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Short communication

Sequence characterization of a Peruvian isolate of *Sweet potato chlorotic stunt virus*: Further variability and a model for *p22* acquisition

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ABSTRACT

Sweet potato chlorotic stunt virus (SPCSV) is probably the most important virus infecting sweetpotato worldwide, causing severe synergistic disease complexes with several co-infecting viruses. To date only one isolate (Ug), corresponding to the EA strain has been completely sequenced. It was later shown to be unusual in that, in contrast to most isolates, it encoded an additional p22 protein at the 3′ end of RNA1. We report the complete sequence and genome organization of a Peruvian isolate of SPCSV (m2-47) as determined by siRNA deep sequencing. We confirm that the ORF encoding p22 is lacking from m2-47 and all tested Peruvian and South American isolates, whereas additional isolates containing *p22* were identified from Uganda. Other potentially important genomic differences such as two small ORFs encoding putative small hydrophobic proteins instead of one, upstream the *hsp70h* gene and a more divergent sequence at its RNA1 3′-UTR in contrast to SPCSV isolates that contain *p22* are discussed and a model for recent acquisition of *p22* in Uganda is proposed. A role for *p22* as a pathogenicity enhancer of SPCSV is also provided by complementary expression of *p22* in transgenic sweetpotato plants.

Sweet potato chlorotic stunt virus (SPCSV) is a bipartite member of the family *Closteroviridae*, genus *Crinivirus*. It is phloem-limited, whitefly transmitted and among the largest of the single-stranded positive-sense RNA viruses with a total genome size of ~17.6 kbp (Dolja et al., 2006; Kreuze et al., 2002). SPCSV has a worldwide distribution including the main sweetpotato production areas of the crop in Africa and the Americas (Gibson et al., 1998; Gutierrez et al., 2003; Kokkinos and Clark, 2006; Valverde and Moreira, 2004) and severe diseases have been associated with mixed infections with SPCSV in Africa (Gibson et al., 1998; Mukasa et al., 2006), Israel (Milgram et al., 1996), Central America (Valverde and Moreira, 2004) and South America (Di Feo et al., 2000; Gutierrez et al., 2003). Experimentally these mixed viral infections are characterized by an accumulation of only the co-infecting virus or viruses. The most dramatic example is the disease caused by the interaction of SPCSV with the potyvirus *Sweet potato feathery mottle virus* (SPFMV; Potyvirus) which causes 'sweetpotato virus disease' (SPVD), the economically most important disease of this crop, associated with yield losses of >60% (Gibson et al., 1998; Gutierrez et al., 2003; Untiveros et al., 2007). SPCSV can be distinguished into two distantly related strains EA and WA based on serology and nucleotide sequence data (Hoyer et al., 1996b, Vetten et al., 1996; Tairo et al., 2005). Only one complete genome of SPCSV has been sequenced so far, corresponding to isolate Ug of the EA strain (Kreuze et al., 2002).

Interestingly, partial sequence analyses of other strains suggest that the genome of Ug isolate is unusual in that it contains a *p22* ORF at the 3′ end of RNA1 (Cuellar et al., 2008). Isolates showing a similar genome organization to Ug, which we hereby designate strain EA^{p22}, have so far been reported only from Uganda while isolates lacking *p22* and corresponding to both EA and WA strains have been reported in Peru, Israel, and Tanzania, suggesting that the latter is more widely distributed (Cuellar et al., 2008). Another unusual feature of the Ug isolate is that, unlike other criniviruses, the 3′-UTR of RNA1 and RNA2 are almost identical (99%).

The protein encoded by *p22* has been shown to have RNA silencing suppression (RSS) activity (Kreuze et al., 2005) and was able to transiently increase viral titers of SPFMV after inoculation in sweetpotato (Cuellar et al., 2009). In *Nicotiana benthamiana*, p22 induces local necrosis when ectopically expressed in leaves by agroinfiltration and systemic necrosis when expressed from a viral vector (Kreuze et al., 2002). RNA silencing has a role in virus defense in plants (Baulcombe, 2004) and p22 may thus increase the severity of symptoms and virus interactions involving SPCSV. Indeed, co-infection of SPFMV with SPCSV isolates containing *p22* cause more

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severe symptoms (systemic necrosis) than co-infection of SPFMV with SPCSV isolates lacking *p22* in the indicator plant *Ipomoea setosa* (Cuellar et al., 2008). However, because isolates involved in these experiments have not been sequenced it cannot be excluded that this is caused by other factors than the *p22* gene.

Together with isolate Ug, isolate m2-47 is commonly used in experimental studies of viral synergisms in sweetpotato (Cuellar et al., 2009; Untiveros et al., 2007). In this study we determine the complete genome sequence of m2-47 isolate (Genbank ID: HQ291259 for RNA1 and HQ291260 for RNA2), confirming the absence of *p22* and also identifying other potentially important genomic differences. Through complementary expression in a transgenic plant we show evidence for a role of *p22* in the pathogenicity of SPCSV. We further show that *p22* is lacking from all available Peruvian and South American isolates and provide a model as to the mode of acquisition of *p22*.

SPCSV m2-47 was originally isolated from severely diseased sweetpotato plants growing in the Cañete valley of the southern coast of Lima by whitefly transmission on the indicator plant *I. setosa* (Gutierrez et al., 2003) and maintained by lateral grafting in an insect-proof greenhouse. Additional isolates used in this work (Table 1, samples 1–7) were isolated in the same way. Indicator plants were tested by NCM-ELISA for SPFMV, *Sweet potato virus G* (SPVG, Potyvirus), Sweet potato virus 2 (SPV2, Potyvirus), *Sweet potato latent virus* (SPLV, Potyvirus), *Sweet potato mild mottle virus* (SPMMV; *Ipomovirus*), *Sweet potato mild speckling virus* (SPMSV), *Sweet potato chlorotic fleck* (SPCFV, *Carlavirus*), C-6 virus, *Cucumber mosaic virus* (CMV, *Cucumovirus*) and Sweet potato caulimo-like virus (SPCV, *Caulimoviridae*) to confirm their absence. The plants were also checked to confirm the absence of begomoviruses by PCR (Li et al., 2004). Isolates 8–19 (Table 1; Untiveros et al., 2008) were maintained in sweetpotato and analyses were done on total RNA from these samples. 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 (Nanodrop, Thermo Scientific) and agarose gel electrophoresis. For isolate m2-47, RNA was lyophilized and sent to Fasteris Life Sciences SA (Plan-les-Ouates, Switzerland) for processing and high throughput sequencing (deep sequencing) on the Illumina Genome Analyzer II and short RNA viral sequences were assembled *in silico* using the programs Velvet and MAQ (http://maq.sourceforge.net) as previously described (Kreuze et al., 2009). Partial sequences of SPCSV isolates from Kisoro and Masaka were determined in a similar way, except that they were sent as a batch RNA prep with several other samples. To verify the integrity of the m2-47 genome assembled *in silico*, overlapping PCR products covering the span of the SPCSV genome were amplified using specifically designed primers and cloned using the pGEM-T easy cloning system (Promega) and *Escherichia coli* DH5α chemically competent cells. The sequence of two or more clones obtained from independent PCR reactions were confirmed by Sanger sequencing (Macrogen). Extension of these contigs, including sequences obtained by RT-PCR, was done using the program ContigExpress included in the Vector NTI package (Invitrogen). Transmembrane helices in p6 proteins were predicted with the TMHMM program (http://www.cbs.dtu.dk/services/TMHMM-2.0/) as previously described (Kreuze et al., 2002). Phylogenetic analyses were performed using the MEGA4 package (Kumar et al., 2008). To confirm the distinct 3′-UTR of RNA1 of m2-47 it was amplified using a custom RLM-RACE protocol as follows: ∼5 μg of RNA was incubated with 100 μM of Modban linker (5′rAppCTG TAG GCA CCA TCA AAT/3ddC/3′; IDT), 40 U of RNaseOUT (Invitrogen), 1x T4 RNA ligation buffer in a total volume of 20 μl and heated at 65 °C for 5 min. The reaction was then transferred to ice, 20 U of T4 RNA ligase (NEB) were added and incubated at 20 °C for 4 h. Then 7.5 μl of the RNA ligation mix was used for reverse transcription with the BanOne primer (5′-ATT GAT GGT GCC TAC AG-3′) and Superscript III reverse transcriptase (Invitrogen) according to the manufacturers recommendations at 50 °C for 55 min, followed by denaturation at 70 °C for 10 min and a treatment with 10 U of RNaseH (Invitrogen) at 37 °C for 20 min. Five microliters of the cDNA mix was then used for 35 cycles of PCR amplification using the BanOne and P7-F (5′-TTG ATG TGG CIC TAC TTT GGT-3′) primers with Platinum® Taq polymerase (Invitrogen) according to the manufacturers recommendations. A specific fragment of the expected size of ∼310 bp was obtained, purified from gel, cloned into *E. coli* and sent for Sanger sequencing as described above. To determine the RNA1 and RNA2 3′-UTRs of remaining SPCSV isolates (Table 1), RNA from all samples were 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 M-MLV reverse transcriptase (Invitrogen) at 37 °C for 1 h. The reaction was stopped by heating at 70 °C for 10 min and diluted fivefold with sterile water. Five microliters of the reaction (cDNA) was used as template for PCR using primers P7-F or SPCSV2-7242: (5′-ATT GAT GAG AAA TAA GCA CCG C-3′) and Crini-3UTR-R: (5′-TTT TTG AGI TTT TAI AAT ACA CAC-3′) flanking the *p22* insertion region. Samples lacking p22 produced a band of ∼250 bp. We confirmed the identity of the band of all isolates by restriction digestion (*not shown*) and of two EA and EA^{p22} isolates each by sequencing (Table 1). The 5′ region of RNA2 was also amplified and sequenced from seven isolates (Table 1) to confirm the presence of the *p6-SHP* (see below) using the primers SPCSV2-44f (5′-TAA GCT CGT ATC ATT GGT TGT CGT CA-3′) and SPCSV2-1046r (5′-GAC CTT CAT CGT ACC CCC GAC-3′). To study a possible role of p22 during SPCSV infection we used non-transgenic and *p22*-expressing (under control of the phloem specific RolC promoter) transgenic sweetpotato lines of cultivar 'Huachano' (Cuellar et al., 2009) and quantified the accumulation of m2-47 by triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) (Karyeija et al., 2000).

The genome of SPCSV m2-47 consisted of approximately 8637nt for RNA1 and 8219nt for RNA2 and shared a total nt sequence identity of 98% with isolate Ug. The genome organization of m2-47 isolate differed from that of isolate Ug in three main ways (Fig. 1).

The absence of a p22 gene in RNA1: Northern blot results using a probe for RNase3 (*not shown*) and previous partial sequence characterization of SPCSV m2-47 indicating the absence of *p22* (Cuellar et al., 2008) is confirmed in this work. Results herein also indicate that *p22*-containing isolates are not common in other regions of South America (Table 1). In contrast, RNA samples collected from infected sweetpotatoes collected in the districts of Kisoro and Masaka in Uganda contained *p22*. Isolates of EA^{p22} have so far been reported only in the Mpigi province in Uganda (Cuellar et al., 2008; Kreuze et al., 2002). The presence of *p22* in isolates from other districts in Uganda further suggests that EA^{p22} is more common than previously known (Cuellar et al., 2008). Furthermore, the suspected presence in Kenya of isolates lacking *p22* (Hoyer et al., 1996a) is confirmed in this study (isolate Africa10, Table 1).

An additional ORF encoding a small hydrophobic protein (SHP) upstream the hsp70h gene: Isolates m2-47 and Ug contain a *p6* gene at the 5′ end of RNA2 showing 100% nt and aa identity. Although similar small ORFs found in the Closteroviridae encode SHP, no evidence of transmembrane domains in p6 have been detected (Kreuze et al., 2002; and results not shown), and instead the SHP is represented by the p7 on RNA1. However, in comparison to isolate Ug, SPCSV m2-47 contains an additional ORF of predicted 6 kDa (p6-SHP) with characteristic transmembrane helices predicted in its central region (Fig. 1). The p6-SHP showed no similarity in sequence to p6. A single nucleotide substitution (A575 → T) creates a stop codon interrupting a hypothetical *p6-SHP* in the corresponding region of isolate Ug. If not interrupted, this protein would show 88%