Analysis of gene content in sweet potato chlorotic stunt virus RNA1 reveals the presence of the p22 RNA silencing suppressor in only a few isolates: implications for viral evolution and synergism

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**INTRODUCTION**

Viruses belonging to the family **Closteroviridae** have single-stranded, positive-sense RNA genomes that are the largest among plant viruses (Dolja et al., 2006). These viruses share a conserved set of ‘core’ genes, but they also show a high level of variability in gene content at the 3’ end of the monopartite genomes in the genera **Closterovirus** and **Ampelovirus** and at the 3’ end of RNA1 in viruses of the genus **Crinivirus**, which have bipartite genomes (Dolja et al., 2006). Gene content and organization of the variable 3’-proximal genomic region have evolutionary implications (Karasev et al., 1996; Dolja et al., 2006), which are interesting not least because some of the genes in the 3’ region encode RNA silencing suppression (RSS) proteins (Reed et al., 2003; Lu et al., 2004; Kreuze et al., 2005; Chiba et al., 2006). RSS proteins combat the fundamental antiviral resistance that is based on RNA silencing in plants (Lindbo & Dougherty, 2005). The functional similarities of the RSS proteins contrast with their very low or non-significant sequence homology with other viral and host proteins (Lakatos et al., 2006; Mérai et al., 2006). It has been suggested that these characteristics might indicate a relatively recent acquisition of the genes for RSS proteins in viral genomes to counteract evolving eukaryotic host defence responses to viral pathogens (Voinnet, 2005).

Sweet potato chlorotic stunt virus (SPCSV; genus **Crinivirus**, family **Closteroviridae**) is a phloem-limited, whitefly-transmitted, bipartite virus that acts synergistically with several unrelated viruses also infecting sweetpotato (*Ipomoea batatas*) (Gibson et al., 1998; Karveija et al., 2000; Kokkinos & Clark, 2006; Mukasa et al., 2006; Univeros et al., 2007). Among the synergistic interactions, co-infection of SPCSV with sweet potato feathery mottle virus (SPFMV; genus **Potyvirus**, family **Potyviridae**) causes the severe sweet potato virus disease (SPVD), economically the most devastating disease affecting sweetpotatoes (Milgram et al., 1996; Gibson et al., 2006). RSS proteins combat the fundamental antiviral resistance that is based on RNA silencing in plants (Lindbo & Dougherty, 2005). The functional similarities of the RSS proteins contrast with their very low or non-significant sequence homology with other viral and host proteins (Lakatos et al., 2006; Mérai et al., 2006). It has been suggested that these characteristics might indicate a relatively recent acquisition of the genes for RSS proteins in viral genomes to counteract evolving eukaryotic host defence responses to viral pathogens (Voinnet, 2005).

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et al., 1998; Gutierrez et al., 2003). SPVD is manifested as a drastic increase in symptom severity and yield reduction accompanied by an increase in SPFMV titres of up to 600-fold, whereas the titres of SPCSV are reduced in comparison with plants infected with SPCSV alone (Karyeija et al., 2000; Mukasa et al., 2006). Due to the synergistic interactions with other sweetpotato viruses, further understanding of the molecular biology of SPCSV has become of immense importance. Viral synergism is often found to be associated with the action of RSS proteins (Pruss et al., 1997). In a Ugandan isolate of SPCSV (SPCSV-Ug), two proteins involved in RSS are encoded by genes at the 3′ end of RNA1 (Kreuze et al., 2005). They are expressed from subgenomic RNAs (sgRNAs) that accumulate at high levels early in infection in the young, systemically infected leaves (Kreuze et al., 2002). One of these proteins (p22) has been shown to act alone in suppressing silencing of the green fluorescent protein reporter gene mRNA in leaves of Nicotiana benthamiana, but the suppression activity was further enhanced by co-expression of the other SPCSV RNA1-encoded protein, RNAseq3 (Kreuze et al., 2005). This two-component RSS system is speculated to play a role in the synergism of SPCSV with other sweetpotato viruses and the development of SPVD.

There is limited information about the genetic variability of SPCSV, all of which is based on analysis of the nucleotide sequences of genes encoding the plant heat-shock protein-like Hsp70h protein and coat protein on RNA2. The Hsp70h gene sequences can be used to place SPCSV isolates into two phylogenetically distinct groups (Fenby et al., 2002; IsHak et al., 2003; Tairo et al., 2005), which also correlate with serological differences (Hoyer et al., 1996b; Gibson et al., 1998; Alicai et al., 1999). According to these molecular criteria, SPCSV isolates Unj2 and Mis1 from distant locations in Tanzania (Tairo et al., 2005) and the Ugandan isolate SPCSV-Ug, for which the complete sequence is available (Kreuze et al., 2002), belong to the so-called East African (EA) strain whose distribution is largely confined to East Africa. Recently, a few SPCSV isolates serologically related to the EA strain were found in Peru (Gutiérrez et al., 2003). However, SPCSV isolates from Nigeria, Israel and the USA have been found to be related to each other (Hoyer et al., 1996b; Pio-Ribeiro et al., 1996; Vetten et al., 1996) and assigned to the strain WA, named according to the original description of SPCSV as a ‘chlorotic stunt’-causing agent in sweetpotatoes in Nigeria, West Africa (Schaefers & Terry, 1976). Closterovirus-like particles were later detected in sweetpotatoes showing chlorotic stunt symptoms in Nigeria (Winter et al., 1992), Israel (Cohen et al., 1992) and Kenya (Hoyer et al., 1996a, b). The virus in these plants was named sweet potato sunken vein virus. However, The International Committee for Taxonomy of Viruses has recommended the name SPCSV (Gibson et al., 1998; Fauquet & Fargette, 2003; Fauquet et al., 2005), which is followed here.

Little is known about the possible sequence variability of RNA1 in SPCSV. The aim of this study was to characterize the 3′-end sequences of RNA1 in a few selected SPCSV isolates of the EA and WA strains with different geographical origins. The data showed that a few but not all EA strain isolates of SPCSV contained the gene for the p22 RSS protein, whereas the other isolates lacked a 767 nt region of RNA1 that included the p22 gene. This first report on intraspecific variability in gene content of members of the family Closteroviridae also indicates that the gene for RSS protein p22 is not essential for synergism between SPCSV and SPFMV, or for the development of SPVD.

**METHODS**

**Virus isolates.** The isolate SPCSV-Tug2 was obtained from an infected sweetpotato plant in Mpihi, Uganda, in 2005 (kindly provided by Arthur Tugume, Makerere University, Uganda). It originated in the same district as the previously characterized isolate SPCSV-Ug isolated in the 1990s (Alicai et al., 1999) and has been used in a few previous studies (Karyeija et al., 2000; Kreuze et al., 2002, 2005). SPCSV-Mis1 was obtained from Misurwgi district in the Lake Victoria basin in Tanzania and SPCSV-Unj2 from Unguja on Zanzibar Island on the Tanzanian coast of the Indian Ocean (Tairo et al., 2005). SPCSV-m2-47 was isolated from an infected sweetpotato plant grown in the Cañete valley, Peru (Gutiérrez et al., 2003). SPCSV-I, previously described as SPSSV in Israel (Cohen et al., 1992; Milgram et al., 1996), was kindly provided by Professor Gadi Loebenstein (The Volcani Center, Bet Dagan, Israel). The isolate of SPFMV (SPFMV-Nam1) used in this study was originally obtained from an infected sweetpotato plant in Uganda and belongs to the EA strain of SPFMV (Karyeija et al., 2000; Kreuze et al., 2000).

Seeds of Ipomoea setosa and pathogen-free in vitro plantlets of sweetpotato (I. batatas cv. Tanzania) were obtained from the International Potato Center (CIP; Lima, Peru). Viruses were maintained and experiments carried out in these plants in an insect-proof greenhouse (temperature 24–26 °C, relative humidity 70 %) under natural daylight extended to 16 h by illumination with high-pressure sodium lamps (light intensity 150–200 μmol s⁻¹ m⁻² at the level of plant height). Viruses were transmitted to new plants by graft inoculation. Infected plants were propagated by rooting stem cuttings.

**Serological detection of SPFMV.** SPFMV was detected by double antibody sandwich (DAS)-ELISA as described previously (Gibson et al., 1998). In brief, 150 mg leaf material was ground in a polystyrene bag with 600 μl extraction buffer [PBS containing 5 % Tween 20 and 2 % polyvinylpyrrolidone (M, 40 000)]. The homogenate was transferred to a 1.5 ml Eppendorf tube and spun at 6000 g for 2 min. Aliquots of the supernatant (100 μl) were transferred to a microtitre plate (Greiner Laborteknik) previously coated with rabbit polyclonal antibodies to SPFMV coat protein (provided by CIP) and incubated at 4 °C overnight. After washing twice for 3 min each, 100 μl alkaline phosphatase-conjugated anti-SPFMV antibody was added to each well and the plate was incubated at 37 °C for 3 h and washed as before. The colour reaction was developed using p-nitrophenyl phosphate (Sigma) as the substrate. Absorbance (405 nm) was recorded using a Benchmark Microplate reader (Bio-Rad Laboratories).

**RNA isolation.** Total RNA was isolated from 400 mg fresh Ipomoea leaves using Trizol (Invitrogen) following the manufacturer’s instructions. RNA was resuspended in 250 μl sterile Milli-Q water (Sigma-Aldrich). The amount and quality of the RNA were checked...
using a spectrophotometer (8543 UV-Visible, Agilent Technologies) and agarose gel electrophoresis, respectively.

**Real-time PCR.** Primers and real-time PCR conditions for amplification of SPFMV were as described by Mukasa et al. (2006). RNA samples were treated with DNase I (Promega) at 37°C for 30 min and the reaction was stopped as instructed by the manufacturer. The RNA was then reverse-transcribed in a reaction mix (20 μl) containing 200 ng random hexamer primers, 10 mM dithiothreitol, 0.5 mM dNTPs, 20 U RNasin (Promega) and 400 U Moloney murine leukemia virus reverse transcriptase (Promega) at 37°C for 1 h. The reaction was stopped by heating at 70°C for 10 min and diluted fivefold with Milli-Q water. The cDNA (5 μl) was used as template for real-time PCR, which was carried out in a total reaction volume of 25 μl. The reaction mix contained SYBR Green QPCR Master Mix (Finnzymes) and 1.0 μM each primer. The 26S rRNA gene of *I. batatas* (GenBank accession no. A972410) was used as an internal control and amplified using primers as described previously (Mukasa et al., 2006). Each sample was loaded in triplicate on a 96-well optical plate (Applied Biosystems) and scanned using the ABI Prism 7000 Sequence Detection System (Applied Biosystems).

**Cloning and sequence analysis.** cDNA was prepared from total RNA extracted from *I. setosa* leaves of SPCSV-infected plants as described above. The reaction mix (15 μl) for PCR-based amplification of SPCSV sequences contained 200 μM dNTPs, 0.5 μM each primer, 0.02 U high-fidelity Phusion DNA polymerase (Finnzymes) and 1.0 μl cDNA. Amplification was carried out in a Mastercycler gradient thermocycler (Eppendorf). Primers for amplification of regions of RNA1 were designed according to the putatively conserved regions of the RNA-dependent RNA polymerase (RdRp) gene and the 3′-untranslated region (3′ UTR) of *cis*-viruses, and the RNA1 sequence of SPCSV-Ug (GenBank accession no. A428554). To amplify the region from the RdRp gene to the 3′ UTR (Fig. 1a). The forward primer RdRp-F (5′-CAANACNAANGAATTGAACAT-3′; designed according to the sequence of SPCSV-Ug) and the degenerate reverse primer SVV-R3 (5′-TTTTTGAGNTTTTATANACACA-3′) were used to amplify a region from the 3′-proximal region of the RdRp gene to the middle of the 3′ UTR, which corresponded to nt 7197–9277 in SPCSV-Ug.

SPCSV isolates Is, Tug2 and m2-47 were assigned to a strain (EA or WA) by analysis of their Hsp70/70h gene sequences. For isolates Tug2 and m2-47, a 404 nt fragment of the Hsp70/70h gene was amplified using primers CL43U and CL43L, as described by IsHak et al. (2003). For isolate Is, primers WAhsp-F1 (5′-CKATGAA GCTCCTTCTGAAATC-3′) and WAhsp-R1 (5′-TTATTCTTCTGAAAGGAA ACTC-3′) were used to amplify the corresponding Hsp70/70h fragment. These primers were designed based on sequences of the WA strain isolates available in databases.

Amplification products were cloned using a PCR-Blunt cloning system (Invitrogen) in *Escherichia coli* DH5α cells. Two or more clones obtained in independent PCRs were sequenced from each isolate and genomic DNA from the *I. batatas* strain. The sequences were confirmed using the CLUSTAL W algorithm available in the AlignX program (VectorNTI-v9 package; Invitrogen).

**Northern blot hybridization.** Total RNA (10 μg) was separated in a 5.5% formaldehyde-containing denaturing agarose gel and blotted onto Hybond-NX membrane (Amersham Biosciences) overnight by capillary transfer. The RNA was fixed to the membrane by exposure to UV light for 1 min and pre-hybridized in a solution containing 50% formamide (Sigma), 5× SSPE, 5% SDS, 2.5× Denhardt’s solution and 1 mg herring sperm DNA (Sigma) ml⁻¹. Probes complementary to the RNase3 and p22 coding sequences were prepared and labelled with 32P [UTP] (Amersham) by in vitro transcription of the genes cloned in plasmids under the T7 or SP6 polymerase promoter. Hybridizations were carried out in a fresh batch of pre-hybridization solution containing 25 μl of the in vitro transcription reaction at 48°C overnight. The following day, membranes were washed at 68°C twice in 5× SSC with 0.5% SDS and twice in 0.2× SSC with 0.5% SDS. Membranes were exposed to X-ray film (Kodak) for 4, 16 or 48 h before being developed using an X-OMAT 1000 automated developer (Kodak).

### RESULTS

**Most SPCSV isolates lack the gene for the p22 silencing suppressor**

Cloning and sequencing of the 3′-proximal region of RNA1 from the SPCSV isolates Tug2 from Uganda, Unj2 and Mis1 from Tanzania, Is from Israel and m2-47 from Peru revealed that only isolate Tug2 contained the whole region (nt 7197–9219) previously described in another Ugandan isolate, SPCSV-Ug. All other isolates were missing 767 nt, which corresponded to nt 8453–9219 in RNA1 of SPCSV-Ug (Fig. 1). This region in SPCSV-Ug and SPCSV-Tug2 starts 29 nt upstream from the beginning of the sgRNA for the p22 RSS protein and ends 38 nt downstream from the stop codon of the p22 open reading frame (ORF) (Fig. 1b). Hence, these isolates were lacking the gene for the p22 RSS protein. Otherwise, the genetic structure of the sequenced part of RNA1 was conserved among the six isolates. All contained the ORFs for RNase3 and p7 (Fig. 1a) and no additional sequence insertions or deletions were detected.

To rule out any possible ambiguities due to RT-PCR or cloning, viral RNA was detected by Northern blot analysis using probes for RNase3 (Fig. 2a), p22 (Fig. 2b) and RdRp (data not shown). The youngest systemically infected leaves of *I. setosa* were tested, as a previous study on SPCSV-Ug showed that these tissues contained the highest amounts of the sgRNAs for RNase3 and p22 early in infection (Kreuze et al., 2002). Detection with the probe for RNase3 revealed a shift in the sizes of the genomic RNA1 and sgRNA for RNase3 in isolates Unj2, Mis1 and Is (Fig. 2a; lanes 1, 3 and 4, respectively) compared with SPCSV-Ug (Fig. 2a, lane 2), which indicated the absence of a genomic region downstream from the RNase3 gene in isolates Unj2, Mis1 and Is. Subsequent rehybridization of the membrane with a probe for p22 showed a signal in isolate Ug but no detectable signal in isolates Unj2, Mis1 and Is (Fig. 2b). These data were consistent with a lack of the p22-containing region in the three isolates. The lack of signal for p22 was not due to smaller amounts of viral RNA in the RNA samples as neither overexposing the X-ray films nor using higher RNA concentrations revealed a signal (data not shown). Taken together, the data indicated that the p22 gene was present only in the two Ugandan isolates of SPCSV.

A putative defective RNA (dRNA; ~3.5 kb) was consistently detected using the probe for p22 (Fig. 2b, filled...
Fig. 1. Genomic structure and sequences of the 3′-proximal region of RNA1 in isolates of SPCSV. (a) Schematic presentation of ORFs in RNA1 indicated as open arrows. The complete sequence of RNA1 in SPCSV-Ug has been described in a previous study (Kreuze et al., 2002). The sequences corresponding to the region of RNA1 shown in the lower drawing were determined in isolates Tug2, Unj2, Mis1, m2-47 and Is in this study. (b) Sequence alignment corresponding to the region framed in (a). The coding region for p7 is indicated. The beginning of the sgRNA sequence for p22 and the beginning and end of the coding region for p22 are indicated. Numbers indicate the positions of the region absent in isolates Unj2, Mis1, m2-47 and Is compared with the sequences of Ug and Tug2. P-Pro, putative papain-like cysteine proteinase; MTR, methyltransferase domain; HEL, helicase domain; RdRp, RNA-dependent RNA polymerase; RNase3, an RNase III endonuclease; p7, putative 7 kDa hydrophobic protein; p22, 22 kDa RNA silencing suppressor protein.
Fig. 2. Detection of genomic RNA1 and sgRNAs with a probe for RNase3 (a) and p22 (b) in four isolates of SPCSV by Northern blot hybridization in the uppermost systemically infected leaves of I. setosa 4 weeks post-inoculation. The systemically infected top leaves of plants from three independent experiments were analysed. The blot was first probed for RNase3, and then stripped and hybridized with a probe for p22. Lanes: H, healthy plant; 1, isolate Unj2; 2, isolate Ug; 3, isolate Mis1; 4, isolate Is. In both panels, the open arrowheads indicate the position of the genomic RNA1, whereas line arrows indicate the positions of sgRNA for RNase3. The asterisk in (a) indicates the position of a non-specific band that corresponds in size to rRNA. The filled arrowhead in (b) indicates a putative dRNA homologous to the p22 sequence but not to RNase3. The dashed arrow in (b) indicates the sgRNA for p22. rRNA was used as a loading control. Molecular size markers (kb) are indicated on the left.

Genetic variability of SPCSV isolates

Phylogenetic analysis of partial Hsp70h gene sequences is used to assign isolates of SPCSV to the two relatively distantly related strains, EA and WA. Isolates Ug, Unj1 and Mis1 included in this study have been shown to belong to the EA strain (Tairo et al., 2005). Strain identification of isolates Is, m2-47 and Tug2 was carried out in this study and showed that m2-47 and Tug2 belonged to strain EA, whereas isolate Is belonged to strain WA (Fig. 3a).

Grouping of the SPCSV isolates according to the Hsp70h sequences of RNA2 was fully consistent with the sequence differences observed in RNA1. This was indicated by phylogenetic analysis (Fig. 3b) of the sequences that flanked the unique p22-containing insert in isolates Ug and Tug2 (Fig. 1b) and also of the differences in the RNase and p7 genes (Table 1) and the 3’-proximal region of the RdRp gene and the 3’ UTR sequences (data not shown) of RNA1. The nucleotide and deduced amino acid sequences of RNase3 in the EA strain isolates Ug, Tug2, Unj2, Mis1 and m2-47 were almost identical (98–99 and 97–100 %, respectively) compared to isolate Is (83 and 80–82 %, respectively). The nucleotide and amino acid sequences of the putative p7 ORF were more variable, showing identities of 97–98 and 92–98 % among the EA strain isolates, which in turn showed identities of 74–76 and 59–61 %, respectively, with the WA strain isolate Is (Table 1).

SPCSV isolates act synergistically with SPFMV in co-infected plants

Northern blot analysis (Fig. 2) revealed that the p22-encoding isolate SPCSV-Ug accumulated in much higher titres in the young leaves of I. setosa than the isolates Mis1, Unj2 and Is lacking the p22 gene, as found consistently in three experiments. As Tug2 was available only in cv. Tanzania co-infected with SPFMV, and m2-47 was obtained as cDNA from Peru for molecular analysis, these isolates were not included in the experiments. Co-infection of the p22-encoding isolates Ug or Tug2 with SPFMV caused a severe disease, which was eventually lethal to plants of I. setosa at 20 days post-inoculation. In contrast, co-infection with SPFMV and the other SPCSV isolates caused severe mosaic and leaf malformation but no necrosis in I. setosa. Infection of I. setosa with SPFMV or SPCSV alone caused only mild symptoms of vein clearing and chlorosis in leaves, respectively. Therefore, all tested isolates of SPCSV acted synergistically with SPFMV in I. setosa, as indicated by markedly increased symptom severity, but the symptoms were more severe with the p22-encoding isolates. Differences in virus accumulation were not tested because extensive necrosis in some virus combinations in contrast to others was expected to make comparisons unreliable.

All isolates of SPCSV tested also act synergistically with SPFMV in sweetpotato plants (cv. Tanzania). Severe symptoms of leaf malformation (Fig. 4) and stunting
characteristic of SPVD developed following co-infection with SPCSV and SPFMV. In contrast, plants infected with SPFMV alone showed no symptoms, and those infected with SPCSV alone displayed only mild chlorosis of the upper leaves and some purpling of the lower leaves. Differences in accumulation of SPFMV were estimated by DAS-ELISA and quantitative (real-time) PCR in symptomatic leaves of the same developmental stage of sweetpotato plants 3–4 weeks post-inoculation. ELISA

Table 1. Amino acid sequence identity (%) of RNase3 (220 aa), p7 (55 aa) and Hsp70h (128 aa) (upper diagonal) and nucleotide sequence identity (660, 165 and 384 nt, respectively) (lower diagonal) among the SPCSV isolates analysed in this study

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Fig. 3. Variability of SPCSV isolates. (a) Phylogenetic tree based on partial hsp70h gene sequences, which allows assigning of the isolates to the two main strains (EA and WA). Arrowheads indicate the isolates included in this study. GenBank accession numbers and country of origin (from top to bottom): S1EA-11a (AJ010914.1; Uganda), S1EA-11b (AJ010915.1; Uganda), S1EA-19a (AJ010920.1; Uganda), S1EA-13a (AJ010916.1; Uganda), Mis1 (AJ783447.1; Tanzania), S1EA-13b (AJ010917.1; Uganda), Bkb1 (AJ783446.1; Tanzania), Ug (AJ428555; Uganda), S2EA-25b (AJ010765.1; Uganda), S2EA-4a (AJ010762.1; Uganda), S2EA-39a (AJ010766.1; Uganda), Tug2 (EU124489; Uganda), S2EA-25a (AJ010764.1; Uganda), S2EA-39b (AJ010767.1; Uganda), S1EA-22b (AJ010919.1; Uganda), Tar2 (AJ783448.1), m2-47 (EU124488; Peru), Unj2 (AJ783449.1; Tanzania), Mad2 (AJ278651.1; Madagascar), Córdoba (AY729021.1; Argentina), WA-Nig (AJ278652.1; Nigeria), WA2-Nig (AJ278653.1; Nigeria), Egypt1 (AJ153811.1; Egypt), White Bunch (AF260321.1; USA), Is (EU124487; Israel). (b) Phylogenetic tree based on the sequences of RNA1 of the six SPCSV isolates included in this study. The flanking sequences of the p22-containing insert of isolates Ug and Tug2, which are shown in Fig. 1(b), were used for the analysis. The bars in (a) and (b) represent 0.02 Kimura nucleotide units. Numbers at the nodes indicate bootstrap values.

Fig. 4. Synergistic interactions between different isolates of SPCSV (Unj2, Ug, Mis1 and Is) and SPFMV in sweetpotato (I. batatas cv. Tanzania). Plants infected with SPFMV were side-graft-inoculated with scions from SPCSV-infected plants. Symptoms of SPVD including reduced growth, malformation and chlorosis of leaves were apparent in plants co-infected with the different isolates of SPCSV and SPFMV 4 weeks post-inoculation. Leaves of healthy plants and plants infected with SPFMV or SPCSV alone did not display any symptoms.
indicated highly increased titres of the SPFMV antigen in doubly infected plants compared with the plants infected with SPFMV only in which the virus was barely detectable (Fig. 5). Real-time PCR revealed that SPFMV accumulated at up to 500-fold higher titres in SPVD-affected plants compared with the plants infected with each SPCSV isolate alone. The titres of isolates Unj2 and Mis1 were not significantly affected (Table 2). In sweetpotato plants infected with the isolates Unj2, Ug and Mis1 alone, all isolates accumulated at similar titres (Table 2).

Table 2. Comparison of the amount of SPFMV and SPCSV RNA by real-time PCR in plants co-infected with SPFMV and SPCSV or infected with SPFMV only

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<th>Sample</th>
<th>Target virus</th>
<th>ΔCt(T)</th>
<th>2^−ΔΔCt</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>SPFMV</td>
<td>26.07</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>SPFMV + Unj2</td>
<td>17.20</td>
<td>468.96</td>
</tr>
<tr>
<td></td>
<td>SPFMV + Ug</td>
<td>19.40</td>
<td>102.06</td>
</tr>
<tr>
<td></td>
<td>SPFMV + Mis1</td>
<td>17.07</td>
<td>514.37</td>
</tr>
<tr>
<td></td>
<td>SPFMV + Is</td>
<td>17.18</td>
<td>476.61</td>
</tr>
<tr>
<td>II</td>
<td>SPCSV</td>
<td>18.20</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>SPCSV + Unj2</td>
<td>22.34</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>SPCSV + Ug</td>
<td>17.41</td>
<td>1.10</td>
</tr>
<tr>
<td></td>
<td>SPCSV + Mis1</td>
<td>17.98</td>
<td>1.00</td>
</tr>
<tr>
<td>III</td>
<td>SPCSV</td>
<td>17.32</td>
<td>1.58</td>
</tr>
<tr>
<td></td>
<td>Ug</td>
<td>17.54</td>
<td>1.36</td>
</tr>
</tbody>
</table>

Hence, isolates of SPCSV were able to act synergistically with SPFMV in sweetpotato plants, regardless of the presence or absence of the p22 gene.

Titres of SPFMV were less elevated (~100-fold) in sweetpotato plants co-infected with SPCSV-Ug compared with the 500-fold elevation in plants co-infected with the SPCSV isolates Unj2, Mis1 or Is (Table 2). Real-time PCR data indicated that the titres of isolate Ug were greatly decreased (33-fold) in plants co-infected with SPFMV, whilst the titres of isolates Unj2 and Mis1 were not significantly affected (Table 2). In sweetpotato plants infected with the isolates Unj2, Ug and Mis1 alone, all isolates accumulated at similar titres (Table 2).

DISCUSSION

The high overall sequence identities and conservation of sequences in RNA1 and RNA2 of the EA strain isolates of SPCSV contrasted with the unexpected difference in gene content of RNA1. Only two isolates (Ug and Tug2) carried the 767 nt sequence containing the ORF for p22, whilst this region was absent from the other three EA strain isolates (Mis1, Unj2 and m2-47) and also the WA isolate Is. The ORF for p22 is also probably absent from a previously reported Kenyan isolate of SPCSV (Hoyer et al., 1996a) although no sequence data are available for direct comparison. The Kenyan isolate was reported to contain only an ORF for a putative 30 kDa protein at the end of the RNA1 sequence (Hoyer et al., 1996a). An ORF for a putative 30 kDa protein would also be predicted for RNA1 of isolates Ug, Tug2, Unj2, Mis1 and m2-47 (but not Is) due to an AUG translation initiation codon at nt 7481 (according to the sequence of SPCSV-Ug; Kreuze et al., 2002). However, this codon is upstream from the determined 5′ end of the sgRNA for the 27 kDa RNA3 (Kreuze et al., 2002) and most likely is not used. Hence, whilst it cannot be verified further due to unavailable sequence data, it seems that the Kenyan isolate (Hoyer et al., 1996a) encodes RNA3 but no p22. Taken together, characterization of the 3′-proximal sequences of SPCSV isolates provides the first example of intraspecific variability in gene content in viruses of the family Closteroviridae.

The results of this study show that isolates of a virus species can vary with regard to the presence of genes encoding RSS proteins. Previous studies using chimeric viruses have shown that, whilst some viral RSS proteins are important for efficient RNA accumulation in certain hosts, they are dispensable for virus replication and systemic movement (Peremysoy et al., 1999; Qu & Morris, 2002; Silhavy et al., 2002; Stenger et al., 2005; Scholthof, 2006). Closteroviruses such as citrus tristeza virus encode several RSS proteins (Lu et al., 2004), which may also explain how SPCSV isolates still show high levels of virulence as well as synergism with SPFMV in the absence of the p22 RSS protein. Indeed, p22 was not essential for the ability of SPCSV to act synergistically with SPFMV and for the consequent
increases in SPFMV accumulation by several hundred fold and the development of the severe symptoms of SPVD. These results were consistent with previous studies on isolates Is (Milgram et al., 1996) and m2-47 (Gutiérrez et al., 2003; Untiveros et al., 2007), which cause SPVD in plants co-infected with various isolates of SPFMV. Isolate Tug2 is maintained in a sweetpotato plant also infected with SPFMV and affected by SPVD (unpublished data). Hence, isolates of SPCSV act synergistically with SPFMV in sweetpotato plants, regardless of the presence or absence of the p22 gene. This is an important finding as it means that any effort to engineer pathogen-derived resistance to SPCSV and SPVD in sweetpotato should not rely on p22 as the transgene and the target sequence.

The data showed that the antagonistic effect of SPFMV, resulting in a decrease in SPCSV accumulation (Mukasa et al., 2006), was most pronounced towards SPCSV-Ug, which encodes p22. The titre of this isolate was decreased 33-fold in full-grown sweetpotato leaves co-infected with SPFMV, whereas the titres of the two other EA strain isolates Mis1 and Unj2 lacking the p22 gene remained unaffected. In I. setosa, the p22-encoding isolates Ug and Tug2 caused necrotic symptoms and eventually lethal necrosis following co-infection with SPFMV, which made assessment of SPCSV titres unreliable. Necrosis caused by Ug and Tug2 may be due to a higher level of accumulation in infected tissues of I. setosa compared with other isolates, or perhaps the activities of p22 in tissues infected with heterologous viruses. For example, chimeric potato virus X (genus Potexvirus) expressing SPCSV p22 causes lethal necrosis in N. benthamiana plants, whereas the same vector virus causes only mosaic symptoms in the absence of p22 (Kreuze et al., 2005). The mechanisms behind the different interactions of the p22-containing SPCSV isolates compared with others in host plants co-infected with SPFMV require further study.

Recombination whereby functional units from homologous or non-homologous sources are brought together lies behind the processes that lead to gene gain (duplication and extensive gene divergence; gene shuffling and horizontal gene transfer) in plant and animal viruses (Lai, 1992; Worobey & Holmes, 1999). Viruses of the family Closteroviridae belonging to three different genera share homologous conserved genes and genome structures, but also show a high level of variability in gene content at the 3’ end of the genomic RNA (closteroviruses and ampeloviruses) and RNA1 (criniviruses) (Aguilar et al., 2003; Dolja et al., 2006). This is illustrated in Fig. 6 for five different criniviruses, which contain a diversity of genes with low or no detectable sequence homology among each other in the 3’-proximal region of RNA1. All of these genes are expressed from sgRNAs, which in turn have been implicated in the diversification of the closteroviruses through recombination and gene gain (Bar-Joseph et al., 1997). How can the variability for the presence and absence of the ORF for p22 in SPCSV be explained? SPCSV-Ug was isolated by whitefly transmission from an infected sweetpotato plant in the late 1990s (Alicai et al., 1999). Its full sequence was reported in 2002 (Kreuze et al., 2002) and the sequence of the 3’-proximal region of RNA1 was reconfirmed in this study. The virus has been maintained in living plants of sweetpotato and I. setosa since its isolation, but no mutants lacking p22 have been observed. An independent isolate, Tug2, was obtained from a sweetpotato plant in Uganda in 2005 and, as reported here, carries the p22 gene. The characterized RNA1 sequences of isolates Ug and Tug2 are highly similar but not identical. Notably, the high sequence conservation of the two Ugandan EA strain isolates in the p22 and flanking regions, an identical size and position of the unique...
sequence compared with other EA and WA strain isolates, and the distinct geographical location of the p22-encoding isolates suggest recent incorporation of this coding region into the SPCSV genome. An alternative scenario would be that p22 was deleted from an early genetic line of SPCSV before diversification of the EA and WA strains. Spontaneous natural deletions of viral sequences in plant viruses have been attributed to errors in RNA replication, such as a ‘copy choice’ mechanism where the RNA polymerase dissociates and primes elsewhere on the template during RNA synthesis (Simon & Bujarski, 1994). Partial or complete deletion of ORFs has been reported, e.g. for potato mop-top virus (genus Pomovirus) (Torrance et al., 1999; Sandgren et al., 2001) and tobacco rattle virus (genus Tobravirus) (Hernandez et al., 1996), especially upon serial passage by mechanical inoculation under experimental conditions where the genomic regions involved in transmission of the virus by vectors become redundant and can be deleted. The loss of functionally redundant sequences is also observed with viral genomes engineered to become gene vectors and from which the inserted heterologous sequences are often deleted during an extended period of virus replication and spread in the host (see, for example, Chung et al., 2007). However, the p22 gene and its flanking sequences seem to be stable in SPCSV-Ug, and p22 is probably not redundant. The two isolates encoding the p22 RSS protein accumulated in higher amounts than the other isolates in young systemically infected leaves but did not induce more severe symptoms in I. setosa plants infected with SPCSV alone. In the field, higher titres of SPCSV might indirectly enhance virus transmission via better virus acquisition by the whitefly vectors, which would provide a selective advantage to the p22-encoding isolates. In conclusion, it is less likely that the p22-containing region was independently and identically deleted from SPCSV isolates of two genetically distinct strains and isolates that are geographically widely distributed in East Africa, Israel and Peru. Therefore, the evidence best fits the theory of recent acquisition of p22 by some EA isolates of SPCSV, in line with recombination-mediated gene gain, which is a frequent phenomenon in the evolution of members of the family Closteroviridae (Dolja et al., 2006).

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REFERENCES


