Genetic Variability and Evolutionary Implications of RNA Silencing Suppressor Genes in RNA1 of Sweet Potato Chlorotic Stunt Virus Isolates Infecting Sweetpotato and Related Wild Species

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Abstract

Background: The bipartite single-stranded RNA genome of Sweet potato chlorotic stunt virus (SPCSV, genus Crinivirus; Closteroviridae) encodes a Class 1 RNase III (RNase3), a putative hydrophobic protein (p7) and a 22-kDa protein (p22) from genes located in RNA1. RNase3 and p22 suppress RNA silencing, the basal antiviral defence mechanism in plants. RNase3 is sufficient to render sweetpotato (Ipomoea batatas) virus-susceptible and predisposes it to development of severe diseases following infection with unrelated virus. The incidence, strains and gene content of SPCSV infecting wild plant species have not been studied.

Methodology/Principal Findings: Thirty SPCSV isolates were characterized from 10 wild Ipomoea species, Hewittia sublobata or Lepistemon owariensis (family Convolvulaceae) in Uganda and compared with 34 local SPCSV isolates infecting sweetpotatoes. All isolates belonged to the East African (EA) strain of SPCSV and contained RNase3 and p7, but p22 was not detected in six isolates. The three genes showed only limited genetic variability and the proteins were under purifying selection. SPCSV isolates lacking p22 synergized with Sweet potato feathery mottle virus (SPFMV, genus potyvirus; Potyviridae) and caused severe symptoms in co-infected sweetpotato plants. One SPCSV isolate enhanced accumulation of SPFMV and caused severe symptoms in co-infected sweetpotato plants. A new whitefly-transmitted virus (KML33b) encoding an RNase3 homolog (<56% identity to SPCSV RNase3) able to suppress sense-mediated RNA silencing was detected in I. sinensis.

Conclusions/Significance: SPCSV isolates infecting wild species and sweetpotato in Uganda were genetically undifferentiated, suggesting inter-species transmission of SPCSV. Most isolates in Uganda contained p22, unlike SPCSV isolates characterized from other countries and continents. Enhanced accumulation of SPFMV and increased disease severity were found to be uncoupled phenotypic outcomes of RNase3-mediated viral synergism in sweetpotato. A second virus encoding an RNase3-like RNA silencing suppressor was detected. Overall, results provided many novel and important insights into evolutionary biology of SPCSV.

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Introduction

Sweet potato chlorotic stunt virus (SPCSV; genus Crinivirus; family Closteroviridae) is a phloem-limited virus [1], semi-persistently transmitted by whiteflies [2,3] and known to occur in most areas where sweetpotatoes (Ipomoea batatas Lam.) are grown. The virus has a bipartite, single-stranded, positive-sense RNA genome [4]. Isolates can be identified as being the ‘East African’ (EA) and ‘West African’ (WA) strain by phylogenetic analysis of their Hsp70h gene located on RNA2
and also by analysis of RNA1 sequences [8]. Strain EA is common in sweetpotatoes grown in East Africa and is also the only strain known to occur there [6,9]. There are only a few reports on its occurrence outside Uganda, e.g., in Kenya, Tanzania, China and Peru [7,8,10]. In contrast, strain WA occurs more widely in sweetpotatoes and has been detected in West and South Africa, Egypt, Israel, Spain, China, Peru, Argentina, and the United States [7,10-18].

The genome organization of SPCSV has many similarities to other criniviruses. Proteins implicated in virus multiplication are encoded by RNA1, whereas the genes for proteins needed for viral movement, encapsidation and vector transmission are located in RNA2 [19]. However, the genome of SPCSV possesses unique features especially concerning the gene content of RNA1. Downstream from the open reading frame (ORF) for the replicase, there is a 686 nucleotides (nt) long ORF for a Class 1 RNase III enzyme (RNase3, 27 kDa), an ORF (171 nt) for a putative, unique hydrophobic protein (p7), and an ORF (575 nt) for a 22-kDa protein (p22) [20]. These genes are not found in other *Closteroviridae* members and other RNA viruses. Furthermore, comparison of the fully characterized isolate SPCSV-Ug from Uganda, consisting of RNA1 (9407 nt) and RNA2 (8223 nt) [20], with isolate m2-47 from Peru shows that they are 98% identical, but m2-47 lacks the p22 gene [8,10].

The 3′-proximal part of RNA1 constitutes an interesting genomic region for study owing to its unique functions. RNase3 and p22 are most likely expressed from the respective m′GpppN-capped subgenomic RNAs (sgRNA), which have been detected in SPCSV-infected plants [4]. RNase3 exhibits endoribonuclease activities similar to the Class 1 RNase III of *Escherichia coli* [20] and can suppress gene silencing induced by sense transcripts [21]. It is alone sufficient to eliminate antiviral defence to unrelated viruses in *RNase3* transgenic sweetpotato plants and predisposes sweetpotato to development of the severe sweetpotato virus disease (SPVD) following infection with Sweet potato feathery mottle virus (SPFMV; *Potyvirus, Potyviridae*) [21]. On the other hand, the p22 protein is not needed for development of SPVD [8,21], although it is a ‘strong’ silencing suppressor. It can efficiently suppress gene silencing induced with dsRNA (hairpin RNA) and prevent cell-to-cell and long distance movement of the silencing signal when expressed at the site of silencing induction, something that RNase3 is unable to do [20]. It is plausible that p7 is expressed, but perhaps only temporarily and/or in low amounts from a non-capped sgRNA, as found with the 30K movement protein of *Tobacco mosaic virus* (genus *Tobamovirus*) [22]. The hydrophobic p6 protein of Beet yellows virus needed for intercellular virus transportation (genus *Closterovirus*) [23].

The 3′-proximal part of RNA1 has been characterized in 27 SPCSV isolates [4,8,10], all of which are from cultivated sweetpotato. Four isolates (from Argentina, Brazil and Israel) belong to the WA strain, whereas 23 isolates belong to the EA strain and include one isolate from Kenya, four isolates from Uganda, two isolates from Tanzania, and 16 isolates from Peru. Only the four EA strain isolates from Uganda contain p22. It seems reasonable to hypothesize that the variability in gene content (p22) is due to recombination-mediated gene loss or gain operating on RNA1. Furthermore, it would seem worthwhile to study whether or not RNase3 and p22 are subjected to evolutionary selective pressures adjusting their abilities to break down host resistance. Detection of molecular signatures of selection pressure on the putative p7 protein might provide information as to whether or not this gene (protein) is of importance to SPCSV. However, these issues could not be addressed comprehensively unless a representative collection of SPCSV was available from sweetpotato and the putative wild host species. Reports on natural infection of wild *Ipomoea* species with SPCSV are not available, but many wild *Ipomoea* spp. can be infected with SPCSV experimentally [24,25].

The aim of this study was to characterize isolates of SPCSV infecting wild species and sweetpotatoes in Uganda in order to understand evolutionary processes shaping the virus population. In East Africa, including Uganda, several biannual and perennial wild species of *Ipomoea* and other genera of *Convolvulaceae* occur naturally, also as weeds in sweetpotato fields [26-28], but whether they are naturally infected by SPCSV has not been investigated. Therefore, a survey of SPCSV isolates in wild species and cultivated sweetpotatoes was carried out in Uganda and a number of the detected virus isolates were characterized for RNase3, p7 and p22 sequences. Whether the EA strain isolates of SPCSV show biological variability in terms of symptom induction and ability to synergise SPFMV in sweetpotato plants was also tested.

**Results**

Wild plants of family *Convolvulaceae* (genera *Ipomoea*, *Hewititia* and *Lepistemon*) were collected from a wide range of plant communities from different parts of Uganda in 2007, as previously described [28]. The wild plants sampled were mostly growing in close proximity to sweetpotato fields or grew as weeds in the field. The wild plants were identified using keys of Verdcourt [26], as described [28], and further characterized by DNA barcoding of the chloroplast gene *matK* [29]. Sweetpotato plants were also sampled from gardens in whose vicinity wild plants were collected. Furthermore, healthy seedlings of *I. setosa* were placed outdoors in shrubs next to a sweetpotato field in Mpigi district and were infected with SPCSV following natural transmission of the virus with whiteflies from the surrounding SPCSV infected wild plants or sweetpotatoes. Plants were established for further study by rooting cuttings in an insect-proof greenhouse at Makerere University Agriculture Research Institute, Kabanyolo (MUARIK).

**Phylogenetic Relationships of SPCSV Isolates**

SPCSV was detected by TAS-ELISA from plants belonging to 12 wild species (*Ipomoea acuminata, I. cairica, I. obscura, I. repens, I. rubens, I. sinensis, I. spathulata, I. stenobasis, I. tenuirostris, I. wightii, Hewititia sublobata and Lepistemon owariensis*) growing in their natural habitats in 13 different geographical regions of Uganda. A total of 30 SPCSV isolates from different wild hosts and representing the 13 different regions (Table 1) were selected for further study along with 34
isolates of SPCSV detected in sweetpotatoes in the same 13 regions of Uganda.

Sequences of the 3'—proximal region of RNA1 in the SPCSV isolates were obtained by reverse transcription, amplification by PCR using a high-fidelity DNA polymerase and direct sequencing of the PCR products without cloning. The ORF for RNase3 was of identical length (684 nt; 228 aa) in all SPCSV isolates characterized in this study, including the 64 new isolates from Uganda, the two isolates detected in local sweetpotato cultivars in Central America (HN1 in Honduras and A1GT in Guatemala) also characterized in this study, and the six previously characterized isolates from Tanzania, Uganda, Israel and Peru included for comparison (Table 1, Figure 1). The ORF for p7 was 171 nt (57 aa) in all EA strain isolates, but was shorter (165 nt; 55 aa) in the WA strain isolates. The ORF for p22 was of identical length (572 nt; 192 aa) in all isolates containing this gene. However, isolates KML33b, KTK40 (l. sinensis), SOR71 (l. obscura), HOM40, KTK39 and KTK41 (sweetpotato) characterized from Uganda and the two SPCSV isolates from Central America did not contain p22. Similarly, the previously characterized two EA strain isolates of SPCSV from Tanzania, one EA strain isolate from Kenya, the EA strain isolates from Peru and the WA strain isolates did not contain p22 [8,10]. In all SPCSV isolates lacking p22, the gap in RNA1 was identically positioned. It began immediately after the stop codon for p7 in the EA strain isolates and six nt downstream from the stop codon of p7 in the WA strain isolates.

Analysis of the RNA1 sequences using six different methods available in RDP v3.29 for detection of recombination breakpoints provided no evidence of recombination in any SPCSV isolate, as confirmed also with a PHI test (p = 0.999).

The nt sequences of SPCSV isolates characterized in this study were subjected to phylogenetic analysis, including the previously characterized WA strain isolate from Israel and five EA strain isolates from Uganda, Tanzania and Peru for comparison. Results on RNase3 sequences showed that all SPCSV isolates from Uganda belonged to the EA strain (Figure 2), no matter whether infecting wild species or sweetpotato. Only the two SPCSV isolates (HN1 and A1GT) from Central America belonged to the WA strain and formed a separate main clade with the WA isolate Is from Israel (Figure 2).

Phylogenetic analysis of the p7 gene sequences resulted in a similar grouping of isolates to the two SPCSV strains (Figure S1). Only few significant subclusters of two or three isolates were supported by high bootstrap values, and the subclusters differed depending on whether RNase3 or p7 was analyzed. Phylogenetic analysis based on the p22 gene sequences revealed no significant subclusters (data not shown).

Irrespective of the gene used for analysis, there was no phylogenetic congruence of isolates with their host species, geographical origins in Uganda, and whether or not they encoded the p22 gene.

**Nucleotide Diversity and Selection Pressure**

Nt sequence identities of EA strain isolates ranged from 92.5% (p7), 96.0% (p22) and 97.7% (RNase3) to 100% depending on the gene analyzed. The aa sequence identities ranged from 93.1% (p7), 94.8% (p22) and 98.7% (RNase3) to 100%. The p22 protein of isolate MBL16 (from l. acuminata) was distinguished from other isolates by 12 unique aa substitutions (Supporting information, Figure S2). The sequences of WA strain isolates from Honduras and Guatemala were identical to each other and nearly identical to the isolate Is; the nt (aa) sequence identities were 99.4% (98.7%) for RNase3 and 99.4% (100%) for p7. The EA and WA strain isolates showed nt (aa) sequence identities of 83.8-84.3% (81.6-82.5%) for RNase3 and 76.2-79.8% (60.7-66.7%) for p7.

Irrespective of the host species from which the SPCSV isolates were characterized, nt diversity (π) values for each of the three protein coding regions studied were relatively low being 2.2%, 4.3% and 0.71% for the RNase3, p7 and p22 gene, respectively. The non-synonymous nt diversity (πs) was 1.2%, 4.1% and 0.4% for the RNase3, p7 and p22 gene, respectively, whereas the synonymous nt diversity (πs) was 5.6%, 5.5%, and 1.6%, respectively. The value of πs was 4.7 or 4.0 fold lower than the value of πs for the RNase3 and p22 gene, respectively. The value of πs (4.1%) was slightly lower than πs (5.5%) for the p7 protein. The selection pressures suggested by the aforementioned results were assessed by a maximum likelihood framework of codon substitution under model MO which yielded ω values of 0.268, 0.562 and 0.324 for the RNase3, p7 and p22 genes, respectively, indicating purifying selection (Table 2). Heterogeneity of selective pressure was revealed by a test using an M3 vs. M0 LRT which showed that M3 fitted the data significantly better than M0 (Table 2) for all proteins. M3 for RNase3 suggested that 55.5% of sites were evolving under strong purifying selection (ω = 0.072), 43.9% of sites under weak purifying selection (ω = 0.513) and only 0.6% of sites under positive selection (ω = 7.781) (Table 2). The proportions of different site classes under M3 for the p7 and p22 genes are shown in Table 2.

LRTs of nested models M2a vs. M1a showed that M2a was a better fit than M1a for RNase3, p7 and p22 proteins (Table 2) which is consistent with positive selection on some aa sites. Parameter estimates under M2a for the RNase3 showed a large proportion of sites (86.4%) under purifying selection (ω = 0.170), 13.1% under neutral evolution (ω = 1.000) and only 0.5% under positive selection (ω = 9.690). These results were verified in an independent LRT involving M8 vs. M7 in which M8 fitted the data for each gene better than M7 (Table 2). The Närve Empirical Bayes (NEB) inference provided similar results as BEB inference under M3, M2a and M8 (Table 2).

**Interactions Between SPCSV and SPFVMV from Wild Plants in Sweetpotato**

Twenty-four of those 64 isolates of SPCSV, which were characterized at the molecular level, had been initially transmitted with whiteflies from the original 12 wild plants and 12 sweetpotato plants sampled from the field to healthy plants of sweetpotato cv. Tanzania (Table 1). These wild plants belonged to seven species (l. hederifolia, l. obscura, l. rubens, l. setosa, l. sinensis, Hewittia sublobata and Lepidiontomon owariensis) which were amenable for establishment in the greenhouse using cuttings and were hence feasible for the experiment. The whitefly-inoculated plants of cv. Tanzania...
Table 1. Sequence accessions of sweet potato chlorotic stunt virus (SPCSV) isolates characterized for their 3' region of RNA1 from wild plants and cultivated sweetpotato in this study and those retrieved from the database previously characterized from cultivated sweetpotato.

| Isolate   | Serotype | Geographical origin | Sequence accession no. | Host   | Habitat description | Reference          |
|-----------|----------|---------------------|------------------------|--------|---------------------|--------------------|
| ARU54*    | EA       | Arua, Uganda        | GU127660               | I. sinensis | Sweetpotato field   | This study         |
| ARU59     | EA       | Arua, Uganda        | GU127659               | I. stenobasis | Ornamental (20 m)   | This study         |
| BUSH2     | EA       | Bushenyi, Uganda    | GU127638               | I. tenuirostris | Sweetpotato field | This study         |
| BUSH13    | EA       | Bushenyi, Uganda    | GU127637               | I. cairica | Grassland (200 m)   | This study         |
| BUSH42    | EA       | Bushenyi, Uganda    | GU127639               | I. acuminata | Shrubland (50 m)    | This study         |
| BUSH66    | EA       | Bushenyi, Uganda    | GU127635               | I. tenuirostris | Thicket (200 m)    | This study         |
| HOM32     | EA       | Hoima, Uganda       | GU127650               | I. cairica | Abandoned homestead (100 m) | This study |
| HOM53*    | EA       | Hoima, Uganda       | GU127641               | H. sublobata | Sweetpotato field   | This study         |
| HOM76*    | EA       | Hoima, Uganda       | GU127653               | I. hedenfolia | Grassland (50m)   | This study         |
| HOM81     | EA       | Hoima, Uganda       | GU127685               | I. acuminata | Cassava-Maize intercrop (20 m) | This study |
| HOM91     | EA       | Hoima, Uganda       | GU127633               | L. owariense | Previous sweetpotato field (50 m) | This study |
| KAP88     | EA       | Kachchorwa, Uganda  | GU127662               | I. obscura | Grassland (10 m)    | This study         |
| KAP90     | EA       | Kachchorwa, Uganda  | GU127613               | I. spathulata | Shrubland (50 m)    | This study         |
| KML23*    | EA       | Kamuli, Uganda      | GU127658               | I. sinensis | Shrubland (10m)     | This study         |
| KML33a*   | EA       | Kamuli, Uganda      | GU127657               | I. sinensis | Sweetpotato field   | This study         |
| KML33b*   | EA       | Kamuli, Uganda      | GU127599               | I. sinensis | Sweetpotato field   | This study         |
| KNG59     | EA       | Kanungu, Uganda     | GU127629               | L. owariense | Shrubland (50 m)    | This study         |
| KTK40*    | EA       | Katakwi, Uganda     | GU127602               | I. sinensis | Sweetpotato field   | This study         |
| MAS46*    | EA       | Masindi, Uganda     | GU127661               | L. owariense | Thicket (40 m)      | This study         |
| MAS52     | EA       | Masindi, Uganda     | GU127655               | I. tenuirostris | Disturbed habitat (5 m) | This study |
| MAS69*    | EA       | Masindi, Uganda     | GU127652               | H. sublobata | Grassland (70 m)    | This study         |
| MBL16     | EA       | Mbale, Uganda       | GU127649               | I. acuminata | Ornamental (100 m)  | This study         |
| MBL86     | EA       | Mbale, Uganda       | GU127646               | I. acuminata | Grassland (10 m)    | This study         |
| MBL91     | EA       | Mbale, Uganda       | GU127666               | I. acuminata | Disturbed habitat (5 m) | This study |
| RKG77     | EA       | Rakai, Uganda       | GU127631               | I. cairica | Sweetpotato field   | This study         |
| RUK106    | EA       | Rukungiri, Uganda   | GU127640               | I. wightii | Hedge (2 m)         | This study         |
| SET1*     | EA       | Mpigi, Uganda       | GU127664               | I. setosa | Shrubland (30 m)    | This study         |
| SET5*     | EA       | Mpigi, Uganda       | GU127615               | I. setosa | Shrubland (30 m)    | This study         |
| SOR6      | EA       | Soroti, Uganda      | GU127616               | I. repens | Grassland on a rock foot (500 m) | This study |
| SOR45*    | EA       | Soroti, Uganda      | GU127618               | I. rubens | Papyrus swamp (40 m) | This study         |
| SOR71*    | EA       | Soroti, Uganda      | GU127605               | I. obscura | Abandoned sweetpotato field (80m) | This study |
| ARU57*    | EA       | Arua, Uganda        | GU127663               | sweetpotato | Sweetpotato field   | This study         |
| ARU91     | EA       | Arua, Uganda        | GU127612               | sweetpotato | Sweetpotato field   | This study         |
| BUR4      | EA       | Kanungu, Uganda     | GU127647               | sweetpotato | Sweetpotato field   | This study         |
| BUSH1     | EA       | Bushenyi, Uganda    | GU127617               | sweetpotato | Sweetpotato field   | This study         |
| HOM12     | EA       | Hoima, Uganda       | GU127651               | sweetpotato | Sweetpotato field   | This study         |
| HOM40     | EA       | Hoima, Uganda       | GU127606               | sweetpotato | Sweetpotato field   | This study         |
| HOM89     | EA       | Hoima, Uganda       | GU127625               | sweetpotato | Sweetpotato field   | This study         |
| KML4      | EA       | Kamuli, Uganda      | GU127643               | sweetpotato | Sweetpotato field   | This study         |
| KML7*     | EA       | Kamuli, Uganda      | GU127656               | sweetpotato | Sweetpotato field   | This study         |
| KNG2*     | EA       | Kanungu, Uganda     | GU127610               | sweetpotato | Sweetpotato field   | This study         |
| KNG53     | EA       | Kanungu, Uganda     | GU127632               | sweetpotato | Sweetpotato field   | This study         |
| KNG92     | EA       | Kanungu, Uganda     | GU127607               | sweetpotato | Sweetpotato field   | This study         |
| KNG101    | EA       | Kanungu, Uganda     | GU127628               | sweetpotato | Sweetpotato field   | This study         |
| KTK39*    | EA       | Katakwi, Uganda     | GU127604               | sweetpotato | Sweetpotato field   | This study         |
| KTK41*    | EA       | Katakwi, Uganda     | GU127603               | sweetpotato | Sweetpotato field   | This study         |
| MBR2      | EA       | Mbarara, Uganda     | GU127611               | sweetpotato | Sweetpotato field   | This study         |
| MBR70     | EA       | Mbarara, Uganda     | GU127630               | sweetpotato | Sweetpotato field   | This study         |
| MKN29     | EA       | Mukono, Uganda      | GU127654               | sweetpotato | Sweetpotato field   | This study         |
| MPG88*    | EA       | Mpigi, Uganda       | GU127622               | sweetpotato | Sweetpotato field   | This study         |
| MSK7*     | EA       | Masaka, Uganda      | GU127624               | sweetpotato | Sweetpotato field   | This study         |
| MSK62*    | EA       | Masaka, Uganda      | GU127623               | sweetpotato | Sweetpotato field   | This study         |

SPCSV in Wild Species
infected with SPCSV were multiplied by rooting stem cuttings and the new plants were observed for symptoms in the greenhouse. All isolates induced similar symptoms of mild chlorosis in younger leaves and purpling symptoms on older leaves, as shown for isolates HOM76 and SOR71 in Figures 3B and 3D, respectively.

Subsequently, new cuttings were grafted-inoculated with SPFMV isolate RUK73 (obtained from a plant of *I. obscura* in Uganda; [29]) in two experiments. All SPCSV isolates (except SOR71) induced the characteristic symptoms of SPVD including small, crinkled and strap-like leaves and severe retardation of plant growth in co-infected plants, as exemplified with coinfection of HOM76 and SPFMV in Figure 3C. Furthermore, three tested isolates lacking the *p22* gene (KTK39, KTK40 and KTK41) induced similar symptoms of SPVD as those 20 isolates which contained *p22*.

Accumulation of SPCSV and SPFMV RNA was compared between the plants and treatments by dot blot hybridization using different amounts of total RNA extracted from the fully-grown upper leaves of *cv. Tanzania*. The viral RNA was detected with DIG-labelled RNA probes specific to *RNA3* and the CP-encoding region of SPFMV. The titers of SPCSV RNA1 in the leaves of plants co-infected with SPCSV and SPFMV were similar or slightly higher than those in plants infected with SPCSV alone (Figure 3F). The low concentrations of SPFMV RNA were barely detectable in plants of *cv. Tanzania* infected with SPFMV alone, but were greatly enhanced following co-infection with SPCSV, irrespective of the presence or absence of *p22* in the SPCSV isolate (Figure 3G). The relative differences in viral RNA concentrations were reproducible in the two experiments.

Suppression of RNA silencing is supposed to increase virus titers in infected tissues, but virus concentrations were enhanced also in absence of *p22* that is a strong suppressor of gene silencing, including *p22* of isolate Ug for comparison. Silencing of the jellyfish (*Aequorea victoria*) green fluorescent protein (GFP) gene expression was induced by double-stranded (hairpin) RNA in leaves of *Nicotiana benthamiana* using an *Agrobacterium tumefaciens* infiltration assay, as described [31]. All three *p22* proteins supported GFP expression, i.e., suppressed *gfp* silencing, in contrast to expression of *β-glucuronidase* (GUS) included as a negative control (Figure 4). These results were consistent with previous studies indicating that suppression of RNA silencing by *p22* is dispensable for development of SPVD or enhancement of the titers of co-infecting viruses unrelated to SPCSV [21].

SPCSV isolate SOR71 was an exception, because the titers of this isolate both in single infection and double infection with SPFMV were too low to generate detectable signals within the
Figure 1. Alignment of the 43 different RNase3 protein amino acid (aa) sequences of Sweet potato chlorotic stunt virus. Groups of isolates containing identical RNase3 aa sequences are represented each by a single isolate. Numbers on top of the alignment indicate the aa positions with reference to isolate Ug (AJ428554) whereas numbers on the right indicate the number of the last amino acid at that position for each isolate. Numbers at the bottom of the alignment indicate aa positions in the new unknown virus (KML33b) related to SPCSV and detected in this study. The class 1 RNase III signature motif at aa positions 36-44 in SPCSV isolates (aa 42-50 in KML33b) is boxed. Two aa sites predicted to be under positive selection are indicated with black shades. Names of isolates characterized from wild plants are in bold.

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Figure 2. Phylogenetic analysis of genes coding for RNase3 of *Sweet potato chlorotic stunt virus* and the corresponding sequence of an unknown related virus (KML33b) detected in this study. The branch of KML33b is not fully depicted. Names of isolates characterized from wild plants are indicated in bold, whereas the ten SPCSV isolates lacking the p22 gene are indicated with a black triangle (▲). Numbers at branches represent bootstrap values of 1000 replicates. Only bootstrap values of ≥ 50% are shown. Scale indicates Kimura units in nucleotide substitutions per site [55].

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time of exposure used (Figure 3F, samples 4a and 4b). Nevertheless, SOR71 was readily detected in these plants by RT-PCR and sequencing of the PCR products (data not shown). The plants co-infected with SOR71 and SPFMV (Figure 3E) did not develop SPVD symptoms, but symptoms remained mild and similar to those caused by SOR71 alone (Figure 3D). However, SPFMV titres were enhanced to the same extent as with other SPCSV isolates (Figure 3G, sample 4).

Due to all these unexpected results on SOR71 (which lacks p22), RNase3 of SOR71 was tested for its ability to suppress RNA silencing using the aforementioned agroinfiltration assay. However, because RNase3 suppresses only sense-mediated gene silencing [21], i.e., caused cosuppression or “weak gfp silencing” [31], the gfp gene was overexpressed by agroinfiltration in leaves of transgenic N. benthamiana constitutively expressing gfp (line 16c) [32] and RNase3 of SOR71 was coexpressed in the same leaf tissue from another A. tumefaciens strain. RNase3 of the previously characterized SPCSV isolate Ug [20] and β-glucuronidase (GUS) were constitutively expressing N. benthamiana (strain). RNase3 of the previously characterized SPCSV isolate Ug [20] and ß-glucuronidase were used as controls. A. tumefaciens strain. RNase3 of SOR71 was coexpressed in the same leaf tissue from another A. tumefaciens strain. RNase3 of the previously characterized SPCSV isolate Ug [20] and ß-glucuronidase (GUS) were included as a positive and a negative control, respectively. Results of the two experiments showed consistently that RNase3 of SOR71 and Ug supported expression of GFP fluorescence and suppressed sense-mediated silencing with similar efficiency (Figure 4).

Table 2. Parameter estimates, log-likelihood (lnL) values, ω-ratio (dω/d0), and likelihood ratio test (LRT) statistics under six different maximum likelihood models of codon substitution that were used to investigate selection pressures exerted on the RNase3, p7 and p22 proteins encoded by the 3’ region of Sweet potato chlorotic stunt virus genomic RNA1.

| Protein | Modelsa | Parameter estimatesb | ω-ratio | Log-likelihood | LRT statisticc | Positively selected |
|---------|---------|----------------------|---------|---------------|---------------|-------------------|
| RNase3  | M0      | ω = 0.268            | 0.268   | −2164.398     | p < 0.001     | 594*              |
|         | M3      | p0 = 0.555, p1 = 0.439 (p2 = 0.006), ω0 = 0.072, ω1 = 0.513, ω2 = 7.781 | 0.309 | −2149.580     | p < 0.001     | 594*              |
|         | M1a     | p0 = 0.857 (p1 = 0.143), ω0 = 0.157 (ω1 = 1.000) | 0.278 | −2153.607     | not allowed   |                   |
|         | M2a     | p0 = 0.864, p1 = 0.131 (p2 = 0.005) | 0.323 | −2150.100     | p < 0.05     | 595, 1594**       |
|         | M7      | ω0 = 0.170, ω1 = 1.000, ω2 = 9.690 | 0.287 | −2155.777     | not allowed   |                   |
|         | M6      | p0 = 0.995 (p1 = 0.005) | 0.310 | −2149.542     | p < 0.01     | 595, 1594*        |
| p7      | M0      | ω = 0.562            | 0.562   | −624.805      | none          |                   |
|         | M3      | p0 = 0.133, p1 = 0.832 (p2 = 0.035), ω0 = 0.000, ω1 = 0.527, ω2 = 5.966 | 0.652 | −808.709      | p < 0.0001   | 32G**, 47C*       |
|         | M1a     | p0 = 0.851 (p1 = 0.149), ω0 = 0.271 (ω1 = 1.000) | 0.379 | −817.282      | not allowed   |                   |
|         | M2a     | p0 = 0.863, p1 = 0.102 (p2 = 0.035) | 0.661 | −808.994      | p < 0.001   | 32G**, 47C        |
|         | M7      | ω0 = 0.403, ω1 = 1.000, ω2 = 6.015 | 0.465 | −817.970      | not allowed   |                   |
|         | M6      | p0 = 0.965 (p1 = 0.035), ω0 = 0.000, ω1 = 6.123 | 0.664 | −809.176      | p < 0.001    | 32G**, 47C        |
| p22     | M0      | ω = 0.324            | 0.324   | −1413.955     | None          |                   |
|         | M3      | p0 = 0.890, p1 = 0.087 (p2 = 0.022), ω0 = 0.249, ω1 = 0.249, ω2 = 7.551 | 0.414 | −1400.157     | p < 0.001    | 5A*, 31L, 160L, 161L** |
|         | M1a     | p0 = 0.880 (p1 = 0.119), ω0 = 0.170 (ω1 = 1.000) | 0.269 | −1407.356     | not allowed   |                   |
|         | M2a     | p0 = 0.977, p1 = 0.000 (p2 = 0.022) | 0.413 | −1400.217     | p < 0.001    | 5A*, 31L, 160L, 161L** |
|         | M7      | ω0 = 0.249, ω1 = 1.000, ω2 = 7.538 | 0.316 | −1409.453     | not allowed   |                   |
|         | M8      | p0 = 0.977 (p1 = 0.022), ω0 = 0.990, ω1 = 6.009, ω2 = 7.597 | 0.415 | −1400.196     | p < 0.001    | 5A*, 31L, 160L, 161L** |

a The models are according to Yang et al. [57] (M0, M3, M7, M8), Wong et al. [59] and Yang et al. [58] (M1a, M2a).
b The numbers of parameters for the different models were 1 (M0), 2 (M1a), 4 (M2a), 5 (M3), 2 (M7), or 4 (M8).

c LRT statistics of M3 vs. M0 are tests of heterogeneity of selection pressures among codon sites, while M2a vs. M1a and M8 vs. M7 are tests of positive selection, all of which assess LRT statistic (−2lnL) against a chi-square distribution with the degrees of freedom (df) equal to the difference in the number of parameters between the nested models under comparison.
d Positively selected amino acid sites at posterior probabilities P > 95.0 (*) or P > 99 (**) are shown. Identification of amino acids under positive selection is based on Naïve empirical Bayes (Neb) (under M3) or Bayes empirical Bayes (BEB) inference (under M2a or M8).

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Figure 3. Symptoms and virus accumulation in single or double infections with Sweet potato chlorotic stunt virus (SPCSV) and Sweet potato feathery mottle virus (SPFMV) in sweetpotato plants of cv. Tanzania. (A) A plant infected with SPFMV (isolate RUK73) shows no obvious virus symptoms 6 weeks post-inoculation. (B) Chlorosis and purpling of older leaves induced by SPCSV isolate HOM76. (C) Typical symptoms of sweetpotato virus disease (SPVD) including retarded growth, severe leaf strapping and puckering induced by co-infection with SPCSV isolate HOM76 and SPFMV. (D) Chlorosis and purpling of older leaves induced by SPCSV isolate SOR71, and (E) similar symptoms in a plant co-infected with SOR71 and SPFMV. Young leaves develop normally in (D) SOR71-infected plants and (E) plants co-infected with SOR71 and SPFMV. In both cases the plants display only mild chlorosis typical of SPCSV infection, which indicates that SOR71 is not able to induce SPVD in co-infection with SPFMV. (F) SPCSV RNA detected by dot blot hybridization with a digoxigenin-labelled RNA probe specific to the RNase3 gene in (a) plants infected with SPCSV alone or (b) plants co-infected with the SPCSV and SPFMV. The amounts of total plant RNA dotted on the membrane are indicated. The SPCSV isolates tested were 1, KTK39; 2, KTK40; 3, KTK41; 4, SOR71; 5, MAS69; 6, SET5; 7, MSK7; 8, TOR16; and 9, HOM76. Isolates 1 to 4 are lacking the p22 gene. Note that SOR71 (4a and 4b) accumulates at very low concentrations in sweetpotato leaves and is barely detectable. H, non-inoculated healthy plant of cv. Tanzania. (G) SPFMV RNA detected by dot blot hybridization with a digoxigenin-labelled RNA probe specific to the CP-encoding region. Samples 1 to 9 are those co-infected with SPCSV and SPFMV and tested for SPCSV (i.e., samples 1b to 9b) in (F). Three additional samples co-infected with SPCSV (10, MAS46; 11, TOR14; and 12, MPG88) and SPFMV were included. Note that SOR71 synergises SPFMV, which is detected by the enhanced SPFMV concentrations (sample 4) as compared to the samples (FM) from cv. Tanzania infected with SPFMV only. H, non-inoculated healthy plant of cv. Tanzania.

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A New Putative Crinivirus encodes an RNase3-like Suppressor of Silencing

One plant of *I. sinensis* was co-infected with two distinct virus isolates, which were designed as KML33a and KML33b (Table 1). KML33a was nearly identical to other EA strain isolates of SPCSV (Figures 1 and 2). In contrast, the nt (aa) sequence of RNase3 in KML33b was only 58.1-59.5% (53.0-55.6 %) identical to the EA strain isolates of SPCSV and 56.3% (50.9-51.3%) identical to the WA strain isolates (Figures 1 and 2). Furthermore, the RNase3 of KML33b was predicted to contain six additional aa residues at the N-terminus and two additional aa residues at the C-terminus, compared to that of SPCSV (Figure 1). The ORF for p7 was of identical length in KML33b and the SPCSV isolates, and the level of sequence similarity of p7 in KML33b and SPCSV isolates was comparable with the similarity of RNase3 nt and aa sequences. KML33b contained no ORF for p22. The corresponding gap in the genomic sequence of RNA1 started at an identical position but was 20 nt shorter as compared with SPCSV isolates. The low identity of KML33b to SPCSV and other viral sequences available in databases suggested that KML33b is a new virus related to viruses in genus *Crinivirus*.

The RNase3-like protein of KML33b was tested for its ability to suppress sense-mediated gene silencing (cosuppression), as described above. Leaves of the *gfp*-transgenic *N. benthamiana* line 16c were co-infiltrated with two *Agrobacterium* strains, one expressing *gfp* to achieve cosuppression (silencing) of *gfp* and another expressing RNase3 of isolate SOR71 (*I. obscura*), Ug, or the RNase3-like protein of the new virus KML33b (*I. sinensis*). Leaves were illuminated with UV light and photographed from the underside with a digital camera 6 days postinfiltration. Hence, the KML33b RNase3 protein was able to suppress silencing.

Discussion

Twelve new natural host species for SPCSV were detected in this study. Natural occurrence of SPCSV in wild species has not been reported, but under experimental conditions, the WA strain of SPCSV is able to infect two of the species (*I. acuminata* and *I. wightii*; [24], which were found infected with EA strain isolates of SPCSV in the wild in Uganda.

Analysis of the RNase3, p7 and p22 gene sequences in a large number of SPCSV isolates from 12 wild species and sweetpotatoes in Uganda revealed nearly identical nt diversity indices and provide little phylogenetic evidence for
diversification of the virus population. The observed similarities between virus isolates and lack of phylogenetic congruence with their wild and cultivated hosts [33,34] suggested that SPCSV isolates in Uganda are exchanged frequently between hosts in the field via transmission by the whitefly vectors. Similar observations have been made for Sweet potato mild mottle virus (SPMMV; genus Ipomovirus; Potyviridae) [35] and SPFMV [29] in the wild species of Convolvulaceae and cultivated sweetpotatoes in Uganda, Rice yellow mottle virus (RYMV; genus Sobemovirus) in cultivated rice and wild graminaceous species in East, Central and West Africa [36], and African cassava mosaic virus and East African cassava mosaic cameroon virus (genus Begomovirus; Geminiviridae) in cassava and various wild hosts in west Africa [37]. Furthermore, no isolate of the WA strain of SPCSV was detected in Uganda, which is consistent with previous studies on SPCSV in East Africa [5,6,9,10]. The two SPCSV isolates detected in sweetpotato samples from Central America belonged to the WA strain and are the only isolates besides the four isolates from Argentina, Brazil and Israel [8,10] which have been characterized for RNA1 sequences.

Purifying selection was implicated on the majority of amino acids in SPCSV RNase3, which may be important for maintaining the reactive sites as well as the conformation necessary for enzymatic activities as proposed for some non-viral RNase III enzymes [38]. However, two amino acids were under positive selection in RNase3. They could be functionally important because the residue at position 159 is located in the C-terminal double-stranded RNA binding domain (dsRBD) and the other one at position 95 is in the N-proximal catalytic endoribonuclease domain (endND) of Class1 RNase III enzymes [39,40] (Figure 1). Hence, positive selection might translate into more efficient dsRNA binding and/or dsRNA cleavage, e.g., targeting different types of double-stranded small RNAs which RNase3 is able to cleave [21]. It is noteworthy that the positively selected amino acid residues 95Y and 159H in RNase3 found in the majority of EA strain isolates are not encoded by the three isolates of the WA strain but replaced by serine and arginine, respectively, in these isolates. The significance of this difference may become apparent with more isolates of the WA strain analyzed in future.

The important function known for SPCSV RNase3 is its ability to eliminate antiviral defence, which also predisposes sweetpotato plants to development of severe disease symptoms following co-infection with heterologous viruses [21]. SPVD is the most harmful example of the viral synergism mediated by RNase3 and manifests in sweetpotato plants co-infected with SPCSV and SPFMV. This study demonstrated that isolates of SPCSV infecting wild plant species incite SPVD when transmitted to SPFMV-infected sweetpotato. However, isolate SOR71 detected in Ipomoea obscura was exceptional in that it did not incite SPVD symptoms although accumulation of SPFMV was enhanced to similar levels as with other SPCSV isolates in co-infected plants. The low titers of SOR71 were not the likely reason for its deviance from other isolates in induction of SPVD because quite minute titers of SPCSV are sufficient to enhance accumulation of SPFMV and induce SPVD, as shown in transgenic sweetpotatoes exhibiting highly efficient RNA-silencing based resistance against accumulation of SPCSV [41]. The results with SOR71 are very significant because they show that enhancement of SPFMV titer can be uncoupled from induction of SPVD.

Because experimental evidence shows that RNase3 alone is responsible for the two phenotypic outcomes of the SPCSV-mediated synergism [21], the unique response in SOR71-infected sweetpotatoes could be linked to unique features of RNase3 in SOR71. However, the only difference which distinguishes the RNase3 of SOR71 from other SPCSV isolates is that a methionine residue is replaced with an isoleucine at position 54 in the catalytic domain adjacent to the RNase III signature motif (Figure 1). The data suggest a hypothesis on a dual mechanism by which the SPCSV-SPFMV synergism might be induced. The RNA-binding activity of RNase3 may contribute to the silencing suppression enforced by the HC-Pro protein of SPFMV, and the additive effects of the two silencing suppressors may overcome antiviral defence which limits accumulation of SPFMV in cv. Tanzania. This scenario is supported by siRNA binding being a common strategy for silencing suppression by viral proteins, including HC-Pro [42,43]. The catalytic activity of RNase3 shown to be required for silencing suppression might, in turn, manipulate or modulate cellular RNA biogenesis and homeostasis to facilitate accumulation of SPCSV to the typical titers in sweetpotato, something that SOR71 fails to do. RNase3 activity in sweetpotato cells seems to be specifically targeted to a component of the antiviral defence system because plants over-expressing RNase3 display no discernible developmental or physiological phenotypes, except loss of resistance to viruses [21]. Therefore, the severe symptoms of SPVD which significantly differ from the much milder symptoms caused by SPCSV may be an ‘accidental’ consequence of the general loss of antiviral defence caused by RNase3, which allows other viruses disturb the physiology of co-infected plants in a manner which they alone are not able to.

A novel virus isolate carrying predicted ORFs for proteins homologous to the RNase3 and p7 of SPCSV was detected in I. sinensis and designated as KML33b. It was found co-infecting the wild plant with an EA strain isolate of SPCSV and both were transmitted by whiteflies to healthy sweetpotato cv. Tanzania. The sequences of KML33b were highly divergent from SPCSV isolates and showed < 60% nt and aa sequence identity. The RNase3-like protein of KML33b was able to suppress sense RNA-mediated gene silencing, similar to RNase3 of SPCSV. The finding is significant because, so far, SPCSV was the only RNA virus known to carry a gene for an RNase III-like protein that suppresses RNA silencing [8,20,21]. Other genomic and biological features of KML33b remain a topic for future study.

Positive selection detected on residues 32G and 47C of the p7 protein suggests that it is important at some stage of the viral infection cycle and may correspond to host adaptation of SPCSV. The functions of the p7 protein in SPCSV genome are not well understood, which hampers more specific attempts to predict the biological significance. The two aa positions occupy hydrophobic residues in all isolates characterized and hence contribute to hydrophobicity which is characteristic to many
small proteins encoded by members of family Closteroviridae [19]. Presuming that p7 is involved in viral cell-to-cell movement, similar to the hydrophobic p6 protein of Beet yellows virus (Closterovirus) [23,44], positive selection of specific residues of p7 may occur to adapt the virus to, e.g., the multitude of different host species available for SPCSV in Uganda. The p22 gene was present in the majority and absent only from few (7.7%) EA strain isolates characterized from Uganda. This finding is intriguing, because previous studies comparing 23 EA strain isolates from Tanzania, Kenya and Peru found p22 only in the four Ugandan isolates [8,10]. Furthermore, the WA strain isolates from Israel and South and Central America did not contain p22. No molecular signatures for recombination were detected in the studied region of RNA1. Moreover, SPCSV isolates showed no phylogenetic congruence for the RNase3 and p7 genes according to whether or not they encode p22, indicating that variability in RNase3 and p7 and acquisition of p22 have been driven by independent evolutionary processes. Positive selection was implicated on four aa sites of p22, which may indicate some measure of selective advantage in RNA1 silencing suppression or other yet unidentified functions of p22 in the EA strain. Purifying selection was found on 97.8% of the amino acids of p22 and has also been implicated on the evolution of other silencing suppressor proteins such as p23 and p20 encoded by Citrus tristeza virus (genus Closterovirus) [45]. Taken together, these data support the hypothesis that p22 provides an advantage to the EA strain isolates prevailing in Uganda. Data about the gene content in RNA1 of SPCSV in other countries of the Lake Victoria zone in East Africa is lacking almost completely and the incidence of SPCSV isolates containing p22 remains a subject of an interesting future study. However, in light of the current information, it seems likely that SPCSV has acquired p22 from an unknown organism in East Africa.

The current study has extended the knowledge of genetic variability and evolution of SPCSV by including many isolates from previously unknown wild host species in East Africa and characterizing a large number of SPCSV isolates for the 3'-terminal part of RNA1, which encodes proteins of conceivable importance for viral virulence. Results on selective pressures directed on the studied ORFs provided evidence that RNase3, p7 and p22 are mainly under purifying selection. Positive selection on a few amino acids in each protein implied that SPCSV isolates are under continuous adaptation, presumably due to the large diversity of hosts they infect in the field. The study of viral diversity in the wild species also revealed a previously unknown virus which is related to SPCSV and carries an RNA silencing suppressor homologous to RNase3. Furthermore, experiments using one SPCSV isolate from wild species demonstrated that the two phenotypic outcomes of synergism between SPCSV and SPFMV, namely enhancement of SPFMV accumulation and development of severe symptoms, can be uncoupled. These data provide novel understanding on evolution of the genetically diverse viruses in Closteroviridae and highlight the value of studying virus isolates from a wide range of natural hosts.

Materials and Methods

Collection of Virus Isolates

Wild plants of family Convolvulaceae were collected in 2007, identified and maintained as described by Tugume et al. [28,29,35]. Samples were collected from private land with the owner’s permission. For any other locations, no specific permissions were required for sampling. The field studies did not involve endangered or protected species.

Isolates of SPCSV from 13 wild plants and 12 plants of cultivated sweetpotato were whitefly-transmitted to healthy sweetpotato cv. Tanzania at MUARIK. Whiteflies (Bemisia tabaci Gennadius) were raised and maintained on cassava (Manihot esculenta Crantz) plants in insect-proof cages for three weeks. Single source plants infected with SPCSV were placed into insect-proof cages and 70-100 adult whiteflies were transferred to each. After a 36-h access period, a 3-week old healthy sweetpotato plant cv. Tanzania (raised from a cutting) was added into the cage for 72 h to be inoculated by the whiteflies and then treated with pymetrozine (Fulfill; Syngenta, Greensboro, NC) to kill the whiteflies. The inoculated sweetpotato plants were tested for SPCSV after 3 wk. Stem cuttings were taken and transferred to University of Helsinki, Finland, where they were grown in an insect-proof greenhouse (temperature 25-30 °C, relative humidity 70%) under natural daylight extended to 16h by illumination with high-pressure sodium halide lamps (light intensity of 150-200 μmol s⁻¹m⁻²).

Serological and Molecular Detection of SPCSV

Triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) was used for serological detection of SPCSV as described [46]. Polyclonal and monoclonal antibodies to SPCSV were provided by the International Potato Centre (Lima, Peru) and Julius Kühn Institute (Braunschweig, Germany), respectively. Testing was repeated on the plants established in the screenhouse. The SPCSV in wild plants was grafted-transmitted to healthy sweetpotato plants of cv. Tanzania for ease of maintenance and study. The presence of SPCSV in plants grown from the tuberous roots obtained from Central America was tested using molecular methods.

Total RNA was extracted using TRIzol Reagent (Invitrogen Ltd, UK) according to the manufacturer’s instructions from 200 mg of leaf tissue. First-strand cDNA was synthesized on 3 μg of total RNA with random primers and Moloney murine leukemia virus (M-MuLV) reverse transcriptase RNase H’ (Finnzymes Oy, Finland) according to the manufacturer’s instructions. The 3’ genomic region of the RNA1 of SPCSV was amplified by polymerase chain reaction (PCR) using a forward CSVR3-F2 (5'-GTGTTTCTACATGTGTGTGCT-3’) and a reverse CSVp22-R2 (5’-AGGTTATGACTTAGGTATAAAC-3’) primers designed in this study and which were complementary to the genomic positions 7491-7515 and 9241-9265, respectively, of the SPCSV EA strain RNA1 (AJ428554; Kreuze et al., 2002). The primers PolymF4b (5’-CGAATACGTGGTTGTCAA-3’) and WA-R1 (5’-CTCTAGGATAACAACATTAACTC-3’) complementary to genomic positions 7046-7063 and 1270-1291 of SPCSV RNA1 accessions AJ428554 and EU124491, respectively, were used
to amplify the 3' genomic region of two SPCSV isolates from Central America. The PCR mixture and cycling parameters were those recommended for Phusion High-Fidelity DNA Polymerase (Finnzymes Oy, Finland).

Sequence Analyses

The PCR products were purified using a combination of Exonuclease I and Calf Intestine Alkaline Phosphatase (Fermentas) as recommended [47]. The purified PCR products were sequenced directly with the Big Dye Terminator kit version 3.1 on an ABI 3130 XL Genetic Analyzer at Haartman Institute, University of Helsinki, using the PCR primers. Sequencing was done in both directions for each of the two independent amplicons of each isolate. The sequences were compared by BLAST search with the existing sequences available in the NCBI database.

Nucleotide (nt) sequences were aligned using CLUSTALX version 1.83 [46], and examined visually. The ORFs for the RNase3, p7 and p22 genes of SPCSV were translated into amino acid (aa) sequences using the EMBOSS tool (http://www.ebi.ac.uk/emboss/translate/index.html?). Percent nt and aa identities between sequences were computed using the CLUSTALW procedure [49] as implemented in MEGALIGN program of the DNASTAR software package (DNASTAR Inc., Madison WI, USA).

Average nucleotide diversity ($π$), the number of nonsynonymous nt substitutions per nonsynonymous site ($d_{ns}$) and the mean of dN values in all pairwise comparisons of the sequences of the gene (i.e., the nonsynonymous nucleotide diversity, symbolized $π_{ns}$), and the number of synonymous nt substitutions per synonymous sites ($d_{s}$) and the mean of dS values in all pairwise comparisons of the sequences of the gene (i.e., the synonymous nucleotide diversity, symbolized $π_{s}$) for each of the genes RNase3, p7, and p22 were calculated using DnaSP version 5 [50]. The sequence data were analyzed for recombination breakpoints using RDP, Maximum Chi square, Bootscans, Chimera, and Sister Scan (Recombination Detection Program RDP3 Package version 3.29; [51]) and verified using the Pairwise homoplaspy index (PHI) test for recombination [52].

Phylogenetic relationships based on the RNase3, p7 and p22 genes were analyzed using the Neighbor-joining algorithm [53] as implemented in MEGA4 [54] using the Kimura two-parameter nucleotide substitution model [55]. Statistical significance of tree branching was tested by performing 1,000 bootstrap replications.

Analysis of Selection Pressures

Non-synonymous to synonymous nt substitution rate ratio ($ω$) which gives a measure of selection pressure at the protein level was assessed using maximum likelihood codon substitution models implemented in CODEML programme of PAML4 package [56]. Six site models including M0 (one-ratio), M1a (nearly neutral), M2a (positive selection), M3 (discrete), M7 (beta), and M8 (beta&ω) were employed as described [57-59]. Likelihood ratio tests (LRTs) of M3 vs. M0 were used to test the heterogeneity of selective constraints among codons, while two comparisons of M2a vs. M1a, and M8 vs. M7 were used to test for positive selection as described [59]. Where the LRTs suggested positive selection, the Bayes empirical Bayes (BEB) approach [58] was used to identify amino acids under positive selection.

Analysis of Interactions Between SPCSV and SPFMV in Cultivated Sweetpotato and Accumulation of Viruses

Scions of sweetpotato cv. Tanzania singly infected with SPFMV EA strain isolate RUK73 (FJ795761) isolated from I. obscura in Uganda [29] were grafted onto 20 sweetpotato plants of cv. Tanzania raised from cuttings taken from the rootstocks that had been inoculated with different isolates of SPCSV via whitefly transmission. The grafted plants were maintained as above and observed for SPVD symptoms.

Concentrations of SPFMV and SPCSV (RNA1) were estimated by dot blot hybridization analysis, using digoxigenin (DIG) labeled RNA probes for the CP encoding region of SPFMV isolate RUK73 or the RNase3 gene of SPCSV isolate HOM76 (Table 1), respectively. The RNA probes were synthesized by in vitro transcription of the cloned DNA fragments encoding these genes as recommended in the DIG Application Manual for Filter Hybridization (Roche, Mannheim, Germany). Total RNA was extracted from the sixth leaf below the uppermost fully expanded leaf at 6 wk after planting the SPCSV-infected cuttings or 6 wk after top graft-inoculation of SPFMV onto SPCSV-infected rootstock. The quantity and quality of the RNA were measured using a spectrophotometer and the concentrations of RNA samples were equalized. A dilution series was prepared from total plant RNA starting from 2000 ng/μL and 5 μl from each RNA dilution was dotted on positively charged nylon membrane (Hybond-N+, Amersham Bioscience Ltd, UK) and detected using the digoxigenin-labelled RNA probes following the procedures described in the DIG Application Manual for Filter Hybridization (Roche).

Agroinfiltration Assays on Suppression of RNA Silencing

The experiments testing suppression of RNA silencing by p22 and RNase3 were carried out essentially as described by Kreuze et al. [20] and Cuéllar et al. [21]. Agrobacterium tumefaciens (strain C58c1; pGV3850) was transformed with binary vectors (pA35Shp200) each expressing one of the proteins under Cauliflower mosaic virus 35S promoter, and the 5’ untranslated region (5’UTR) of Potato virus A (PVA) was used for translation enhancement [20]. GUS was included as a negative control. Leaves of N. benthamiana were co-infiltrated with Agrobacterium strains for expression of the gfp gene for green fluorescent protein (GFP), gfp-specific double-stranded (hairpin) RNA to induce “strong gfp silencing” [31], and a third construct to express the putative silencing suppressor, as described previously [20]. Alternatively, leaves of transgenic N. benthamiana plants (line 16c) constitutively expressing gfp [32] were co-infiltrated with a mixture of two Agrobacterium strains, one expressing gfp to achieve cosuppression (silencing) of gfp and another expressing the putative silencing suppressor protein. Cultures of A. tumefaciens were diluted to OD$_{590}$=0.25 or OD$_{600}$=0.50 for the two types of experiments, respectively. Plants were grown from seeds in growth rooms under
controlled conditions (16 h photoperiod at 18/22 °C, 70% relative humidity; 200 μE m⁻²s⁻¹ light intensity) and given fertiliser (0.3 g/l) (16:9:22 N:P:K; Yara, Espoo, Finland) at every watering. The first 3-4 fully expanded leaves were agroinfiltrated.

Supporting Information

Figure S1. Phylogenetic analysis of genes coding for p7 of Sweet potato chlorotic stunt virus and the corresponding sequence of an unknown related virus (KML33b) detected in this study. Names of isolates characterized from wild plants are indicated in bold, whereas the ten SPCSV isolates lacking the p22 gene are indicated with a black triangle (▲). Numbers at branches represent bootstrap values of 1000 replicates. Only bootstrap values of ≥ 50% are shown. Scale indicates Kimura units in nucleotide substitutions per site [55]. (PDF)

Figure S2. Alignment of the 24 different p22 protein amino acid sequences of Sweet potato chlorotic stunt virus. Groups of isolates containing identical p22 aa sequences are represented each by a single isolate. The aa sites predicted to be under positive selection (black shades) and the 12 unique aa substitutions in isolate MBL16 (arrows) are pointed out. Numbers on top of the alignment indicate the aa positions with reference to SPCSV isolate Ug (AJ428554). Names of isolates from wild plants are in bold.

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Author Contributions

Conceived and designed the experiments: AKT SBM PRR JPTV. Performed the experiments: AKT RA IW. Analyzed the data: AKT RA IW S. JPTV. Contributed reagents/materials/analysis tools: AKT IW SBM JPTV. Wrote the manuscript: AKT IW SBM JPTV.

References

1. Karyeja RF, Kreuze JF, Gibson RW, Valkonen JPT (2000) Synergistic interactions of a potyvirus and a phloem-limited crinivirus in sweetpotato plants. Virology 269: 26-36. doi:10.1006/viro.1999.0169. PubMed: 10725195.
2. Sim J, Valverde RA, Clark CA (2000) Whitefly transmission of sweet potato chlorotic stunt virus. Plant Dis 84: 1250. doi:10.1094/PDIS-84-5-0510.
3. Gamarna HA, Fuentes S, Morales FJ, Glover R, Maluphy C et al. (2010) Bemisia afer sensu lato, a vector of Sweet potato chlorotic stunt virus. Plant Dis 94: 510-514. doi:10.1094/PDIS-94-5-0510.
4. Kreuze JF, Savenkov EI, Valkonen JPT (2002) Complete genome sequence and analysis of the subgenomic RNAs of Sweet potato chlorotic stunt virus reveal several new features for the genus Crinivirus. J Virol 76: 9260-9270. doi:11.128/JVI.76.18.9260-9270.2002. PubMed: 12186910.
5. Alcina T, Fenby NS, Gibson RW, Adipala E, Vetten HJ et al. (1999) Occurrence of two serotypes of sweetpotato chlorotic stunt virus in East Africa and their associated differences in coat protein and HSP70 homologue gene sequences. Plant Pathol 48: 718-726. doi: 10.1046/j.1365-3059.1999.00422.x.
6. Taio F, Mukasa SB, Jones RAC, Kullaya A, Rubaihayo PR et al. (2005) Unraveling the genetic diversity of the three main viruses involved in sweetpotato virus disease (SPVD), and its practical implications. Mol Plant Pathol 6: 199-211. doi:10.1111/j.1364-3703.2005.00267.x. PubMed: 20565651.
7. Qin Y, Zhang Z, Qiao Q, Zhang D, Tian Y et al. (2013) Molecular variability of sweet potato chlorotic stunt virus (SPCSV) and five potyviruses infecting sweet potato in China. Arch Virol 158: 491-495. doi:10.1007/s00705-012-1503-8. PubMed: 23053527.
8. Cuellar WJ, Taio F, Kreuze JF, Valkonen JPT (2008) Analysis of gene content in Sweet potato chlorotic stunt virus RNA1 reveals the presence of p22 RNA silencing suppressor in only few isolates: implications to viral evolution and synergism. J Gen Virol 89: 573-582. doi:10.1099/vir.0.83477-0. PubMed: 1819389.
9. Antua V, Barg E, Adipala E, Gibson RW, Vetten HJ (2008) Further evidence for limited genetic diversity among East African isolates of Sweet potato chlorotic stunt virus. J Phytopathol 156: 181-189. doi:10.1111/j.1439-0434.2007.01338.x.
10. Cuellar WJ, Cruzado KR, Fuentes S, Univeros M, Soto M, Kreuze JF (2011) Sequence characterization of a Peruvian isolate of Sweet potato chlorotic stunt virus: further variability and a model for p22 acquisition. Virus Res 157: 111-115. doi:10.1016/j.virusres.2011.01.010. PubMed: 21262289.
11. Hoyer U, Mais E, Jelkmann W, Lesemann DE, Vetten HJ (1996) Identification of the coat protein gene of a Sweet potato sunken vein closterovirus isolate from Kenya and evidence for a serological relationship among geographically diverse closterovirus isolates from sweetpotato. Phytopathology 86: 744-750. doi:10.1094/Phyto-86-744.
12. Vetten HJ, Hoyer U, Mais E, Lesemann DE, Jelkmann W (1996) Serological detection and discrimination of geographically diverse isolates of sweet potato sunken vein closterovirus. Phytopathology 100 (Suppl, abstract no 891A).
13. Carey EE, Gibson RW, Fuentes S, Machmud M, Mwangi ROM et al. (1999) The causes and control of virus diseases of sweetpotato in developing countries: is sweetpotato virus disease the main problem? In: CIP program report. Lima, Peru: International Potato Center. pp. 1997-248.

14. Fenby NS, Foster GD, Gibson RW, Seal SE (2002) Partial sequence of HSP70 homologue gene shows diversity between West African and East African isolates of Sweet potato chlorotic stunt virus. Tropical Agric 79: 26-30.
15. Gutiérrez DL, Fuentes S, Salazar L (2003) Sweet potato virus disease (SPVD): distribution, incidence, and effect on sweet potato yield in Peru. Plant Dis 87: 297-302. doi:10.1094/PDIS.2003.87.3.297.
16. Ishak JA, Kreuze JF, Johansson A, Mukasa SB, Taio F et al. (2003) Some molecular characteristics of three viruses in sweet potato virus disease-affected sweet potato plants in Egypt. Arch Virol 148: 2449-2460. doi:10.1007/s00705-003-0187-5. PubMed: 14848298.
17. Valverde RA, Lozano G, Navas-Castillo J, Ramos A, Valdés F (2004) First report of Sweet potato chlorotic stunt virus and Sweet potato feathery mottle virus infecting sweetpotato in Spain. Plant Dis 88: 428. doi:10.1094/PDIS.2004.88.4.428B.
18. Sivparsad BJ, Gubba A (2012) Molecular resolution of the genetic variability of major viruses infecting sweet potato (Ipomoea batatas L.) in the province of KwaZulu-Natal in the Republic of South Africa. Crop Protect 41: 49-56. doi:10.1016/j.cropro.2012.04.020.
19. Dolja VV, Kreuze JF, Valkonen JPT (2006) Comparative and functional genomics of closteroviruses. Virus Res 117: 38-51. doi:10.1016/j.virusres.2006.02.002. PubMed: 16529837.
20. Kreuze JF, Savenkov EI, Cuellar W, Li X, Valkonen JPT (2005) Viral class 1 RNase III involved in suppression of RNA silencing. J Virol 79: 7227-7238. doi:10.1128/JVI.79.11.7227-7238.2005. PubMed: 15890961.
21. Cuellar WJ, Kreuze JF, Rajamäki ML, Cruzado KR, Univeros M, Valkonen JPT (2009) Elimination of antiviral defense by viral RNase III. Proc Natl Acad Sci U S A 106: 10354-10358. doi:10.1073/pnas.0806042106. PubMed: 19515815.
Detection of viruses in sweetpotatoes from Honduras and Guatemala

Conrad C, Rauhut R (2002) Ribonuclease III: new sense from

Sweet potato feathery mottle virus show spatial differences in virus variability. Curr Opinion Plant Biol 3: 336-340. doi: 10.1016/S1368-7274(02)00089-0. PubMed: 12061487.

Alzhanova DV, Hagiwara Y, Peremyslov VV, Dolja VV (2000) Genetic analysis of the cell-to-cell movement of beet yellows closterovirus. Virology 268: 192-200. doi:10.1006/viro.1999.1055. PubMed: 10863341.

Cohen J, Franck A, Vetten HJ, Lesemann DE, Loebenstein G (1992) Purification and properties of closterovirus-like particles associated with a whitely transmitted disease of sweetpotato. Ann Appl Biol 121: 257-268. doi:10.1111/j.1744-7348.1992.tb03438.x.

Karyjeva RF, Gibson RW, Valkonen JPT (1998) Resistance to sweetpotato virus disease (SPVD) in wild East African Ipomoea. Ann Appl Biol 133: 39-44. doi:10.1111/j.1744-7349.1998.tb05000.x.

Verdoucq B (1983) Convolvulaceae. In CE Hubbard Mille-Redhead: Flora of tropical East Africa. London: Whitefriars Press Ltd.. pp. 1-161.

Blundel M (1992) Collins Photo Guide. Wild flowers of East Africa. London: Harper Collins.

Tugume AK, Mukasa SB, Valkonen JPT (2008) Natural wild hosts of Sweet potato feathery mottle virus show spatial differences in virus incidence and virus-like diseases in Uganda. Phytopathology 98: 257-268. doi:10.1094/PHYTO-98-6-0640. PubMed: 18944287.

Tugume AK, Cuéllar WJ, Mukasa SB, Valkonen JPT (2010) Molecular genetic analysis of virus isolates from wild and cultivated plants show East Africa as a hotpot for the evolution and diversification of Sweet potato feathery mottle virus. Mol Ecol 19: 3139-3156. doi:10.1111/j.1365-294X.2010.04682.x. PubMed: 20609081.

Kashif M, Pietilä S, Artola K, Jones RAC, Tugume AK et al. (2012) Incidence and virus-like diseases in Uganda. Phytopathology 98: 3149-3157. PubMed: 15448378.

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