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Genomic diversity of sweet potato geminiviruses in a Brazilian germplasm bank

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ABSTRACT

Begomoviruses cause major diseases of sweet potato worldwide impairing considerably the yields of this important food staple. Since sweet potato plants are vegetatively propagated and globally transported, they are prone to accumulate and disseminate geminiviruses. Effective diagnostic tools are, therefore, desirable. We studied the genomic diversity of geminiviruses present in naturally-infected sweet potato accessions belonging to a Brazilian germplasm bank collection. Fifty-five samples from different sweet potato accessions displaying geminivirus-like symptoms were analyzed by combining rolling circle amplification (RCA) with restriction fragment length polymorphism (RFLP) and sequencing. The restriction enzyme *Msp*I (*Hpa*II) revealed diverse band patterns in 55 samples and digestion with *Bam*HI, *Sst*I or *Pst*I resulted in full-length sweet potato geminivirus DNAs of about 3 kb in 46 samples. In addition, smaller fragments were identified as either viral "Defective DNAs" (D-DNAs) or mitochondrial plasmid DNAs. The diversity of sweet potato-associated geminiviruses was found to be very high under Brazilian conditions. Representative viral full-length DNAs have been cloned and sequenced yielding two new tentative species, three strains and several variants of previously described sweet potato geminiviruses. Sequence comparisons identified footprints of recombination in their genomes underscoring the risk of generating new geminiviruses in vegetatively propagated germplasm bank material. The sites of recombination were found in conjunction with predicted hairpin structures. We propose diagnostic routines to screen germplasm bank material for geminiviruses by the rapid and reliable RCA/RFLP as the technique of choice.

1. Introduction

Geminiviruses are important plant pathogens consisting of circular single-stranded DNA (ssDNA) packed into twin-shaped icosahedral particles (Böttcher et al., 2004; Zhang et al., 2001). Four genera of geminiviruses have been described, which differ in genome organization, host range and insect vector (reviewed by Jeske, 2009). Sweet potato-infecting geminiviruses are monopartite, belong to the genus *Begomovirus*, and multiply in several species of the family *Convolvulaceae* as well as in the experimental host *Nicotiana benthamiana* after insect transmission (Lotrakul et al., 1998, 2003). Their symptoms vary in different sweet potato [*Ipomoea batatas* (L.) Lam] cultivars and *Ipomoea* species, but even when appearing symptomless they may cause severe losses in sweet potato production (Clark and Hoy, 2006). Two main routes of their distribution have been described: transmission by the insect vector *Bemisia tabaci* (Genn.) and vegetative propagation (Valverde et al., 2004). The latter might lead to an accumulation of these viruses, often accompanied by RNA viruses, especially species belonging to the genus *Potyvirus*. In total, about 20 virus species from distinct virus families are known to infect sweet potatoes (reviewed by Valverde et al., 2007).

Begomoviruses infect *Ipomoea* plants worldwide and have been described in Japan, Israel, Peru, Italy, Spain, China, Taiwan, Korea, Kenya and the United States of America (Banks et al., 1999; Briddon et al., 2006; Cohen et al., 1997; Fuentes and Salazar, 2003; Li et al., 2004; Lotrakul et al., 1998, 2002; Lozano et al., 2009; Luan et al., 2007; Onuki and Hanada, 1998). Here we provide the first detailed description of these viruses for Brazil.

The current diagnosis of sweet potato viruses is based on immunodetection (Onuki et al., 2000), polymerase chain reaction (PCR) (Kokkinos and Clark, 2006; Li et al., 2004) and hybridization as well as grafting to susceptible indicator hosts such as *I. setosa* (Lotrakul et al., 1998; Valverde et al., 2007). As these techniques need a priori knowledge on the viruses, unknown geminiviruses and symptomless variants may escape detection. With rolling cir-

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cle amplification (RCA)-aided diagnosis using the bacteriophage Phi29 polymerase, these problems are reliably circumvented. All small circular DNAs in a sample are amplified and easily identified by RFLP (Haible et al., 2006; Homs et al., 2008; Owor et al., 2007b; Schubert et al., 2007) irrespectively of their origin and whether they are already known. RCA amplified viral DNAs can further be efficiently used for cloning of geminiviral full-length genomes (Ferreira et al., 2008; Inoue-Nagata et al., 2004; Wu et al., 2008) and biolistic inoculation (Haible et al., 2006; Jeske et al., 2010; Knierim and Maiss, 2007).

Many geminiviruses accumulate defective DNAs (D-DNAs) in host plants which consists of approximately half the virus genome and less (reviewed by Patil and Dasgupta, 2006). Some of them interfere with the multiplication of their parental viruses and are, hence, called defective interfering DNAs (DI-DNA) (Frischmuth and Stanley, 1991; Stanley et al., 1990). The presence of DI-DNAs may ameliorate symptom expression and have, therefore, been extensively investigated in order to develop non-conventional resistance strategies (Frischmuth et al., 1997; Frischmuth and Stanley, 1994). D-DNAs have been detected in begomovirus-infected sweet potatoes. They were preferentially amplified by PCR using universal primers (Briddon and Markham, 1994) and complicated cloning of geminiviral full-length sweet potato DNAs (Lotrakul and Valverde, 1999; Lotrakul et al., 2002).

In addition to geminiviral circular DNA, some plant species harbour small circular plasmid DNAs of mitochondrial origin (mtP) which have been detected during the search for cytoplasmic male sterility causes, but for which no function has been assigned until now (Backert et al., 1995; Benslimane et al., 1994; Hansen and Marcker, 1984). These mtPs have been recently detected during RCA diagnosis of Beet curly top virus in sugar beet (Homs et al., 2008). Here, we report on novel mitochondrial plasmid (mtP) DNAs from sweet potato which may be used as ideal size markers and positive-controls for the success of RCA and allow the identification of the host plant species, for which a peculiar pattern of mtP fragments can be expected (T.P., unpublished data).

Because of vegetative propagation, viruses that accumulate in sweet potato tissues might be spread through germplasm exchange all over the world. Mixed infections of different sweet potato geminivirus species and strains are supposed to occur frequently. Different geminiviruses may enter the same nucleus (Morilla et al., 2004), which is thought to be a major prerequisite for recombination to occur. Geminiviruses replicate by different modes like complementary strand replication (CSR), rolling circle replication (RCR), and recombination-dependent replication (Alberter et al., 2005; Erdmann et al., 2010; Jeske et al., 2001; Jovel et al., 2007; Preiss and Jeske, 2003), the latter of which may explain the high number of recombinant viruses described (Garcia-Andres et al., 2007; Lefeuvre et al., 2007; Monci et al., 2002; Owor et al., 2007a; Padidam et al., 1999; Ribeiro et al., 2007). Some recombination hot spots have been identified in Tomato yellow leaf curl virus which appear on sites where hairpin structures are predicted (Garcia-Andres et al., 2007).

In the present work, the genomic DNAs of two new tentative species of sweet potato begomoviruses, three new strains, several variants and defective fragments obtained using the RCA/RFLP approach are described.

2. Materials and methods

2.1. Sample collection

Plant samples were collected in the sweet potato germplasm bank from CNPH in Brasilia–DF (Brazil). Leaf samples from 55 different sweet potatoes accessions displaying mosaic, veinal and overall chlorosis, yellowing, curling and stunting were collected. The samples were stored on ice for 2 h, then frozen in liquid nitrogen, and stored at −80 °C.

2.2. Nucleic acid extraction

Leaf tissues were ground in liquid nitrogen and extracted following a modified protocol according to Dellaporta et al. (1983). Samples were directly added to 800 μl of pre-warmed 65 °C extraction buffer [0.1 M Tris–HCl pH 8.0; 0.05 M EDTA pH 8.0; 0.5 M NaCl; 1% polyvinylpyrrolidone (PVP) 40,000; 0.07% β-mercaptoethanol] supplied with 50 μl of 20% sodium dodecyl sulphate (SDS). The tubes were shaken vigorously at 65 °C until the tissue became dispersed, and further incubated for 15 min at 65 °C. After cooling down to room temperature for 2 min, 250 μl of 5 M potassium acetate (−20 °C) was added, the mixture gently inverted eight times and incubated for 20 min on ice. Samples were centrifuged in a tabletop centrifuge at full speed for 10 min, supernatants were removed, added to 500 μl of phenol/chloroform/isoamyl alcohol (25:24:1), and incubated for 10 min. After centrifugation (10 min, 8000 rpm, tabletop centrifuge), the aqueous phase was added to 500 μl chloroform, and centrifuged again. Nucleic acids were precipitated by ethanol and washed twice with 70% ethanol (Sambrook and Russell, 2001). The air-dried precipitate was dissolved in 50 μl 10 mM Tris–HCl (pH 8.0).

2.3. RCA/RFLP

Geminivirus infected plants were determined using RCA diagnosis as described by Haible et al. (2006). 3 μl of RCA products corresponding to 300 ng DNA (TempliPhi kit; GE Healthcare, Munich) were digested with 10 U *Msp*I (*Hpa*II), separated on 1.4% agarose gels, and stained with ethidium bromide. Potential enzymes for the release of full-length fragments (*BamH*I, *Pst*I and *Sst*I (*Sac*I) were selected to digest 1.5 μl RCA products.

2.4. Cloning

Bacterial cloning was performed using RCA products as described in Inoue-Nagata et al. (2004). *BamH*I, *Pst*I and *Sst*I were used to clone the DNA of 4 μl digested RCA product into 50 ng of the correspondingly cut pBluescript SKII+ plasmid vector (Stratagene, Amsterdam). Ligation products were transformed into *E. coli* DH5α cells, and plasmid DNAs were isolated (Sambrook and Russell, 2001).

2.5. Sequence analysis

Sequencing was performed using a CEQ 8000 sequencer and the CEQ-DTCS Quick start sequencing kit according to the manufacturer's protocol (Beckman-Coulter, Krefeld). Initial sequencing was done using the M13-forward and M13-reverse primers and followed by primer walking (see Table 1 and Supplementary data, Fig. 1). Partial sequences were combined with the help of cap contig assembly program (Bioedit software package, Hall, 1999). After aligning the nucleotide sequences with Codaln (Stocsits et al., 2005) and ClustalX 2.0.6 (Larkin et al., 2007), recombination sites were identified by TOPALi v2.5 Hidden Markov Model (HMM) (Husmeier and McGuire, 2003; Milne et al., 2004) and RDP software package (Martin et al., 2005). DNA secondary structures were predicted with the help of the thermodynamic properties tool within the oligo analysis plugin in Vector NTI 10 (Invitrogen, San Diego) and the mfold web server (Zuker, 2003). Sequence accessions obtained in this study and those for comparison are listed in Table 1.

Table 1
Begomoviruses used in comparative analysis with their respective sources, origin and sequence accession numbers.

ID	Isolate Name	Abbreviation	Bank CNPH #[a]	Host species	From	Length	Primers[b]	Acc.
1	Sweet potato leaf curl virus-Ceará [Brazil–Fortaleza1] (-CE [BR:For1])	SPLCV-CE	1033	*I. batatas*	Brazil	2841	1–3	FJ969832
2	Sweet potato leaf curl virus- Rio Grande do Sul 1 [Brazil:Tavares1] (-RS1 [BR:Tav1])	SPLCV-RS1	1052	*I. batatas*	Brazil	2845	1/4/5/7/9	FJ969833
3	Sweet potato leaf curl virus-Rio Grande do Sul 2 [Brazil:Rosario1] (-RS2 [BR:Ros1])	SPLCV-RS2	1154	*I. batatas*	Brazil	2829	1–3	FJ969837
4	Sweet potato leaf curl virus- Rio Grande do Sul 2 [Brazil:Machado Assis 1] (-RS2 [BR:Mac1])	SPLCV-RS2	1158	*I. batatas*	Brazil	2841	1–3	FJ969835
5	Sweet potato leaf curl virus- Rio Grande do Sul 2 [Brazil:Porto Alegre 1] (-RS2 [BR:Poa1])	SPLCV-RS2	1043	*I. batatas*	Brazil	2829	1–3	FJ969836
6	Sweet potato leaf curl virus- Rio Grande do Sul 2 [Brazil:Esteio 1] (-RS2-[BR:Est1])	SPLCV-RS2	1078	*I. batatas*	Brazil	2829	1–3	FJ969834
7	Sweet potato leaf curl virus-Rio Grande do Sul 2 [Brazil:Santa Catarina 1]	SPLCV-RS2	1091	*I. batatas*	Brazil	2829	1–3	%)
8	Sweet potato golden vein-associated virus-Paraíba 1 [Brazil:Souza 1] (-PB1 [BR:Sou1]) (-RS2 [BR:Sac1])	SPGVaV-PB1	598	*I. batatas*	Brazil	2834	1–3	FJ969830
9	Sweet potato golden vein-associated virus-Pará [Brazil:Belém 1] (-PA [BR:Bel1])	SPGVaV-PA	663	*I. batatas*	Brazil	2831	5–9	FJ969829
10	Sweet potato mosaic-associated virus-[Brazil:Brasilia 1] (-[BR:BSB1])	SPMaV	1015	*I. batatas*	Brazil	2803	2/6–7/10–11	FJ969831
11	Sweet potato leaf curl virus-[United States of America:Louisiana:1994] (-[US:Lou:94])	SPLCV-US		*I. batatas*	USA	2828		AF104036
12	Sweet potato leaf curl virus-[Italy:Sicily:2002] (-[IT:Sic:02])	SPLCV-IT		*I. indica*	Italy	2830		AJ586885
13	Ipomea yellow vein virus [Spain 98]	IYVV-[ES98]		*I. indica*	Spain	2763		AJ132548
14	Sweet potato leaf curl virus-[Japan:Kumamoto:1998]	SPLCV-[JP:Ku]		*I. batatas*	Japan	2828		AB433787
15	Sweet potato leaf curl virus-[Japan:Miyazaki:1996]	SPLCV-[JP:Mi]		*I. batatas*	Japan	2844		B433786
16	Sweet potato leaf curl virus-[Japan:Kyoto:1998]	SPLCV-[JP:Ky]		*I. batatas*	Japan	2828		AB433788
17	Sweet potato leaf curl virus-[RL7]	SPLCV-[RL7]		*I. purpurea*	China	2799		EU267799
18	Sweet potato leaf curl virus-[RL31]	SPLCV-[RL31]		*I. purpurea*	China	2800		EU253456
19	Sweet potato leaf curl Georgia virus-[United States:Georgia:16] (-[US:Geo:16])	SPLCGV		*I. batatas*	USA	2773		AF326775
20	Sweet potato leaf curl China virus	SPLCCV		*I. batatas*	China	2771		DQ512731
21	Ipomea yellow vein virus Spain:Málaga: IG1: 2006	IYVV-IG1		*I. indica*	Spain	2791		EU839576
22	Ipomea yellow vein virus Spain:Málaga: IG3: 2006	IYVV-IG3		*I. indica*	Spain	2783		EU839577
23	Ipomea yellow vein virus Spain:Málaga: IG5: 2006	IYVV-IG5		*I. indica*	Spain	2783		EU839578
24	Sweet potato leaf curl Canary virus Spain: Canary Islands: BG4: 2002	SPLCCaV-BG4		*I. batatas*	Spain	2806		EF456742
25	Sweet potato leaf curl Canary virus Spain: Canary Islands: BG7: 2002	SPLCCaV-BG7		*I. batatas*	Spain	2807		EF456745
26	Sweet potato leaf curl Canary virus Spain: Canary Islands: BG21: 2002	SPLCCaV-BG21		*I. batatas*	Spain	2807		EU856365
27	Sweet potato leaf curl Canary virus Spain: Canary Islands: BG25: 2002	SPLCCaV-BG25		*I. batatas*	Spain	2807		FJ529203
28	Sweet potato leaf curl Lanzarote virus Spain: Canary Islands: BG27: 2002	SPLCLaV-BG27		*I. batatas*	Spain	2814		EF456746
29	Sweet potato leaf curl Lanzarote virus Spain:Málaga: BG30: 2006	SPLCLaV-BG30		*I. batatas*	Spain	2814		EU839579
30	Sweet potato leaf curl Spain virus Spain: Canary Islands: BG1: 2002	SPLCESV-BG1		*I. batatas*	Spain	2780		EF456742
31	Sweet potato leaf curl Spain virus Spain: Canary Islands: BG5: 2002	SPLCESV-BG5		*I. batatas*	Spain	2779		EF456743
32	Sweet potato leaf curl Spain virus Spain: Spain:Málaga: IG2: 2006	SPLCESV-IG2		*I. indica*	Spain	2781		FJ151200
33	Sweet potato leaf curl virus Spain: Canary Islands: BG6: 2002	SPLCV-ES-BG6		*I. batatas*	Spain	2829		EF456744
34	Sweet potato leaf curl virus Spain: Canary Islands: BG12: 2002	SPLCV-ES-BG12		*I. batatas*	Spain	2829		EU856364
35	Sweet potato leaf curl virus Spain: Canary Islands: BG13: 2002	SPLCV-ES-BG13		*I. batatas*	Spain	2828		EU856366

[a] Accession numbers if applicable.
[b] Used for sequencing in this study, compare Supplementary data, Table 1; % Sequence was identical to SPLCV-RS2-[BR:Est1], not submitted to GenBank.

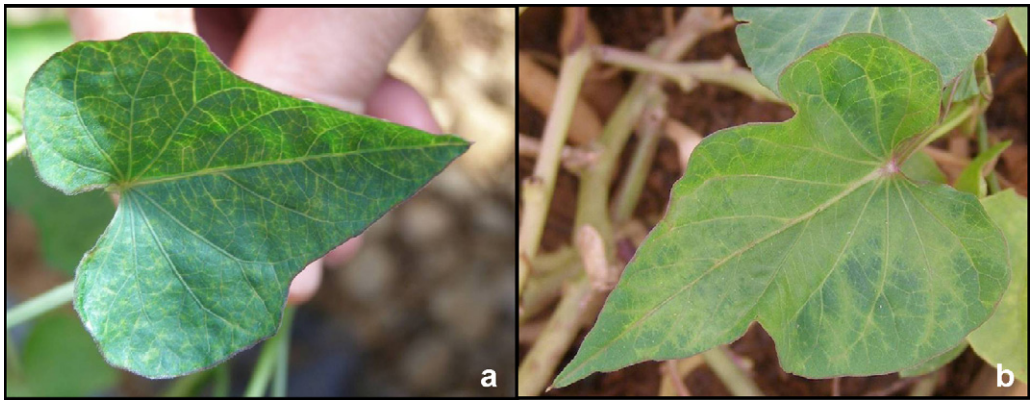

Fig. 1. Golden vein (a) and mosaic (b) symptoms of *Ipomoea batatas* (L.) Lam. plants which were infected with SPGVaV or SPMaV.

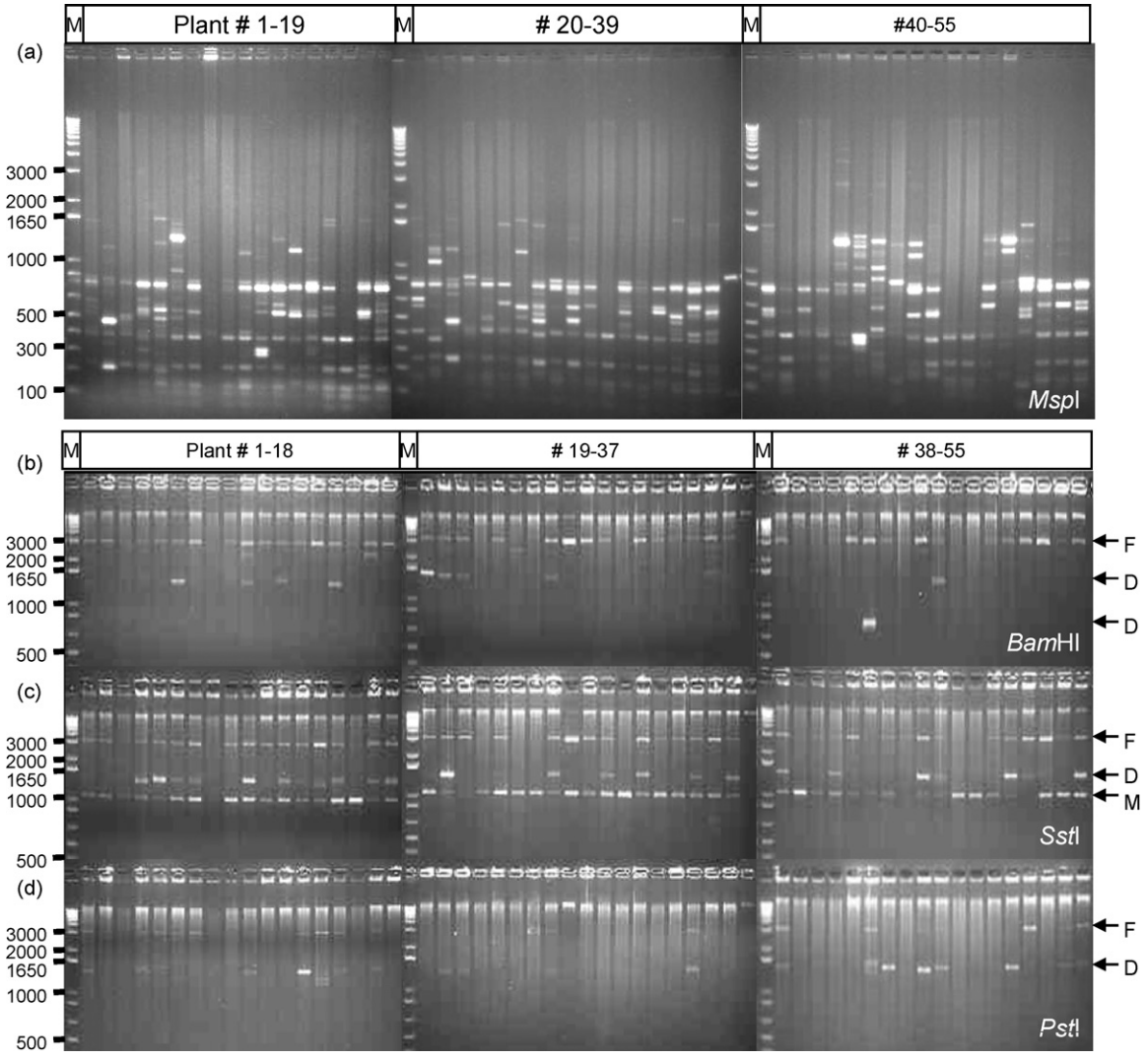

Fig. 2. Diversity of circular DNAs in the investigated symptomatic sweet potato plants. Fifty five leaf samples corresponding to different germplasm accessions (Table 1) were analyzed by RCA/RFLP diagnosis in 1.4% agarose gels: (a) *Msp*I restriction of the RCA product resulted in diverse band patterns in all samples except #8 (Accession CNPH 1035). Fragments (from <100 to 2000 bp) varied in band intensities, which indicates the presence of several independently replicating circles. RFLP with *Bam*HI (b), *Sst*I (c) and *Pst*I (d) revealed full-length fragments (F) of approximately 3000 bp in 46 samples, linearized defective DNA (D) of 1400 bp in 33 samples and 800 bp in sample #43. mtP DNA (M) with a size of 1000 bp was linearized only by *Sst*I in 50 samples. Plant samples were numbered (#) in the order of their collection.

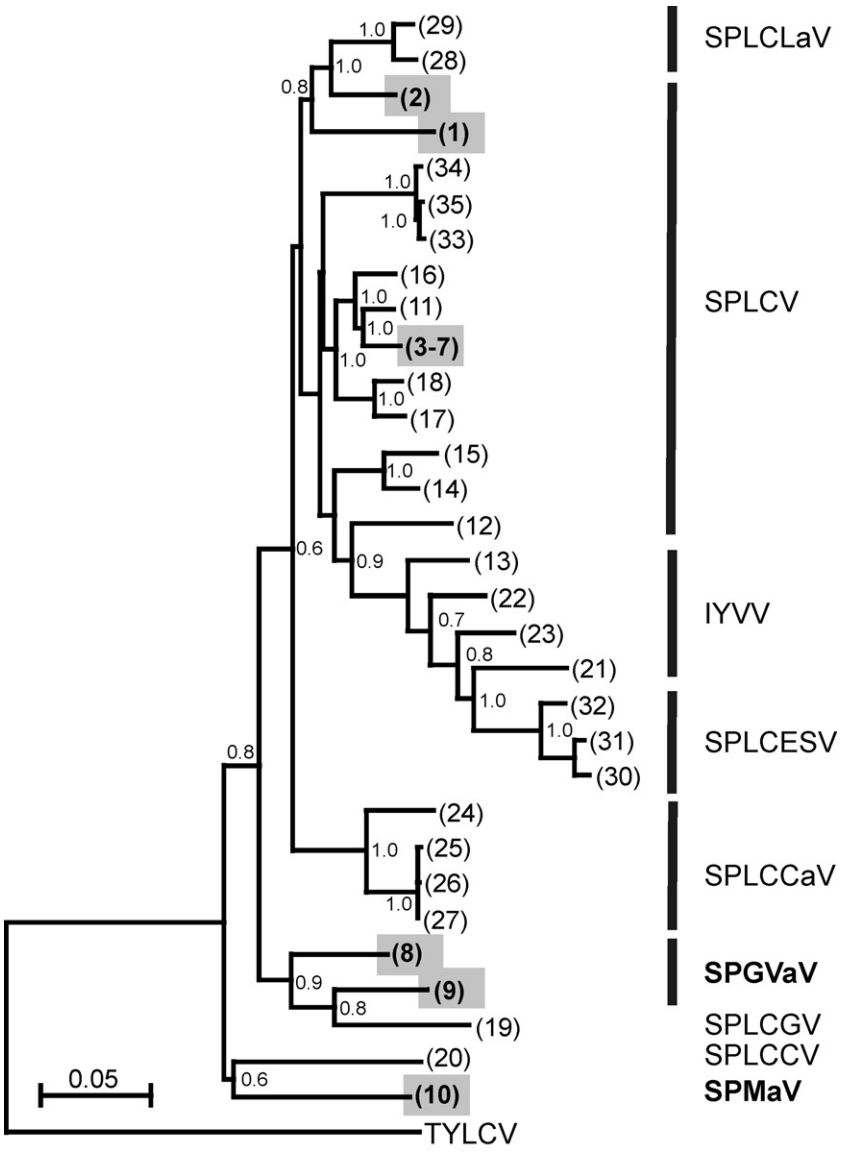

Fig. 3. Phylogenetic tree of the nucleotide sequences derived from 25 sweet potato infecting begomoviruses of the database in addition to the six sequences determined in this study (highlighted and in bold). Tomato yellow leaf curl virus-[Almeria] (AJ489258) was included as outgroup. The tree is based on a multiple alignment using Codaln and Clustal X2.06 programmes with 10,000 bootstrap trials for a NJ tree. Bootstrap values (>0.5) are shown at the branch points. The horizontal branches are proportional to the calculated substitutions per site, whereas the vertical branches are arbitrary. Numbers in brackets refer to the IDs in Table 1.

3. Results

3.1. The diversity of small circular DNAs in sweet potatoes

Symptomatic leaf material (Fig. 1) from 55 different sweet potato accessions was analyzed by RCA/RFLP. After *Msp*I digestion, varying band patterns were obtained (Fig. 2a). Five smaller fragments were present in nearly every sample, which represent mitochondrial plasmids (mtP) as determined by the sequence analysis discussed below. The mtP DNA was cut with *Sst*I and appeared as a prominent band of about 1000 bp in 50 (91%) of the samples (Fig. 2c). Restriction enzymes *Bam*HI, *Sst*I and *Pst*I generated geminiviral full-length fragments of about 3000 bp in 46 out of the 55 symptomatic leaf samples (84%) (Fig. 2b–d). In the other nine samples no geminivirus-specific bands were detectable with all chosen restriction enzymes (Fig. 2a–d). Defective geminiviral DNAs comprising about half the genome size were detected in 33 samples (72% of the infected samples) (Fig. 2b–d).

3.2. Genome analysis of the sweet potato begomoviruses

After bacterial cloning, viral full-length inserts of eleven plasmids were obtained from ten different sweet potato plants and completely sequenced. Two new tentative species, three new virus strains and several sequence variants of sweet potato begomoviruses were identified (Table 1), following the ICTV guidelines with a species demarcation line of 89% sequence identity (SI) for begomoviruses (Fauquet et al., 2008).

The genome sizes ranged from 2803nts (SPMaV) to 2845nts (SPLCV-RS1) with a typical structure for monopartite begomoviruses harbouring four ORFs in the complementary and two ORFs in the viral sense DNA (Table 1, Fig. 4). All isolates contained the conserved nonanucleotide within the origin of replication and bidirectional transcription units (reviewed by Jeske, 2009). Computer analysis predicted positions of ORF V2 start codons at different locations for the different viruses, but considering the conserved CAAT box and TATA box elements, one start codon at alignment position

191 is the most probable one for expressing a 93aa V2 protein.

Since we were unable to re-transmit the cloned DNA to sweet potato plants and to determine their influence on symptoms, we propose provisional names for the novel virus species: sweet potato golden vein-associated virus SPGVaV (Table 1, ID 8, 9 from plant shown in Fig. 1a) and sweet potato mosaic-associated virus SPMaV (Table 1, ID 10 from plant shown in Fig. 1b). Complete names and accession numbers of the most closely related viruses are summarized in Table 1, and we will use here the identification numbers listed there to facilitate reading of the text: SPGVaV is most similar to sweet potato viruses from the US (ID 11), from Brazil (ID 2) and two isolates from Spain (ID 28, 29; ID 30–32) with 88% SI each. It was isolated from two plants revealing two strains with 92% SI to each other. We propose to indicate them by isolate descriptors (PB1 [BR:Sou1], ID 8; PA [BR:Bel1], ID 9). SPMaV (ID 10) is most closely related to SPLCESV (ID 30–32) with 88% SI. Three further isolates are classified as strains of SPLCV: SPLCV-RS1 (ID 2) were similar to viruses from Japan (ID 15, 94% SI) and from Spain (ID 28–29, 93% SI), SPLCV-CE (ID 1) and SPLCV-RS1 (ID 2) were similar to viruses from the US (ID 11, 91% SI). Several isolates share 97% SI with SPLCV-US and are classified as variants collectively named SPLCV-RS2 (ID 3-7). Although isolated from five independent source plants, most sequences showed only differences for two to ten nucleotides, except for SPLCV-RS2 [BR:Mac1] (ID 4) which carries a unique 12 bp insertion at nucleotide position 1315 encoding four in-frame amino acids into ORF C2 as well as ORF C3 (Fig. 4).

Upon phylogenetic analysis, all sweet potato begomoviruses form a distinct cluster which is separated from Old and New World begomo-, topocu-, curto- and mastreviruses (data not shown) supporting the idea to combine them in a new distinct subgenus (Fauquet and Stanley, 2003). Within this subgroup (Fig. 3), SPGVaV (ID 8, 9) clusters with the US isolate SPLCGV (ID 19), whereas SPMaV (ID 10) clusters with the Chinese isolate SPLCCV (ID 20). The other isolates of this study (ID 1, 2, 3–7) belong to SPLCV.

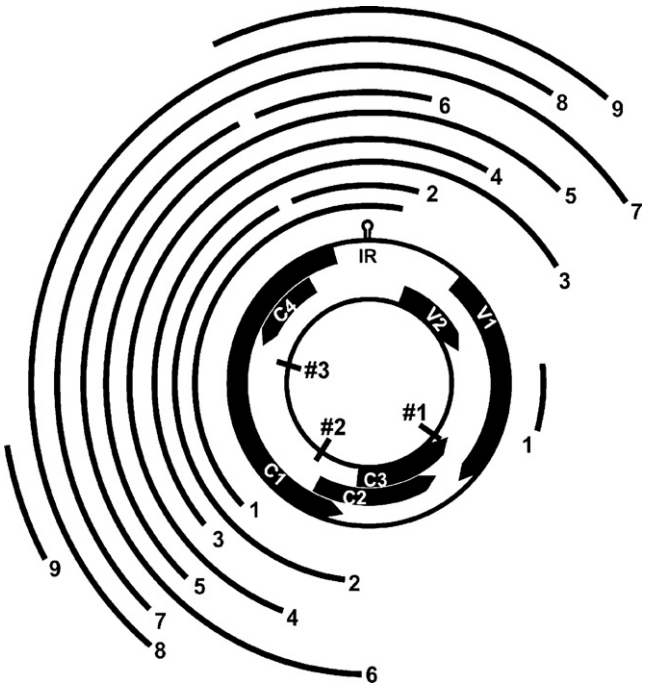

Fig. 4. Genome organization of sweet potato begomoviruses and their defective DNAs. It is identical to that of all monopartite Old World begomoviruses, with V1 and V2 in viral and C1–C4 in complementary strand orientation. Positions of predicted secondary structures and recombination spots are marked with #1, #2 and #3. The sequences retained in the analyzed defective DNAs (D-DNAs) are indicated by black arcs and identification numbers at their start and end (1–9). All D-DNAs share part of the intergenic region (IR) including the hairpin structure and most (except #9) contain ORF C4 and variable portions of ORF C1. In D-DNA-2 and -6, the N-terminus of ORF C1 is disrupted by short deletions and in D-DNA-1 and -9 larger fragments are lacking.

3.3. Defective viral DNAs in sweet potato

In addition to the genomic components, defective derivate DNA was found frequently. Their sizes ranged from 1323 to 1390 bp, except for D-DNA-[9] with 755 bp (Table 2). All D-DNAs share a common region including the hairpin structure of the origin of replication (ori) and usually a truncated ORF C1, only in D-DNA-[4] is ORF C1 intact. Three D-DNAs retained the N-terminus of ORF C2 and four D-DNAs had variable N-terminal parts of ORF V2 and ORF V1 (Fig. 4). Both, D-DNA-[2] and D-DNA-[6] showed a deletion of 27 bp, which was located at the beginning of ORF C1 and altered the N-terminus of Rep (Table 2). Two D-DNAs (-[1]; -[9]) combined two discontinuous sequences.

Some D-DNAs were isolated from plants from which no full-length virus has been cloned so far. However, through multiple sequence comparisons, the potential parent virus can be assigned tentatively (compare Tables 1 and 2): D-DNA-[1] and -[3] were most closely related to ID 3–7, D-DNA-[4] to ID 12, D-DNA-[7] and -[9] to ID 10, D-DNA-[5] to ID 15, and D-DNA-[2] and -[6] to ID 8.

Table 2
Characteristics of sweet potato begomovirus-derived defective DNAs.

D-DNAs				Closest relatives		
Name	Sample[a]	Enzyme[b]	Length (bp)	Name	Sequences % (nt positions)	Accession numbers
SPBV D-DNA-[1]	15/CNPH 1085	*Bam*HI	1356	SPLCV-RS2	1844–118 544–790	GQ268223
SPBV D-DNA-[2]	44/CNPH 981	*Pst*I	1390	SPGVaV-PB1	1560–2540 2570–144	GQ268224
SPBV D-DNA-[3]	20/CNPH 1067	*Sst*I	1383	SPLCV-RS2	1855–408	GQ268225
SPBV D-DNA-[4]	06/CNPH 1239	*Bam*HI	1376	SPLCV-IT	1617–163	GQ268226
SPBV D-DNA-[5]	05/CNPH 1043	*Sst*I	1368	SPLCV-[JP:Mi]	1826-348	GQ268227
SPBV D-DNA-[6]	13/CNPH 1090	*Pst*I	1352	SPGVaV-PB1	1578–2537 2566–123	GQ268228
SPBV D-DNA-[7]	26/CNPH 1168	*Bam*HI	1335	SPLCESV-BG01	1831-383	GQ268229
SPBV D-DNA-[8]	52/CNPH 1015	*Pst*I	1323	SPLCV-CE	1804–289	GQ268230
SPBV D-DNA-[9]	43/CNPH 598	*Bam*HI	755	SPLCESV-BG01	1–331 391–495 1961–2065	GQ268231

[a] Sample number/accession number.
[b] Restriction enzyme used for cloning; % Sequences in the closest relatives with highest similarity to the respective D-DNA.

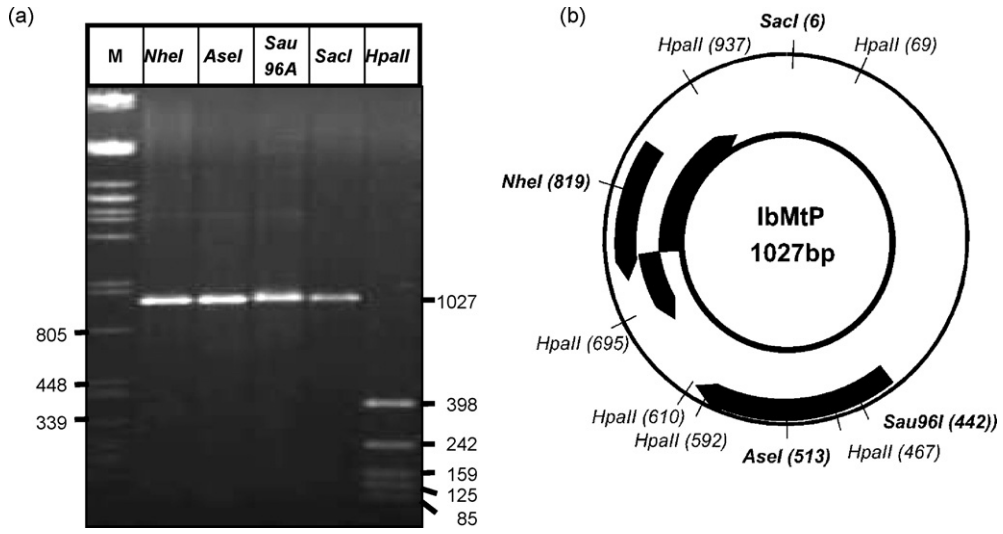

Fig. 5. Characterization of mtP DNA from *Ipomoea batatas* (L.) Lam. (Ib-mtP). The RCA product of the mtP DNA from an uninfected commercially available cultivar was linearized (a) with the restriction enzymes *Nhe*I, *Ase*I, *Sau*96A and *Sac*I which revealed fragments of the same size indicating that only one mtP species was present. *Hpa*II restriction resulted in six fragments with sizes from 18 to 398 bp, whereby the 18 bp fragment is not detectable in this gel system. Genomic map of Ib-mtP derived from sequencing (b), including the positions of the six *Hpa*II sites, the unique enzyme recognition sites in bold, and the predicted small open reading frames (arrows between the circles).

3.4. Mitochondrial plasmid (mtP) DNA of sweet potato

The sweet potato mtP DNA (FN421476) formed a characteristic pattern after restriction digestion with *Hpa*II/*Msp*I in all samples (Fig. 2a). The same pattern was detected in plant material collected in the field as well as in plants raised from commercially available sweet potato roots. In order to prove their endogenous origin, true seeds were grown in a containment greenhouse in Stuttgart without any source of sweet potato geminiviruses. The RFLP was plant species-specific and different for other *Ipomoea* species (data not shown).

Linearized full-length fragments of 1027 bp were obtained with restriction enzymes *Sst*I, *Sau*96I, *Ase*I and *Nhe*I (Fig. 5). Six complete sequences and several further RFLPs (data not shown) yielded identical results, in spite of sampling from different cultivars and geographical regions such as Brazil, Costa Rica, Israel and the USA. Only one clone from Brazil showed three nucleotides deletion. Analysing their translation capacity with standard algorithms revealed only small ORFs (Fig. 5b), a result which is consistent with former investigations on mtPs from various organisms (see Section 4).

3.5. Recombination

Using different computer programmes, precise recombination hot spots have been found, when comparing SPGVaV-PB1, SPGVaV-PA, SPLCGV and SPLCV-US as representative examples (Fig. 6). Interestingly, these recombination spots are located within genomic regions which are conserved in sequence and in secondary DNA structure for nearly all sweet potato geminiviruses (Supplementary data, Figs. 1–3).

For SPGVaV, a recombination spot was predicted within the gap of the small intergenic region (SIR) between the termination sequences of ORFs V1 and C3. A hairpin with a stem of 10 bp (T_m 62.2 °C) was predicted for this sequence (Fig. S1b). It was conserved in several sequences, but three isolates (Supplementary data, Fig. 1a; subset I) shared a deletion of the complete SIR at this position, which led to an overlap of the V1/C3 stop codons. The stop codons of

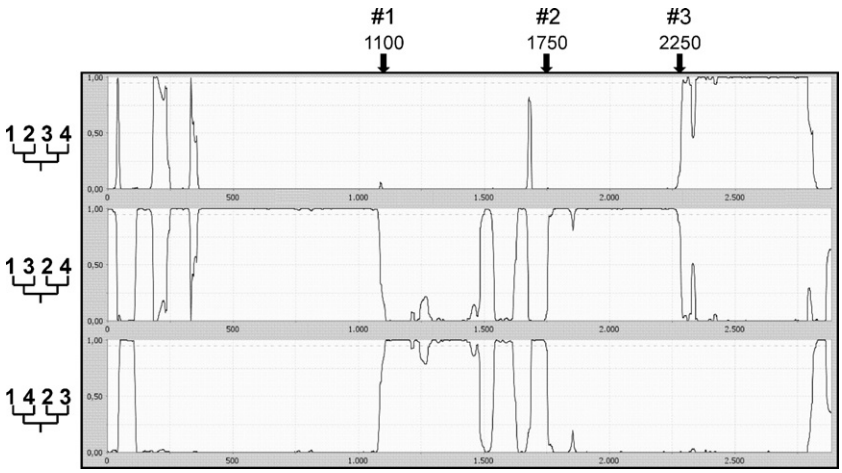

Fig. 6. Mapping of recombination sites by Hidden Markov Model analysis using TOPALI software was performed with SPGVaV-PA (1), SPLCGV (2), SPGVaV-PB1 (3) and SPLCV-US (4). The probability for the basic tree topology (*y* axis) shown at the left side was drawn to the genome position (*x* axis) and longer stretches exceeding the threshold line were taken as indication for a reliable descendent of a sequence portion. The most prominent changes in tree topology were interpreted as the mostly supported recombination sites marked at genome positions 1100 (#1), 1750 (#2) and 2250 (#3).

V1 and C3 for isolates combined as subset II (Supplementary data, Fig. 1a) were located within the stem of the hairpin structure.

A second recombination spot was found within the promoter region for the transcript covering ORFs C2/C3, about 50 bp upstream of the start codon of C2. Although the sequence alignment (Supplementary data, Fig. 2a) showed diversity within this sequence range, hairpins (T_m up to 82.7 °C) were predicted for every isolate (Supplementary data, Fig. 2b). At this recombination spot a co-variation was observed in the stem nt position 7: several isolates (Supplementary data, Fig. 2a) shared a T–A pairing, whereas few a C–G pairing and only SPGVaV-PA contained a T/G mismatch which might be the result of a recent recombination event.

For the third recombination spot at alignment nucleotide position 2237–2256, hairpins (T_m up to 81 °C) were predicted (Supplementary data, Fig. 3) which formed stems of 7 bp and 8 bp and loops of 3 bases.

Some partial nucleotide sequences indicate recombination events between sweet potato begomoviruses and other geminiviruses which had not been found to infect plants of the family *Convolvulaceae*: a portion of SPLCV-CE starting within the ORF C1 (nt 2636) and including nearly the complete ORF C4 (to nt 2278) showed maximal identity (88%) to Tomato yellow distortion leaf virus (FJ174698) from Cuba. The DNA sequence of ORF C4 was most closely related to Tomato pseudo-curly top virus (X84735) from Florida and Rhynchosia golden mosaic virus (EU339938) from Mexico (88% SI each). A second such sequence was detected in the SPMaV genome comprising part of the large intergenic region, starting from nt 2689 and downstream into the ORF C4 to nt 2234, with a maximal identity of 81% SI to Lindernia anagallis yellow vein virus from Vietnam (DQ641701).

4. Discussion

The results have shown that RCA-based diagnosis reliably detected several sweet potato infecting geminiviruses. Sequences of two new tentative virus species have been obtained without prior knowledge of these viruses. In addition, three new strains and several variants of sweet potato leaf curl virus have been identified. The high number of geminivirus-positive samples underlines the epidemic potential of sweet potato begomovirus infection in Brazil, but also the efficiency of this diagnosis for screening sweet potato germplasm bank material.

Direct diagnosis of the infecting viruses by RFLP using *Hpa*II/*Msp*I fragments was complicated, because defective viral DNAs and host plant mtP DNAs were frequent. The function of mtP DNA remains to be resolved, because only four extremely small ORFs (<51 aa) were detectable. The derived protein sequences of these ORFs indicated no similarities to any known protein (for further discussion of this issue see Homs et al., 2008). The sequence of the mtP DNA was conserved within a wide spectrum of sweet potato plant sources, which makes it, however, a predictable component. MtPs of diverse sweet potato germplasm accessions from Brazil and samples from other origins showed identical restriction patterns. Therefore, they may serve as a reliable internal positive-control for the success of the RCA technique.

In contrast, D-DNAs are less predictable, since their RFLP patterns are more diverse, their amounts vary (Fig. 2), and their sequence deletions occur at many positions (Fig. 4). They always share segments of the large intergenic region, of ORFs C4 and C1, whereby the truncations of ORF C1 were not uniform and some D-DNAs contain in addition a part of ORF V2. ACMV D-DNA was associated with isometric particles (Frischmuth et al., 2001). D-DNAs of several geminiviruses are transmissible by grafting or insect vector and may modulate the symptom-strength in plants (reviewed by Patil and Dasgupta, 2006). Whether the D-DNAs of sweet potato infecting geminiviruses share these properties and whether they ameliorate or enhance symptoms have to be determined in the future.

The phylogenetic analysis using recombination detection program (Martin et al., 2005) and Hidden Markov Model analysis (HMM) of Topali v2 (Husmeier and McGuire, 2003; Milne et al., 2004) revealed evidence for several recombination events during the evolution of sweet potato infecting geminiviruses. Some recombination hot spots were found at sequences where hairpin structures can be predicted, as it has been described previously (Garcia-Andres et al., 2007; Lozano et al., 2009). The secondary structures were conserved within nearly all available sweet potato infecting geminivirus sequences and the co-variation (reviewed by Chen et al., 1999) of stem nucleotides within these sequences suggest a biological relevance of these structures. Until now, only the stem loop structure of the origin of replication has been shown to be conserved in all geminiviruses (Hanley-Bowdoin et al., 1999).

Recombination spot #1 (Figs. 4 and 6; at alignment positions 1079–1103) is located between the stop codons of the V1 and C3 ORF, so that no coding region is affected by recombination. Several isolates form a conserved hairpin structure within this region and a 19 bp deletion is located exactly at this position in five sequences. The stem of the hairpin has been derived from an inverted duplication of the stop codons and the polyadenylation signals attached after ORF V1 and ORF C3 in both strand directions. Such a feature is conceivable, if the polymerase was forced to use the nascent strand as template during stalled replication.

Recombination spot #2 (Figs. 4 and 6) is located about 50 bp upstream of the C2 start codon, 25 bp downstream of a conserved possible CAAT box and 150 bp downstream of a highly conserved region which has been also found in Tomato golden mosaic virus to bind plant transcription factors in order to promote transcription of a 0.7 kb complementary sense mRNA (Shung et al., 2006; Tu and Sunter, 2007). This region contains a nuclease hypersensitive site in Abutilon mosaic virus minichromosomes characteristic for a nucleosome free region (Abouzid et al., 1988; Pilartz and Jeske, 1992, 2003). The stem sequence covariates at nucleotide position seven: most of the sequences contain a T–A pair at this position, whereas five sequences a C–G pair. SPGVaV-PA [BR:Bel1] shows here a T/G mismatch which could result from a recent recombination event. Recombination close to spot #1 and spot #3 seem to occur frequently between geminiviral DNAs, in contrast to spot #2 (Lefeuvre et al., 2007).

A particular sequence stretch covering ORF C1 and ORF C4 of SPLCV-CE and SPMaV with no similarity to other sweet potato infecting geminiviruses showed higher similarity to other geminiviruses from Asia and America. The similarity of SPLCV-CE ORF C4 to Tomato pseudo-curly top virus is consistent with the results of Lefeuvre et al. (2007). They found in the same sequence stretch a close relationship between Tomato leaf curl Seychelles virus (AM491778) and SPLCGV, and proposed a topocuviral origin of these sequences for both viruses. Considering the relatively low DNA sequence similarity between SPLCV-CE C4 and Tomato pseudo-curly top virus C4 of only 88%, the ancestral viruses have yet to be discovered or the recombination event may have happened long time ago. Recombination events may be blurred by a fast alteration of geminiviral sequences (Duffy and Holmes, 2008). In nature, sweet potato begomoviruses have only been found in *Convolvulaceae* plants, which, the other way round, have not been described as host for other geminiviruses. Therefore, it remains to be determined, where and how sweet potato geminiviruses had a chance to recombine with other geminiviruses. This event may have happened in still unknown host plant species.

The high frequency of recombination for geminiviruses may result from their genome organization and replication modes. Problems may arise if unidirectional replication is combined with

bidirectional transcription facilitating the collision of transcription and replication complexes (Brewer, 1988; Olavarrieta et al., 2002; Schvartzman and Stasiak, 2004). For geminiviruses, the combination of complementary sense transcription and rolling circle replication may, thus, lead to fragmented progeny DNA which has to be repaired by recombination-dependent replication (as discussed in detail by Jeske et al., 2001). Considering this scenario, it is not surprising that hot spots of recombination occur preferably at control elements of complementary strand transcription (promoter, terminator). Transcription factor binding may stall replication and during repair smaller ssDNA fragments may be generated that invade new strands, even if the target virus DNA has only smaller homologous stretches to the parent virus. The distribution of such possibly aberrant replication products has been mapped during replication studies (Jeske et al., 2001) and during the identification of nuclease-hypersensitive sites in viral minichromosomes (Pilartz and Jeske, 2003). Both results are consistent with the distribution of recombination hot spots of various geminiviruses (Lefeuvre et al., 2007).

Like in all other studies dealing with the experimental transmission problem (Lotrakul et al., 1998, 2003), we were unable to re-infect plants with cloned viral DNAs. Biolistics using RCA amplified multimers on *N. benthamiana* and several *Ipomoea* species failed to obtain systemic infection, as we have afterwards tested by RCA/RFLP diagnosis (data not shown). The reasons for this general failure remain to be determined and demand a cautious naming of the viruses.

In spite of this drawback, the results elucidate the epidemic potential of sweet potato-infecting geminiviruses in germplasm bank material. Routine tests with RCA/RFLP are highly recommended before germplasm bank material, especially of vegetative propagated plants, is distributed. Sweet potato begomoviruses have obviously already been spread by human-driven dissemination all over the world through vegetative propagation and export of their roots as indicated by the sweet potato leaf curl virus occurrence in Asia, America, Africa and Europe. There may also remain a still unexplored diversity of these viruses as suggested by the recent discovery of several new sweet potato infecting geminiviruses in this and other studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2010.02.003.

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