P	(ORF3 aa 764-781)
CSSV:	CCATC**TATAAAT**GAGAG	(nts 6957-6973)	KC**K**C**Y**L**C**DEG**H**FARE**C**P	(ORF3 aa 785-802)
KTSV:	CTCCT**TATATAA**GGGGA	(nts 7371-7387)	EC**K**C**Y**A**C**GELG**H**FASD**C**N	(ORF3 aa 763-780)

Reverse transcriptase

			RNaseH domain	
CaMV:	FCCV**Y**V**DDI**LVFSN	(ORF5 aa 397-410)	ICRYA**SG**S**F**KAAEK	(ORF5 aa 575-588)
FMV:	FCMV**Y**V**DDI**IVFSN	(ORF5 aa 390-403)	ICRYS**SG**S**F**KQAEK	(ORF5 aa 565-578)
CERV:	YCCV**Y**V**DDI**LVFSN	(ORF5 aa 380-393)	ICRYA**SG**S**F**KAAER	(ORF5 aa 555-568)
MMV:	FSLV**Y**V**DDI**IVFSD	(ORF5 aa 390-403)	ICRYS**SG**T**F**KPAEL	(ORF5 aa 565-578)
SbCMV:	ICLA**Y**I**DDI**LIFTK	(ORF5 aa 355-368)	LCKYV**SG**T**F**TDTET	(ORF5 aa 587-600)
PCSV:	ISLA**Y**I**DDI**IVFTK	(ORF5 aa 339-352)	ITKWS**SG**T**F**KPAEE	(ORF5 aa 590-603)
CsVMV:	FIIV**Y**I**DDI**LVFSK	(ORF3 aa 358-371)	LCRYN**SG**T**F**KENEQ	(ORF3 aa 539-552)
TVCV:	NCIV**Y**I**DDI**LLYSR	(ORF3 aa 355-368)	HCRYY**SG**SYTEPQE	(ORF3 aa 533-546)
SPCV:	FILV**Y**I**DDI**LIFSK	(ORF2 aa 354-367)	ICRYN**SG**T**F**KEHEK	(ORF2 aa 533-546)
SPVCV:	NCIV**Y**I**DDI**LLYSK	(ORF3 aa 340-353)	HCRYY**SG**T**F**KDNEK	(ORF3 aa 519-531)
PVCV:	SALV**Y**I**DDI**LLFSE	(ORF1 aa 1536-1549)	ICGFA**SG**K**F**KVSEQ	(ORF1 aa 1708-1721)
RTBV:	FALL**Y**I**DDI**LIASN	(ORF3 aa 1335-1348)	IAGYA**SG**N**F**GEKKT	(ORF3 aa 1515-1528)
ComYMV:	FIAV**Y**I**DDI**LVFSE	(ORF3 aa 1560-1672)	ICAYA**SG**S**F**NPIKS	(ORF3 aa 1740-1753)
BSV:	YIAV**Y**I**DDI**LIFSD	(ORF3 aa 1456-1469)	VCAYA**SG**RYHPIKS	(ORF3 aa 1636-1649)
CSSV:	FIAV**Y**I**DDI**LVFSE	(ORF3 aa 1439-1452)	ICAYA**SG**K**F**GVVKS	(ORF3 aa 1619-1632)
KTSV:	FIAV**Y**V**DDI**LIFSN	(ORF3 aa 1487-1500)	VCAYA**SG**K**F**NPIKS	(ORF3 aa 1665-1678)

Fig. 3. Sequence motifs conserved among members of the family *Caulimoviridae* (SPCV and SPVCV are in bold). Virus sequences are as indicated in Table 1. The numbers on the right of each column indicate the genomic position where each motif is found.

Table 1. Caulimovirid genome sequences used in this work

Name	Abbreviation	GenBank accession no.	Reference
Blueberry red ringspot virus	BRRV	NC003138	Glasheen et al. (2002)
Banana streak virus	BSV	NC008018	Zhuang & Liu, unpublished
Cauliflower mosaic virus	CaMV	NC001497	Franck et al. (1980)
Carnation etched ring virus	CERV	NC003498	Palkovics & Balazs (1996)
Cestrum yellow leaf curling virus	CmYLCV	NC004324	Stavolone et al. (2003)
Commelina yellow mottle virus	ComYMV	NC001343	Medberry et al. (1990)
Cacao swollen shoot virus	CSSV	NC001574	Hagen et al. (1993)
Cassava vein mosaic virus	CsVMV	NC001648	de Kochko et al. (1998)
Figwort mosaic virus	FMV	NC003554	Richins et al. (1987)
Kalanchoe top-spotting virus	KTSV	NC004540	Yang et al., unpublished
Mirabilis mosaic virus	MMV	NC004036	Dey & Maiti (1999)
Peanut chlorotic streak virus	PCSV	NC001634	Richins, unpublished
Petunia vein clearing virus	PVCV	NC001839	Richert-Pöggeler & Shepherd (1997)
Rice tungro bacilliform virus	RTBV	NC001914	Hay et al. (1991)
Soybean chlorotic mottle virus	SbCMV	NC001739	Hasegawa et al. (1989)
Sweet potato badnavirus B	SPBV-B	NC012728	Kreuze et al. (2009)
Sweet potato caulimo-like virus	SPCV	HQ694978	This study
Sweet potato vein clearing virus	SPVCV	HQ694979	This study
Tobacco vein clearing virus	TVCV	NC003378	Lockhart et al. (2000)
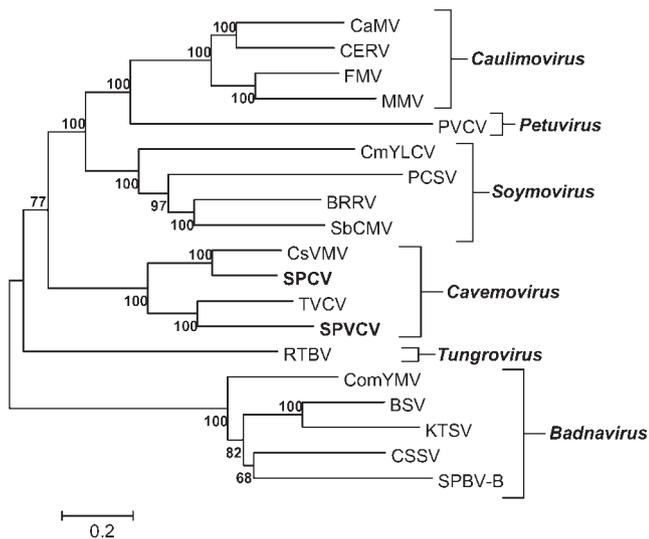
Fig. 4. Phylogenetic reconstruction of the family *Caulimoviridae* based on complete genome sequences. The tree was constructed by neighbour-joining with a bootstrap analysis of 1000 replicates. The genus *Cavemovirus* is split into two branches (SPCV + CsVMV and SPVCV + TVCV) with a high bootstrap value (100 %). The genomes used in this analysis are listed in Table 1. Bar indicates nucleotide substitutions per site (Kimura two-parameter).

(11.3 %) for ORF b, 24.1 % (16.1 %) for ORF c and 20 % (12.3 %) for ORF d. An additional short ORF (e) was found in SPVCV but not in TVCV (Fig. 2). Phylogenetic analyses consistently showed that genes (CP, MP and replicase) of SPCV were more similar to and clustered with those of CsVMV, while those of SPVCV did so with the corresponding encoded proteins from TVCV, similar to results obtained using complete nucleotide sequences (Fig. 4).

Geographical distribution and variability of SPVCV and SPCV

Sweet potato samples originating from different parts of the Americas (190 samples) and collected in Tanzania, Kenya and Uganda (34 samples) were screened for SPVCV and SPCV by PCR using primers targeting the reverse transcriptase domain for SPVCV and SPCV. Only one sample out of 32 from North America was positive (for SPCV) and none of the 93 samples originating from South America were positive by PCR for either virus. In contrast, 13 out of 65 samples (20 %) from Central America were positive for either SPCV or SPVCV: four samples were positive for SPCV (6.1 %) and nine for SPVCV (13.8 %) (Table 2). Sweet potatoes from Guatemala, collected from Northern (Petén) and Southern Districts (Santa Rosa), showed the highest frequency of infection (>40 %) suggesting common occurrence of SPCV and SPVCV in this country. All sweet potato plants that were PCR

positive for SPCV or SPVCV showed symptoms of vein clearing after graft transmission to the indicator plant *I. setosa*. At least two samples from Uganda, one from Kenya and one from Tanzania were infected with SPCV (11.7 %) and one sample each from Uganda and Kenya were infected by SPVCV (5.8 %). Only freeze-dried material was available from African samples, therefore grafting experiments on the indicator plant *I. setosa* could not been carried out with samples from Africa. Phylogenetic analyses of nucleotide sequences corresponding to the reverse transcriptase region for both viruses showed geographical grouping of isolates. SPCV sequences from Africa clustered separately from isolates from the Americas, whereas SPVCV sequences from Africa did not form a separate cluster and grouped together with sequences from Central America (Fig. 5).

DISCUSSION

In sweet potato, several viral synergistic interactions are driven by the crinivirus SPCSV, which can enhance the accumulation of several unrelated RNA viruses (Karyeija, et al., 2000; Di Feo et al., 2000; Mukasa et al., 2006; Untiveros et al., 2007). We now show that this phenomenon extends itself to DNA viruses as well, as these normally symptomless viruses generated vein-clearing symptoms and increased titres in sweet potato following co-inoculation with SPCSV (Fig. 1a). In both cases SPCSV titres decreased (Fig. 1c) similar to what has been previously observed in other synergistic interactions of SPCSV (Mukasa et al., 2006; Cuellar et al., 2008). This is the first report on a synergistic interaction between SPCSV and DNA viruses. We previously showed that the RNase3 protein of SPCSV is sufficient to reproduce its synergistic effect on the accumulation of heterologous RNA viruses (Cuellar et al., 2009). Our experiments here suggest that RNase3 probably also compromises resistance to DNA viruses and that a step in RNA silencing that is common between RNA and DNA viruses is affected by SPCSV.

Genome organization is generally well conserved in the order of domains within a virus genus (van Regenmortel et al., 1997) and differences in sequence conservation and genome organization are used as criteria for genus demarcation among members of the family *Caulimoviridae* (Fauquet et al., 2005). Here we described two distinct caulimovirids found infecting sweet potato and producing similar symptoms in the indicator plant *I. setosa* (Fig. 1a). Both viruses encode genes characteristic of members of the family *Caulimoviridae* (Hohn & Fütterer, 1997; Fauquet et al., 2005) (Fig. 2). Furthermore, full genome phylogenetic analysis (Fig. 4) and additional characteristics such as a high A + T composition and the inverted order of the CP-MP domains (de Kochko et al., 1998; Calvert et al., 1995; Lockhart et al., 2000), place both viruses firmly in the genus *Cavemovirus*. SPVCV and SPCV are distinct from other caulimovirid sequences recently reported in sweet potato (Kreuze et al., 2009).

Table 2. Sweet potato cavemovirus isolates and sequences used in this work

Isolate	Origin	GenBank accession no.	Reference
SPCV Mad1	Madeira	HQ005308	De Souza & Cuellar (2010)
SPCV Cub44	Cuba	HQ698912	This study
SPCV Pan128	Panama, Panama	HQ698913	This study
SPCV Gua138	Guatemala, Petén	HQ698914	This study
SPCV Gua154	Guatemala, Santa Rosa	HQ698915	This study
SPCV Mex183	Mexico	HQ698916	This study
SPCV Uga20	Uganda, Palisa	HQ698917	This study
SPCV Uga39	Uganda, Apac	HQ698918	This study
SPCV Ken11	Kenya, Siaya	HQ698919	This study
SPCV Tan10	Tanzania, Ilala	HQ698920	This study
SPVCV Dom1	Dominican Republic	HQ694979	This study
SPVCV Jam9	Jamaica, St Catherine Parish	HQ701675	This study
SPVCV Dom16	Dominican Rep., Azúa	HQ701676	This study
SPVCV Gua35	Guatemala, Petén	HQ701677	This study
SPVCV Dom47	Dominican Republic	HQ701678	This study
SPVCV Pan128	Panama, Panama	HQ701679	This study
SPVCV Gua129	Guatemala, Petén	HQ701680	This study
SPVCV Gua140	Guatemala, Petén	HQ701681	This study
SPVCV Gua154	Guatemala, Santa Rosa	HQ701682	This study
SPVCV Gua177	Guatemala, Petén	HQ701683	This study
SPVCV Uga22	Uganda, Hoima	HQ701684	This study
SPVCV Ken11	Kenya, Siaya	HQ701685	This study

Apart from ORFs encoding proteins involved in core functions (replicase, MP and CP) SPVCV and SPCV encode a predicted IBP located in a similar genome region and with marginal amino acid sequence similarity (30 and 50 %, respectively) to the P6 transactivator protein of CaMV (Fig. 2). P6 of CaMV is independently expressed via the subgenomic 19S RNA, accumulates at high levels and is the main component of the characteristic subcellular inclusion bodies formed by CaMV and other caulimovirids (Fauquet *et al.*, 2005; Haas *et al.*, 2005; Martinière *et al.*, 2009). Two major types of subcellular inclusion bodies associated with CaMV have been described in the cytoplasm of infected cells: electron-dense inclusion bodies (EDIBs) and electron-lucent inclusion bodies (ELIBs). The former, also referred to as 'virus factories', are where viral proteins are synthesized and most of viral DNA and P6 accumulate, while viral proteins involved in vector transmission accumulate in ELIBs (Haas *et al.*, 2002; Martinière *et al.*, 2009). EDIBs are resistant to phenol treatment, thus protecting the packaged viral DNA, a characteristic used to isolate and identify caulimovirid DNA (Donson & Hull, 1983; Covey *et al.*, 1998). EDIBs have been observed in tissue infected by SPCV (Atkey & Brunt, 1987) and the protocol for viral DNA extraction that we used depends on the formation of EDIBs, thus our results would suggest that the IBP from sweet potato cavemoviruses accumulate similar to the P6 of CaMV. CaMV P6 has also been identified as a pathogenicity determinant (Stratford & Covey, 1989) and later as an RNA silencing suppressor protein (Love *et al.*, 2007). Additional studies are needed to find out if the IBPs of sweet potato cavemoviruses share some of these functions. Additional ORFs are predicted in the genomes of several caulimovirids, including SPVCV and SPCV, with no function assigned to them yet (Fauquet *et al.*, 2005). In SPVCV, four of these small ORFs are found directly upstream of the CP, and downstream of the predicted stem–loop of the 5′ leader sequence (Fig. 2). Interestingly, we were also able to identify such ORFs at a similar position in TVCV. Although they showed little sequence similarity, this genomic organization is another characteristic in common between SPVCV and TVCV (Fig. 2).

Pararetroviruses can move horizontally by insect vectors and members of the genus *Caulimovirus* encode an insect transmission factor (ITF) found in ORF II (Haas *et al.*, 2002). Interestingly, an insect vector has not been identified for any member of the genus *Cavemovirus* (Hohn *et al.*, 2008) including SPCV and SPVCV (Atkey & Brunt, 1987), a finding confirmed by our work reported here, and none of them seems to encode an ITF. In addition, except for successful transmission of an infectious clone of CsVMV using biolistics (de Kochko *et al.*, 1998), mechanical transmission of cavemoviruses has been attempted to several indicator plants with no success (Wambugu, 1991; Lockhart *et al.*, 2000), and this is also consistent with our work reported here. Sweet potato is a vegetatively propagated crop and therefore spread of SPVCV and SPCV is achieved by vertical transmission, although the widespread occurrence of these viruses in Central America but not in South America may be related to the presence of an as-yet-unidentified vector in this region.
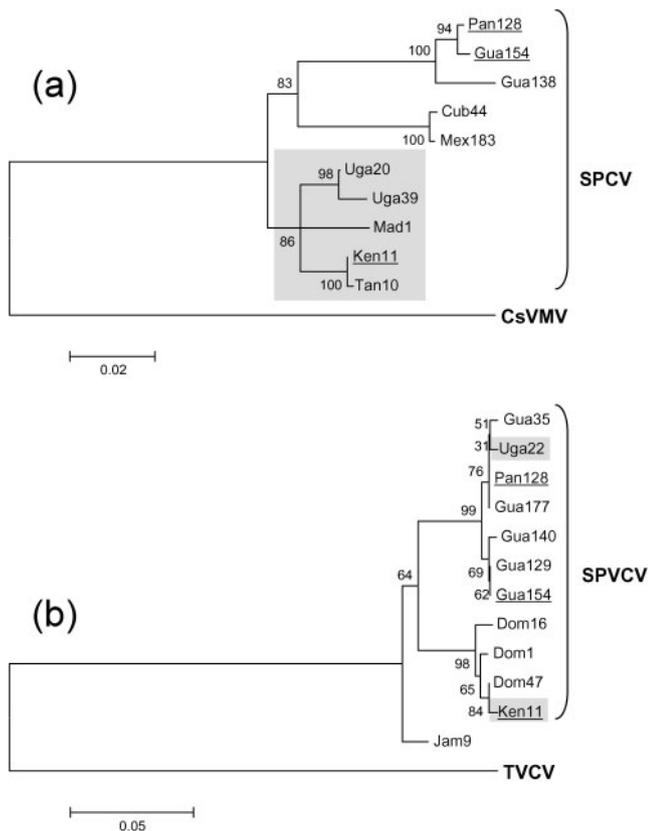
Fig. 5. Neighbour-joining subtrees (1000 bootstrap replicates) of cavemovirus isolates of SPCV (a) and SPVCV (b). A region of the replicase ORF has been used in each case (see Methods) and isolate names are explained in Table 2. Underlined names indicate samples co-infected by isolates of SPCV and SPVCV. The grey box in (a) indicates the separate branch formed by African SPCV isolates. In (b) the African SPVCV isolates do not separate from isolates found in samples from Central America. Bars indicate nucleotide substitutions per site.

Endogenous pararetrovirus sequences (EPRVs) with sequence similarity to members of the family *Caulimoviridae* have been detected integrated in the genome of several plant species. They present deleterious mutations and rearrangements in comparison with analogous active viral sequences (Staginnus & Richert-Pöggeler, 2006; Hohn *et al.*, 2008). Therefore genomic integrity and sequence conservation of functional domains, as shown for SPVCV and SPCV, are important characteristics that help distinguishing EPRVs from active pararetroviruses. PCR tests (De Souza & Cuellar, 2010) and dot-blot hybridizations using a probe targeting a region of the conserved replicase gene suggest that SPVCV-like sequences are not present as integrated sequences in the genome of *I. setosa* or *I. batatas* (Fig. 1b). In addition, analysis of deep sequencing data from different hosts indicates that SPVCV-like (or SPCV-like) sequences are not detected in sweet potato (*I. batatas*) or *I. setosa* (data not shown) in contrast with the ubiquitous presence of TVCV-like sequences in various solanaceous plants (Lockhart *et al.*, 2000; Hohn *et al.*, 2008;

unpublished data). Nevertheless, given that integrated TVCV-like sequences can be detected in some but not all solanaceous species (Lockhart *et al.*, 2000; Hohn *et al.*, 2008), we do not rule out the possibility that integrated SPVCV or SPCV sequences may be present in some *Ipomoea* species.

Reassembling and sequence comparisons of EPRVs found in solanaceous plants show they have a similar genome organization to TVCV and probably have a common phylogenetic origin (Geering *et al.*, 2010). According to this, Geering *et al.* (2010) suggested that TVCV and related EPRVs be classified outside the genus *Cavemovirus* as a distinct genus for which they proposed the name 'Solendovirus' (for *Solanaceae* endogenous virus). Our results support this division as SPVCV forms a phylogenetic sublineage with TVCV (Fig. 4) and has a similar genome organization (Fig. 2).

The PCR protocols described in this and in a previous work (De Souza & Cuellar, 2010) have been very useful for the detection of cavemoviruses in a relatively short time and without the necessity of virus indexing in indicator plants. Our results confirm the presence of SPCV in East Africa (Wambugu, 1991; Aritua *et al.*, 2007) and show for the first time the presence of SPCV in Tanzania and its widespread occurrence in Central America. Although in Guatemala SPCV seems to be quite common there are no previous reports of the virus in this country probably due to a lack of studies on the matter. Our results also indicate that SPVCV is distributed across a similar geographical range as SPCV, including Central America and Africa (Table 2). The higher incidence and variability of SPVCV isolates in Central America as compared with Africa could suggest the virus has recently been introduced to East Africa; accordingly the two isolates found in Kenya (Ken11) and Uganda (Uga22) did not differ sequence-wise from isolates of Central America (Fig. 5b). The formation of a separate cluster of African SPCV isolates (86 % bootstrap value) (Fig. 5a) could suggest that this virus may have been present in this region for a longer time than SPVCV. It is noteworthy that none of the cavemoviruses reported here has yet been detected in South America (none out of 93 samples).

The crinivirus SPCSV has a worldwide distribution (Tairo *et al.*, 2005), including Central America where severe diseases have been associated with its mixed infections (Moreira & Valverde, 2004). Although, detection of SPCV (or SPVCV) in co-infection with SPCSV in the field has not yet been reported, virus-like diseases and particles of unknown aetiology but showing characteristics similar to cavemoviruses have been reported in sweet potato collected in Africa and the Americas (Wambugu, 1991; Tairo *et al.*, 2004; Moreira & Valverde, 2004; Aritua *et al.*, 2007; Sim *et al.*, 2008) and therefore it is likely that SPCSV co-occurs with the cavemoviruses described here more often than previously thought. It is also possible that the clear synergistic symptoms we observed in sweet potato under greenhouse conditions (Fig. 1a) are not as obvious in the

field, or co-infection with other, more common viruses such as SPFMV mask the symptoms caused by SPCV and SPVCV. Future research will have to address the possible impact these viruses have on sweet potato production.

METHODS

Plant material and virus isolates. Plants infected with SPCV or SPVCV were propagated by lateral grafting on *I. setosa* on a monthly basis and kept in an insect-proof screenhouse. Disinfected soil [peat, sand, clay 2:1:1 (v/v) including 3.5 % (P/P) of Pro-mix BX (Les Tourbiéres Premier Ltée)] was used as a substrate for the plants. The virus isolate of SPCV characterized here has been described previously (De Souza & Cuellar, 2010). SPVCV was detected in accession CIP400851 (cultivar name 'Chambrita', collection code 'Sosa 30') during virus indexing at CIP. The infected material came from the Dominican Republic. Additional virus isolates for this study were obtained from CIP 2009 indexing material (Group 25) that included samples from Central and South America. Samples from Africa were kindly provided by Silver Tumwegamire and Willmer Perez. NCM-ELISA tests were used to exclude the presence of co-infecting viruses including SPCSV, SPFMV, sweet potato virus G (SPVG), sweet potato virus 2 (SPV2), sweet potato latent virus (SPLV), sweet potato mild mottle virus (SPMMV; genus *Ipomovirus*), sweet potato mild speckling virus (SPMSV), sweet potato chlorotic fleck (SPCFV; genus *Carlavirus*), C-6 virus and cucumber mosaic virus (CMV; genus *Cucumovirus*). In addition, plant material was found to be negative for begomoviruses by PCR using universal primers SPG1 and SPG2 (Li et al., 2004). For double-infection tests in sweet potato we used the Peruvian isolate SPCSV m2-47 (Gutiérrez et al., 2003).

Propagation of the virus and transmission. Transmission of both viruses by *M. persicae* was attempted by using a 48 h acquisition access period, a density of 30 insects per plant and 48 h inoculation access period on healthy 10 day-old *I. setosa* plants. After inoculation plants were placed in a growth chamber for 8 weeks to evaluate symptom development. Mechanical transmission was tested by sap inoculation on to three carborundum-dusted leaves of the following indicator plants (two plants each): *I. setosa*, *Ipomoea nil*, *Chenopodium amaranticolor*, *Chenopodium quinoa*, *Datura metel*, *Datura stramonium*, *Gomphrena globosa*, *Nicotiana benthamiana*, *Nicotiana debneyii*, *Nicotiana occidentalis*, *Nicotiana tabacum* 'White burley', *N. tabacum* 'Samsun', *Physalis floridiana*, *Physalis peruviana* and *Solanum lycopersicum*.

Isolation of viral DNA. A quick method described by Covey et al. (1998) was used for the isolation and cloning of SPCV and SPVCV genomic DNA from the indicator plant *I. setosa*. Fresh leaf material (400 mg) from symptomatic plants was ground in 2 ml sterile water and the viral DNA was extracted as indicated (Covey et al., 1998). The final pellet obtained after two washings in 70 % ethanol (to remove traces of isopropanol and polyethylene glycol) were resuspended in 50 μl of sterile water. A series of typical bands corresponding to different topological forms of the caulimoviral circular dsDNA (Donson & Hull, 1983) were observed upon electrophoresis in 1 % agarose gels. DNA prepared this way was cut with different restriction enzymes giving an approximate total genomic size of 8 and 9 kbp for SPCV (De Souza & Cuellar, 2011) and SPVCV (Fig. 1d), respectively.

Genome cloning and sequence analysis. To reconstruct the full genome of SPCV and SPVCV fragments produced by different restriction enzymes of the viral DNA preparations obtained as reported previously (De Souza & Cuellar, 2010) were cloned and sequenced. Once the first sequences were obtained, primers were designed to amplify regions overlapping contiguous restriction frag-

ments, which were cloned into plasmid pGEM-T easy (Promega) and sequenced using SP6 and T7 primers (Macrogen). Virus genomic sequences were identified and assembled using software Vector NTI-v9 package of programs (Invitrogen) and (PSI-)BLAST available online from the National Center for Biotechnology Information (NCBI). CLUSTAL_X (Jeanmougin et al., 1998) and MEGA4 (Kumar et al., 2008) were used in sequence comparison and phylogenetic analyses using the neighbour-joining algorithm with representative members of the family *Caulimoviridae* (Table 1). The dataset was subjected to 1000 bootstrap replicates. ORFs included those obtained from the above-mentioned caulimovirid genomes, plus the newly annotated SPCV and SPVCV ORFs. Annotation of caulimovirid ORFs was achieved by similarity search against protein databases and whole genome alignment versus related viruses using GenBank, utilizing the BLASTN and BLASTX programs (NCBI). In both cases, default parameters were employed. In the end, manual examination of annotations was carried out to produce the final versions of SPCV and SPVCV, which were submitted to GenBank (accession nos HQ694978 and HQ694979, respectively). RNA secondary structure predictions were obtained using RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) (Gruber et al., 2008) and pknotsRG (Reeder, et al., 2007) and visualized using PseudoViewer, version 3 (Byun & Han, 2009).

Virus detection. Isolates from the Americas were tested by symptom development, NCM-ELISA and PCR for SPCV and by symptom development and PCR for SPVCV, after graft infection in *I. setosa*. For a quick PCR screening of SPVCV and SPCV in sweet potato, DNA was extracted using a modified NaOH extraction protocol (Wang et al., 1993) from approximately 200 mg leaf tissue. For SPCV, primers were used as described previously (De Souza & Cuellar, 2010). For SPVCV, primers forward: 5′-TGAATGCAAAGACAA-AAACCTA-3′ and reverse: 5′-GATAAACTAACTCCTGCTTCTT-3′ were used to amplify by standard PCR a fragment of 922 and 373 bp, respectively, containing a region of the reverse transcriptase domain. For SPCV NCM-ELISA 100 mg fresh leaf material was collected and homogenized with 2 ml extraction buffer (TBS, 0.2 % sodium sulphite) and detection was carried as reported previously (Gutiérrez et al., 2003). TAS-ELISA for detection of SPCSV was carried out as reported by Karyeija et al. (2000). For detection of SPVCV DNA by dot blot, total nucleic acids were extracted from infected sweet potato and *I. setosa* plants using CTAB (Doyle & Doyle, 1987). The probe was synthesized by conventional PCR using GoTaq Flexi DNA-polymerase (Promega) and PCR DIG-labelled oligonucleotides (Roche) using primers as described above. Total DNA (8 μg per sample) was boiled for 5 min and snap cooled on ice before being blotted on to Hybond-N+ membranes (Amersham) that were previously soaked in 2× saline/sodium citrate (SSC) buffer using a vacuum pump. DNA was cross-linked using a Stratalinker UV2400 (Stratagene). Pre-hybridization [5× standard saline phosphate/EDTA, 5 % SDS, 50 % formamide, 5× Denhardt solution, herring sperm DNA (Sigma) 1 mg ml^{-1}] was at 55 °C for 2 h. Hybridization with 5 μl of probe was at 55 °C overnight with fresh pre-hybridization buffer under the same conditions. The membrane was washed twice in 2× SSC/0.1 % SDS for 5 min at 55 °C and twice again in 0.1× SSC/0.1 % SDS for 30 min at 65 °C. The membrane was blocked for 1.5 h in 10 ml 1× blocking solution [1 % blocking powder (Roche) in maleic acid buffer] at room temperature. The membrane was reacted with the antibody solution (1/10 000 anti-DIG diluted in 1× blocking solution) at room temperature for 30 min with gentle rotation. The membrane was washed three times for 30 min each with washing solution (0.1 M maleic acid, 0.15 M NaCl, 0.3 % Tween 20, pH 7.5) at room temperature. Blots were equilibrated for 5 min in detection buffer (0.1 M Tris/HCl/0.1 M NaCl, pH 9.5). A 1:150 dilution of CSPD-Star Reagent (Roche) in detection buffer was added to the blot. After 5 min incubation at room temperature the membrane was exposed to X-ray films (6 and

12 h approximately) in a dark room and the films were developed according to supplier recommendations (Sigma).

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