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Molecular characterization and an infectious clone construction of sweet potato leaf curl virus (SPLCV) isolated from Korea

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Summary. – Sweet potato leaf curl disease (SPLCD) was primarily identified in sweet potato fields in Korea in 2003, and the complete genomic sequence of sweet potato leaf curl virus (SPLCV) has been cloned. The genome of the Korean SPLCV isolate (SPLCV-KR) comprises 2,828 nucleotides with six open reading frames in DNA-A, similar to a monopartite begomovirus. Additionally, neither the genome B genomic component nor the DNA beta sequence was detected. The results of phylogenetic analysis using the maximum parsimony method showed that SPLCV-KR is more closely related to SPLCV-US (US) than SPLCV-CN (China) and SPLCV-JP (Japan). A tandem repeat dimer of SPLCV-KR was cloned and found to be infectious in sweet potatoes (*Ipomoea batatas*) via biolistic inoculation. The SPLCV-infected sweet potatoes exhibited mild leaf curl symptoms of SPLCD, and the newly-replicated viral DNA was detected via Southern blot analysis. Results of biotic, molecular, and phylogenetic characterization suggest that SPLCV-KR is a new strain of SPLCV and is importantly placed in the evolutionary progression from curtoviruses to begomoviruses.

Keywords: sweet potato leaf curl virus; sweet potato leaf curl disease; phylogenetic analysis; infectious clone; biolistic infection

Introduction

Sweet potato leaf curl virus (SPLCV) is a member of the genus *Begomovirus* (Valverde *et al.*, 2004). Sweet potato-infecting begomoviruses including SPLCV are informally designated as sweepoviruses, since sweepovirus is distinct from the other begomoviruses with regard to their phylogenetic relationship (Fauquet and Stanley, 2003). The complete SPLCV genome was initially cloned in the US by Lotrakul and Valverde (Lotrakul *et al.*, 1998; Lotrakul and Valverde, 1999), and the genomes of the other variants of SPLCV (Ipomoea yellow vein virus (IYVV)), and sweet potato leaf curl Georgia virus (SPLCGV)) (Banks *et al.*, 1999; Lotrakul *et al.*, 2003) were also subsequently cloned. It was found that SPLCV has a single genome with an approximate size of 2.7 kb (A-like genomic component).

Considering its importance in the phylogenesis of the begomoviruses and in sweet potato pathology, research on SPLCV has gradually increased during the last decade. Currently, there are 100 complete genome sequences and more than 20 partial sequences of SPLCV deposited in the NCBI database. SPLCV has been detected in Bangladesh, Brazil, China, India, Italy, Japan, Kenya, Korea, Pakistan, Peru, Spain, Taiwan, the United States, and Trinidad and Tobago (Banks *et al.*, 1999; Briddon *et al.*, 2005; Fuentes and Salazar, 2003; Lotrakul *et al.*, 1998; Lotrakul *et al.*, 2002; Lozano *et al.*, 2009; Luan *et al.*, 2006; Miano *et al.*, 2006; Umaharan *et al.*, 1998; Zhang and Ling, 2011). The complete genome sequences from isolates in seven countries (Brazil, China, Italy, Japan, Korea, Spain, and the United States) are available in the database.

Korea is currently the world′s 20th most productive country for sweet potatoes. The sweet potato (*Ipomoea batatas*

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Abbreviations: SPLCV = sweet potato leaf curl virus; SPLCD = sweet potato leaf curl disease; IR = intergenic region; BCTV = beet curly top virus

L.), a dicotyledonous plant in the family *Convolvulaceae*, originated from the American tropics and is one of the most significant horticultural crops on Earth. In Korea, the partial nucleotide sequence of the *AC1* gene of SPLCV was initially detected from samples collected in 2002 (Lotrakul *et al.*, 2002). RT-PCR assays also detected the presence of SPLCV in sweet potatoes in a Korean fields as one component of a multiple viral infection involving other RNA viruses, such as sweet potato feathery mottle virus (SPFMV) and sweet potato G potyvirus (SPGV) (Kwak *et al.*, 2006).

The existence of SPLCV in Korea implies the potential development of sweet potato disease complexes. The majority of begomoviruses with two-component genomes (bipartite) are found in the New World (America), whereas the Old World viruses (Africa, Middle Asia, and Europe) only harbor one genomic component (monopartite). The Old World begomoviruses including SPLCV have two virion sense-stranded ORFs of the *AV1* (coat protein) and *AV2* (movement) genes to regulate viral movement and systematic transmission, and four complementary sense-stranded ORFs of the *AC1* (replication-related function), *AC2* (transcriptional activation), *AC3* (transcriptional enhancing), and *AC4* (unknown function) genes to regulate viral genome replication and transcription in the monopartite genome. Meanwhile, the New World begomoviruses have a genome (DNA-B) for systematic transmission in addition to the component A-like genome (DNA-A) that is also present in Old World viruses. The begomoviruses are thought to have evolved from an ancient monopartite to current forms by acquisition of foreign genetic material. This evolution implies that the Old World begomoviruses, especially sweepoviruses and legumoviruses, may play an important role as part of the intermediate geminivirus evolution between curtoviruses and begomoviruses (Fauquet and Stanley, 2003). The disease complex of begomoviruses is rapidly expanding from Old World to New World and from New World to Old World as a result of genetic recombination changing the host range.

The establishment of infectious clones of the viruses would provide greater insights into the virus-associated diseases. An infectious clone as a reverse genetic research tool can overcome many restrictions that cannot be experimentally regulated *in vivo*. It would also provide opportunities to screen novel hosts and alternative model hosts against viruses. Artificial infection techniques have been developed via approaches such as *Agrobacterium*-mediated inoculation (Balaji *et al.*, 2004), particle bombardment (Ariyo *et al.*, 2006; Briddon *et al.*, 1998; Rothenstein *et al.*, 2005), and rub inoculation (Rothenstein *et al.*, 2005).

In the present study, we focused on the establishment of an infectious clone from the SPLCV Korean isolate, along with molecular characterization and phylogenetic relationship analysis, since there have not been any studies to date using an infectious clone of sweepoviruses.

Materials and Methods

Sweet potatoes. Symptomatic individuals (curly top) from five lines (263, 388, 445, 508, and 618) of sweet potatoes (*I. batatas* L., Jinhongmi cultivar) (Fig. 1, upper panel; line 508) were transferred from the experimental field of the National Academy of Agricultural Science in Mokpo (for lines 263, 388, and 445; southern Korea) and Yeoju city (for lines 508 and 618; central Korea) to the laboratory in 2003. To adapt to environmental changes after the transfer, the five Jinhongmi cultivars were kept at 24°C under a cool white fluorescent lamp with photoperiods of 10–14 hrs for about a week and then were harvested. The leaves, stems, and roots of the five individual cultivar lines were sampled in 50 ml conical tubes. The samples were immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

Isolation of genomic DNA. Total genomic DNA was extracted from each frozen tissue (leaves, stems, and roots of the five cultivars) as described in a previous study (Dellaporta *et al.*, 1983). DNA samples from each tissue were prepared in duplicate. One was used for Southern blot analysis and the other was used for amplifying, cloning, and DNA sequencing. The concentration and purity of the genomic DNA were assessed by using a spectrophotometer (Biophotometer Plus, USA) with a UVette (Eppendorf, USA). The DNA samples were kept at -80°C until further analysis.

Viral detection by PCR. PCR was conducted using a primer set to detect the viral *AC2* gene to identify SPLCV by sequence analysis, since the *AC2* gene had the lowest sequence homology among six ORFs from the compared begomoviruses (Table 1). The following primer set was designed with the Primer3 program (Rozen and Skaletsky, 2000): 5SPAC2 forward primer, 5′-ATGTCCAATCTCCCTTCTGGA-3′ and 3SPAC2 reverse primer, 5′-AGGCGTTCCAAAATACCAGTC-3′. PCR products of the partial *AC2* gene were ligated into the pGEM-T easy vector (Promega, USA) as recommended in the manual provided by the manufacturer. The nucleotide sequence of the cloned *AC2* gene was then determined (Macrogen, Korea).

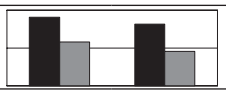
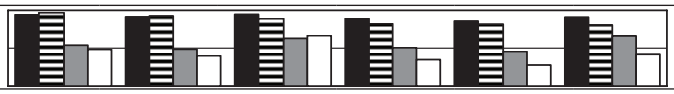
Detection of viral replication by Southern blot analysis. To detect viral replication in the sweet potato samples, total genomic DNA was extracted from the tissues of each of the sweet potato lines, as described above. A partially-amplified *AC2* gene was radio-labeled with [α-32P]-CTP to generate the DNA probe (Lee *et al.*, 2011). Fifteen micrograms of DNA extracted from each tissue were separated on a 0.8% agarose gel and visualized after staining with ethidium bromide. The DNA was transferred to a positively-charged nylon membrane using 5x SSC transfer solution for approximately 16 hrs and covalently linked to the nylon membrane using a UV crosslinker. After the membrane was rinsed, prehybridization and hybridization were conducted at 65°C. After washing and drying the blotted filter, the membrane was exposed to X-ray film (Kodak, USA) under an intensifying screen at -80°C for two days.

Cloning of the SPLCV genome. All enzymatic reactions were performed as recommended by the manufacturer (Takara Bio Inc., Japan), and the general cloning procedure was conducted as described previously (Sambrook and Russell, 2001). For the cloning of the partial SPLCV genome, primer sets were designed with the reference genome

sequence (NC_004650) using Primer3 software. The SPF1 forward primer (5′-CTCGTGCAGTTCTCTTGCTA-3′) and the SPR1 reverse primer (5′-GCAACTGGGATTCCACAAGA-3′) were designed from 2526 bp to 1330 bp for the amplified product named SP1 fragment. The SPF2 forward primer (5′-GTGTATCAGACCCTGCGTTG-3′) and the SPR4 reverse primer (5′-AAACGCGCATTCGCCCTGTCAT-3′) were designed from 1012 bp to 322 bp for the amplified product named SP2 fragment (Fig. 2). Two fragments were amplified from the genomic DNA sample of the sweet potato cultivar line 508, which manifested severe symptoms but harbored less viral DNA in the tissues based on the results of the Southern blot analysis. Gel-purified PCR products were directly ligated with the pGEM-T easy vector and were designated as a pSP1 clone or a pSP2 clone after screening the colonies. To determine the complete genome sequence of SPLCV, triplicate sequencing reactions were conducted using a 3730XL DNA analyzer (Macrogen, Korea). The genome sequence of SPLCV was analyzed by using Lasergene 5.0 (DNAstar, USA) to predict ORFs. Using the predicted ORFs, a BlastN search was conducted. The searched results were analyzed for sequence similarity with SPLCV-KR using Lasergene 5.0 (Table 1). The Blast-matched nucleotide and amino acid sequences were aligned using the ClustalW method in Lasergene and the percent identity was calculated for all of the compared viruses.

Construction of a tandem repeat dimeric clone. An infectious SPLCV-KR clone was constructed as described in Fig. 2. The pSP1 and pSP2 clones were double-digested by *Eco*RV and *Pst*I. The new fragment was created via ligation between the double-digested pSP2 (insert; 2018 bp) and pSP1 fragment (vector backbone; 4450 bp), and designated as the SPLCV 1.2 mer (pSPLCV1.2; 6468 bp, partial tandem repeat, not depicted in Fig. 2). The monomeric clone of SPLCV (pSPLCV 1.0) was obtained via ligation between the *Sac*I-digested pSPLCV1.2 (2828 bp) and pGEM-T easy self-ligated vector (3016 bp). The SPLCV tandem repeat dimeric clone (pSPLCV2.0; SPLCV

Table 1. Sequence similarity between SPLCV-KR and the related geminiviruses

Percent identities [a]		Nucleotides		Nucleotides/Deduced amino acids					
Mean similarities [b]									
Host	Viruses [c] (GenBank)	Full	IR	AV1	AV2 [d]	AC1	AC2	AC3	AC4
Sweet potato	SPLCV-US (AF104036)	97.0	95.8	96.8 /97.9	97.3 /98.0	98.3 /98.7	96.7 /93.7	97.6 /95.4	97.4 /95.1
	SPLCV-ES (EF456744)	92.8	86.6	94.4 /97.5	90.0 /91.5	96.2 /96.5	92.5 /84.7	86.1 /83.7	94.5 /85.3
	SPLCV-JP (AB433787)	95.0	85.3	97.1 /98.6	86.3 /86.3	93.3 /94.9	86.9 /78.3	87.2 /83.0	96.4 /91.2
	IYVV-IT (AJ586885)	91.3	91.4	93.8 /95.4	94.5 /96.7	92.8 /95.7	85.8 /76.7	78.1 /70.6	94.8 /88.2
	SPLCV-BR (FJ969833)	92.6	70.2	97.2 /98.6	84.5 /86.3	97.3 /90.9	94.7 /92.1	95.6 /93.5	85.8 /70.6
	SPLCV-GA (AF326775)	82.4	69.9	91.1 /96.5	95.9 /97.4	92.2 /80.9	84.6 /75.7	77.0 /69.9	77.3 /53.9
	SPLCV-CN (DQ512731)	83.2	71.1	89.0 /95.4	90.9 /92.8	91.9 /66.1	84.1 /76.2	79.9 /77.1	91.3 /81.4
Chili	CLCV-IN (DQ629103)	61.5	51.8	56.1 /53.7	52.2 /43.8	77.3 /69.9	55.9 /39.7	47.0 /28.8	62.8 /34.3
Tomato	TLCV-AU (NC_003896)	61.5	55.5	59.1 /56.8	54.2 /47.1	77.4 /71.2	56.0 /37.6	47.5 /28.1	62.1 /39.2
Ageratum	AYVV-CN (NC_004626)	62.3	51.8	59.1 /56.5	54.0 /43.8	18.0 /70.2	55.0 /38.1	45.0 /28.1	67.0 /47.1
Pepper	PLCV-CN (NC_000882)	59.9	41.6	57.0 /53.3	47.8 /39.9	77.4 /63.4	56.6 /39.7	47.2 /26.8	59.5 /34.3
Sugar beet	BCTV-CA (M02457)	44.9	27.4	38.6 /21.1	35.5 /27.5	68.4 /59.7	30.5 /21.2	41.1 /28.1	79.9 /55.9

[a]Percent identity by nucleotide sequence and amino acid sequence of putative ORFs from Genbank.

[b]The mean similarities indicate the mean percent identity shared with sweepoviruses and other geminiviruses in the graph. The middle line indicates 50% mean similarity, the dark bar (for nucleotide sequence) and the horizontally striped bar (for the amino acid sequence) describe the similarity of the sweepoviruses, and the shaded bar (for the nucleotide sequence) and the empty bar (for the amino acid sequence) describe the similarity of the other viruses described in the table.

[c]AYVV-CN: Ageratum leaf curl virus from China; CLCV-IN: Chili leaf curl virus from India; IYVV-IT: Ipomoea yellow vein virus from Italy; PLCV-CN: Pepper leaf curl virus from China; SPLCV-BR: Sweet potato leaf curl Brazil virus from Brazil; SPLCV-CN: Sweet potato leaf curl China virus from China; SPLCV-ES: Sweet potato leaf curl Eastern Spain virus from Spain; SPLCV-GA: Sweet potato leaf curl Georgia virus from Georgia, United States; SPLCV-JP: Sweet potato leaf curl Japan virus from Japan; SPLCV-US: Sweet potato leaf curl virus from the United States; and TLCV-AU: Tomato leaf curl virus from Australia. The GenBank accession numbers of the genome sequences are given in parentheses.

[d]In the comparison between BCTV-CA and SPLCV-KR, the function of AV2 in BCTV-CA was involved in the regulation of the double-stranded form and single-stranded form of the viral DNA, rather than viral movement. Hence, the AV3 sequence of BCTV-CA was compared with the AV2 sequence of SPLCV-KR.

dimer) was created via ligation between the *Kas*I-digested pSPLCV1.2 and pSPLCV1.0.

Phylogenetic analysis. A phylogenetic tree of geminiviruses was constructed using amino acid sequences of SPLCV-KR and other geminiviruses. For the comparison of each ORF, the 11 species of begomoviruses, which were BlastN-matched with the complete genome sequence of SPLCV-KR, and a species of curtovirus as the out-group were selected. The amino acid sequences of the species were aligned via MUSCLE software (Edgar, 2004). Evolutionary history inferences were generated via the maximum parsimony (MP) method, and phylogenetic analyses were conducted in MEGA4 software (Tamura *et al.*, 2007). The bootstrap consensus tree from 1000 replicates was taken to represent the evolutionary history of the analyzed taxa (Felsenstein, 1985). Using the complete deletion option, all gaps and the positions containing missing data were eliminated from the dataset. The MP tree was generated using the close-neighbor-interchange algorithm with search level 3, in which the initial trees were obtained with the random addition of sequences (ten replicates). The tree was drawn to scale with branch lengths calculated via the average pathway method and were expressed as the number of changes over the entire sequence.

Biolistic infection. Particle bombardment was conducted using a Gene Gun (Helios Gene Gun, Bio-Rad Laboratories, USA) as described in the manual. The five micropropagated sweet potatoes were kindly provided by Dr. Junseol Lee (Mokpo Experiment Station, National Institute of Crop Science; NICS, Mokpo, Korea). The sweet potatoes were transferred from the agar containing MS medium to sterilized soil at the leaf formation stage. The sweet potatoes were grown at 24°C under a cool white fluorescent lamp with photoperiods of 10–14 hrs for a month until infection. Before biolistic infection, one leaf of the micropropagated sweet potatoes was prepared for genomic DNA as described above. The DNA samples were tested using PCR analysis with the AC2-specific primer sets to rule out prior infection with SPLCV. After growth, we selected individuals that did not show relevant symptomatic development and were not positive by PCR to be used as target samples for biolistic infection. Total five sweet potatoes were infected using gold particles (0.5 μm in diameter) mixed with 0.5 μg of dimeric infectious clones (pSPLCV2.0) at a pressure of 250 psi. The plants were analyzed by Southern blot hybridization to test SPLCV replication and multiplication 4 weeks after inoculation. Newly developed five leaves from each infected plant were harvested, and genomic DNA was extracted. A tandem repeat dimeric clone of SPLCV (pSPLCV2.0) was employed as a positive control and as the DNA template to amplify the *AC2* partial sequence as a probe for Southern blot analysis as described above.

Results

Isolation of SPLCV and sequence analysis

The *AC2* partial sequence of SPLCV was amplified from the sweet potato cultivar Jinhongmi using PCR analysis (data not shown). The results of Southern blot analysis demonstrated that SPLCV replicated in the tissues of four sweet potato lines, but not in line 388 (Fig. 1, lower panel). Viral DNA accumulation differed in each line, and no clear correlation was detected between symptom severity and the amounts of viral DNA. Sweet potato line 508 had the most severe symptoms on its leaves than the other lines, but it proved difficult to identify symptoms of viral infection in the roots and stems of the sweet potato plant, even though the root tissues contained more viral DNA than the leaves and stems of most of the sweet potato lines (Fig. 1, lower panel). Unlike the line 508, the line 263, 388, 445, 618 did not show the typical symptoms in their leaves (data not shown). The root tissues from lines 263 and 618 produced a clear band representing the open circular (OC) replicating DNA form on Southern blot analysis. However, the leaf and stem tissue samples did not produce a visible OC band. The DNA was extracted from the cultivar exhibiting the most severe symptoms (line 508) and was used as the source for genomic cloning of the SPLCV-KR and further analysis.

The complete genomic sequence of SPLCV-KR was determined via a triplicate sequencing reaction using internal sequencing primers in both directions (GenBank accession number: FJ560719). The DNA-A-like sequence of SPLCV-KR was composed of 2828 nucleotides, and six ORFs were predicted from the determined sequence. Like other whitefly-transmitted geminiviruses, SPLCV-KR harbors four complementary-sense ORFs (C1, C2, C3, and C4) and two virion-sense ORFs (V1 and V2) (Fig. 2). A potential hairpin loop structure within the intergenic region (from 2682 bp to 131 bp) was conserved, as shown in the other viruses. The sequence alignment of the intergenic region of the sweepoviruses (Fig. 3) demonstrated that the iterative *cis*-element (iteron), which is expected to bind and to form a nick by Rep protein (AC1) on the intergenic region of SPLCV-KR, is located before the ten base pairs from the 5′-end of the TATA box, which is the same as that of SPLCV-US (5′-TTGGAGAC-3′), but different from the sequences of IYVV-IT, SPLCV-CN, and SPLCV-JP (5′-TTGGTGAC-3′) and SPCLV-GA (5′-TTGGTGTC-3′). The other iteron was identified after three base pairs from the 3′ end of the TATA box as a form of the inverted repeat (Fig. 3) and was also identical to the sequence of SPLCV-US (5′-TGTCTCC-3′). Unlike SPLCV-KR, the iteron sequence was identified as 5′-TGTCACC-3′ in SPLCV-CN and IYVV-IT and 5′-TGT CCACC-3′ in SPLCV-BR. In particular, SPLCV-GA harbors a variable sequence (5′-TGGGACAC -3′) in this position. The iteron of SPLCV-KR was identical in terms of arrangement and position in relation to the location of the TATA box among the compared sweepoviruses, with the exception of SPLCV-BR and SPLCV-GA. Except for the *cis*-element region near the TATA box and the conserved hairpin loop region (about 100 base pairs), the IRs of these viruses were highly variable in sequence similarity.

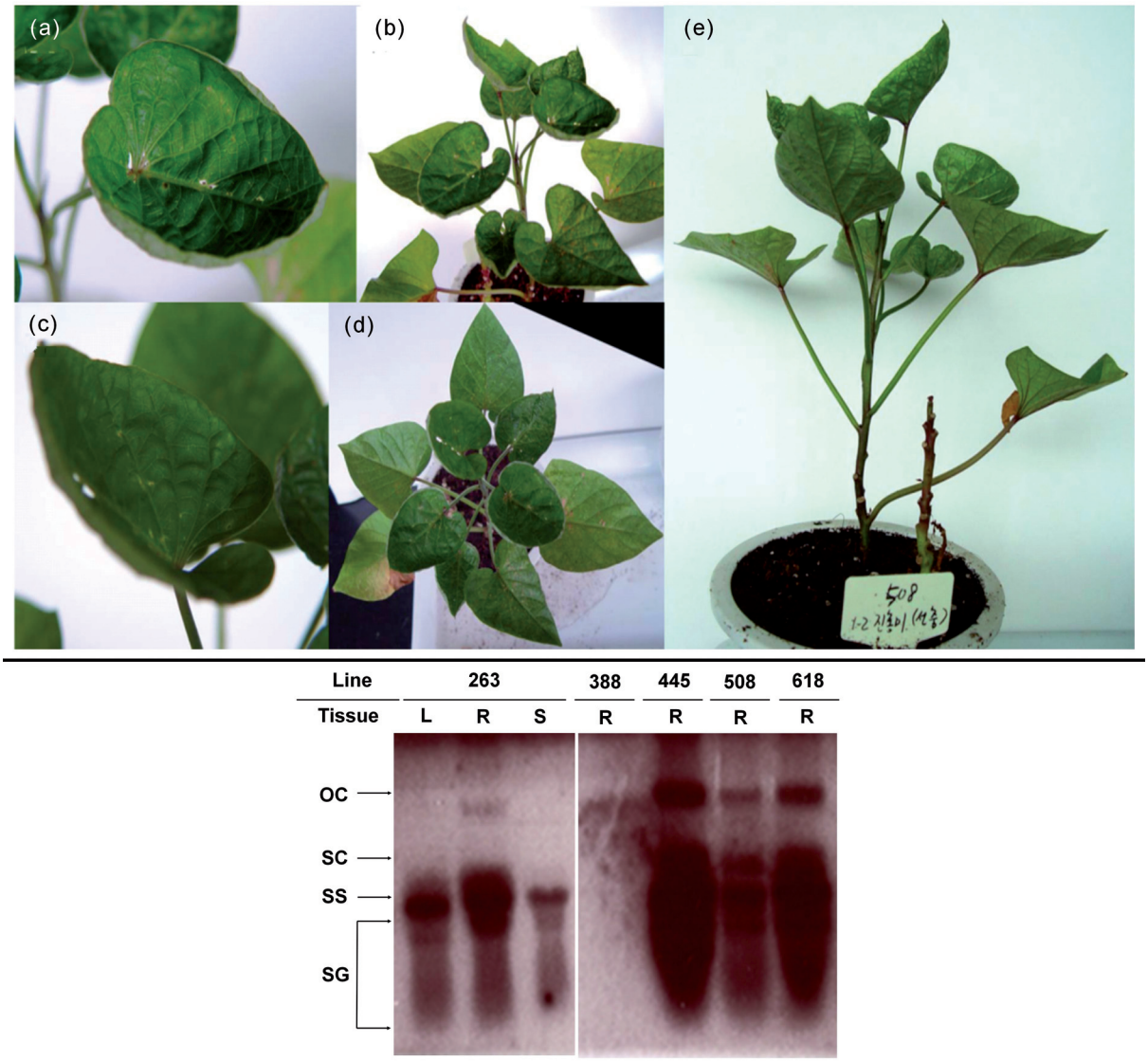


Fig. 1

Sweet potato (*I. batatas* L.) Korean cultivar Jinhongmi (508 Jinhongmi, Sunchang) exhibited geminivirus-infected curly top leaf symptoms and was positive for viral replication

Upper panel. A sweet potato with the typical leaf curl symptom. (a) Severe leaf curl and vein-thickening symptoms; (b) yellow, curly leaves; (c) crumple characteristics; (d) perpendicular view; e.) overview of the symptomatic sample (curly top symptoms) Lower panel. Viral replication detection by Southern blot analysis. Variable replicating forms of the SPLCV genome were detected in sweet potato cultivars (lines 263, 445, 508, and 618) and tissues (L: leaves, R: roots, and S: stem): open circular (OC), supercoiled (SC), single-stranded (SS) and subgenomic (SG).

The results of the BlastN search demonstrated that SPLCV-KR is highly similar to the other sweepoviruses (Table 1). In a comparison of the genome sequences, the nearest begomovirus in terms of sequence similarity was SPLCV-US (97.0%), and SPLCV-CN (83.2%) was the least similar among the sweepoviruses, although it was closest in terms of geographical distribution. IYVV-IT was less identical (91.3%) than SPLCV-US. The genome sequences of begomoviruses that infect other hosts were less than 60% similar to that of SPLCV-KR. The IR sequence similarity was more variable than the similarities of the genome sequences and the amino acid sequences among the compared viruses. The IR of SPLCV-US was only 95.8% identical to that of SPLCV-KR, even though the genomic sequence identity was greater than 97.0%. The amino acid sequences and nucleotide sequences of important proteins in the viral cycle (AC1 and AV1) were more than 96.8% identi-

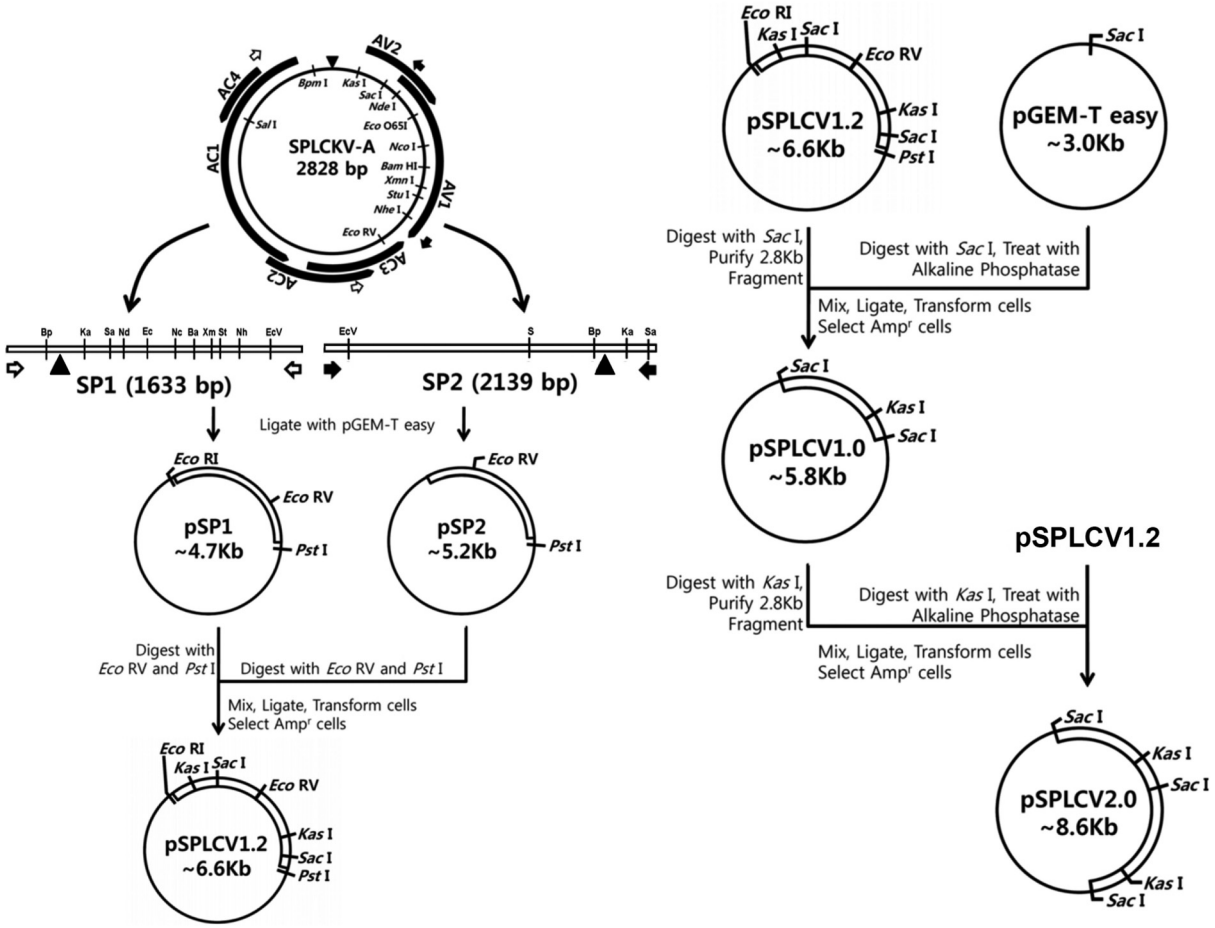


Fig. 2

Description of schematic genome map and construction of tandem dimeric SPLCV clone in the pGEM-T easy vector (pSPLCV2.0)

SPLCV-KR is composed of 2828 nucleotides. The dark arrow bar represents the protein coding regions. The closed and empty arrows indicate the binding regions of the primer set for positional cloning, the inverted triangle denotes the location of the replication start site on the invariant nona-nucleotides (TATTAAT|AC), and unique restriction sites are shown. Restriction sites were abbreviated as follows. Ba; *Bam*HI, Bp; *Bpm*I, Ec; *Eco*O65I, EcV; *Eco*RV, Ka; *Kas*I, Nc; *Nco*I, Nd; *Nde*I, Nh; *Nhe*I, S; *Sal*I, Sa; *Sac*I, St; *Stu*I, Xm; *Xcm*I.

cal to those of SPLCV-KR. However, the IR of IYVV-IT was similar (91.4%) to the results for genomic sequence similarity, as shown by amino acid and nucleotide sequence similarity. The IRs of SPLCV-GA and SPLCV-CN were approximately 70% identical to that of SPLCV-KR. Additionally, the IR of the other host-infecting begomoviruses was less than 55.5% identical to that of SPLCV-KR. Seven sweepoviruses (IYVV-IT, SPLCV-BR, SPLCV-CN, SPLCV-ES, SPLCV-GA, SPLCV-JP, and SPLCV-US) shared at least 53.9% to 98.7% identity with SPLCV- KR, depending on the similarity of the amino acid sequences. The *AV1* gene had the highest levels of amino acid sequence similarity, from 95.4% to 98.6%, among the compared viruses. However, the similarity of *AC2* and *AC3* was substantially low, from 66.1% to 95.4%. The similarity of the amino acid sequences demonstrated that SPLCV-KR shares very little identity with other host-infecting geminiviruses, except for the amino acid sequences of C1 and V1, as described in the results of a survey conducted in China (Luan *et al.*, 2007) (Table 1).

Phylogenetic analysis

Patterns of sequence similarities among the sweepoviruses were also detected in the phylogenetic relationships of each ORF (Fig. 4). Beet curly top virus (BCTV) was used as an out-group for the analysis, since it is involved in the curtoviruses even though it has seven ORFs. The function of the V2 protein in BCTV has not been reported in any

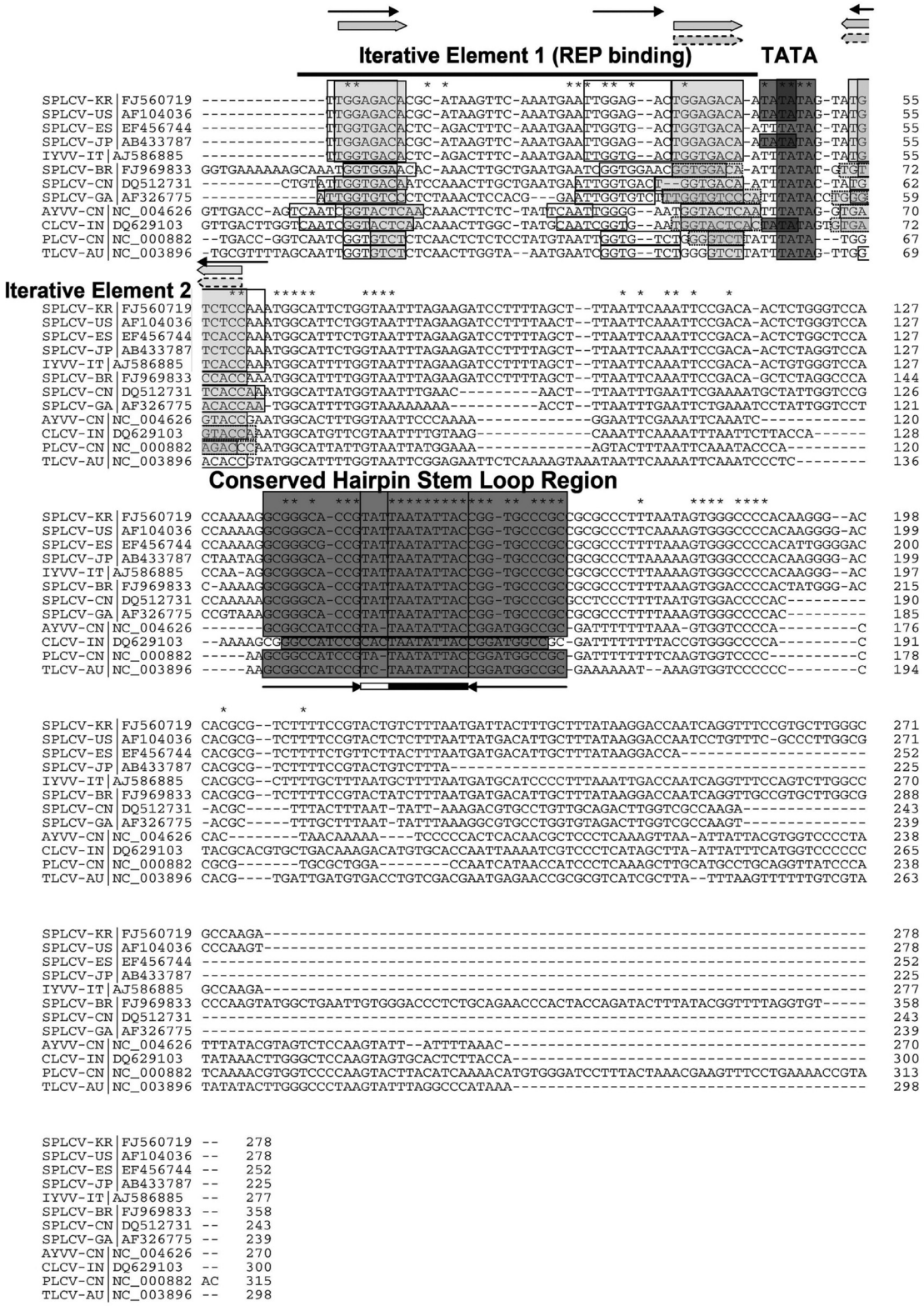


Fig. 3

Intergenic region (IR) of the Korean sweet potato leaf curl virus isolate (SPLCV-KR)

Nucleotide sequence alignment of the intergenic region among the compared geminiviruses. An asterisk indicates the conserved sequence in the alignment and gaps are represented by dashes. An arrow shows the direction and kind of repeated sequences.

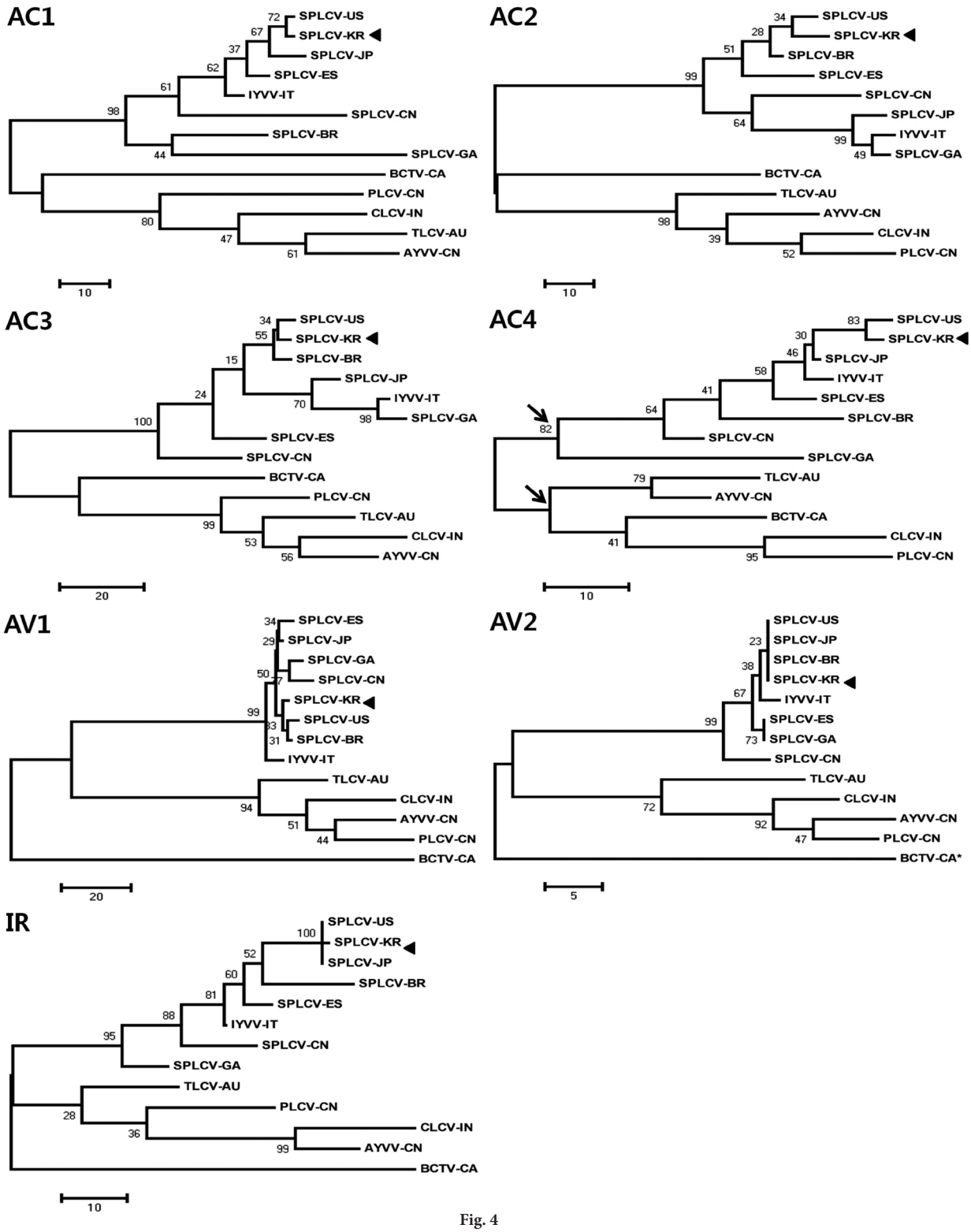


Fig. 4

Phylogenetic relationships among SPLCV-related geminiviruses determined using deduced amino acid sequences from putative ORFs and nucleotide sequences from IRs

The compared ORFs were deduced from the geminiviruses listed in Table 1. The underbar indicates the parsimony distance on the tree and the asterisk (*) denotes AV3 of BCTV-CA.

other geminivirus subgroups, and it differs from that of the AV2 protein of monopartite begomoviruses, which regulates the movement. Interestingly, the V2 protein of BCTV is not a homolog of AV2 or V2 of other geminiviruses because it has a unique function in controlling the proportions of viral ssDNA and viral dsDNA. Therefore, the V3 rather than V2 of BCTV was compared. The viruses with lower sequence identity were basically separated from the highly similar viruses as described in the results of the sequence similarity assays (Table 1). The phylogenetic relationship of the AC4 protein for the SPLCV-GA did not show similar patterns that were shown in the phylogenetic analysis of other viral proteins. The AC4 protein of SPLCV-GA was firstly separated from the ancestor, and only then AC4 proteins of the other viruses were separated. However, SPLCV-GA was more closely branched with the other sweepoviruses in the case of the phylogenetic relationship with the other viral proteins. SPLCV-GA was usually branched with SPLCV-CN in the phylogenetic tree of each ORF; however, interestingly, SPLCV-GA was closer to SPLCV-KR and SPLCV-US than to SPLCV-CN in the phylogenetic tree of the AV2 protein.

Biolistic infection

Challenging the infectious clones with low pressure (250 psi) helium gas did not induce the typical symptoms of leaf curl disease observed in the original sample with SPLCD (Fig. 1), but did result in mild symptoms of leaf curl disease (Figs 5a-e). The majority of symptoms were observed in the veins of leaves, and the symptoms included vein thickening, crumpling, vein clearing, and curling. The newly developed leaves exhibited variable symptoms, and some of these symptoms were similar to what was observed in the IYVV-infected leaves and SPLCV-infected *Ipomoea setosa* (Fig. 5b) (Banks *et al.*, 1999, Zhang and Ling, 2011). Newly-developed leaves (Figs 5b and d) had more severe symptoms than the other leaves (Figs 5a, c, and e) in the same plant. Additionally, the results of Southern blot analysis of the leaves presenting symptoms revealed replicating DNA bands from the geminiviruses (Fig. 5, lower panel).

Discussion

In the past decade, the genomic sequences of SPLCV have become available from Brazil (Zhang and Ling, 2011), China (Luan *et al.*, 2007), Italy (Banks *et al.*, 1999), Japan (unpublished; Genbank accession no. AB433786.1), Spain (Lozano *et al.*, 2009), and the US (Lotrakul *et al.*, 1998), and extensive surveys have been conducted in other countries. One Korean study surveyed sweet potato fields to detect SPLCD, but the SPLCV-infected samples were detected at a significantly lower frequency than other virus-infected samples (Kwak *et al.*, 2006). Additionally, a few putative samples from fields were found to be infected by different RNA viruses as well as SPLCV by RT-PCR detection assays (Kwak *et al.*, 2006). Therefore, the results of that detection study suggest that SPLCV could potentially pose as a serious threat to sweet potato agriculture in South Korea, since SPLCD caused by SPLCV has not significantly influenced Korean agriculture so far. The results of this study also suggest that sweet potatoes infected with multiple viruses might serve as a source of transmission of numerous viral diseases via whiteflies and as a reservoir for viral recombination and mutation. Thus, we attempted to identify the full genomic sequence of SPLCV in Korea, to analyze its phylogenesis, and to establish an infectious clone for further applications.

Our comparison of amino acid sequences showed that SPLCV-KR substantially differs from SPLCV-CN (DQ512731) (Luan *et al.*, 2007), but is very similar to SPLCV-US (AF104036) (approximately 97% similarity of the genome sequence) (Table 1). The majority of monopartite geminiviruses addressed in the BlastN results of this study demonstrated that the C1 protein (homologue of Rep: replication-associated protein) had the highest levels of similarity. In contrast, the amino acid sequences of the C2 protein (homolog of TrAP: transcriptional activator protein) and C3 protein (homolog of TEn: transcriptional enhancer protein) among the sweepoviruses were highly variable (Table 1). Therefore, we analyzed the phylogenetic relationships of each ORF using the MP method to clarify these differences (Fig. 4). All protein-coding sequences of SPLCV-KR were more closely aligned with SPLCV-US than with the other viruses. However, according to inference from the sequence comparison of AC2 and AC3 among the sweepoviruses, it appears that SPLCV-KR and SPLCV-US may have evolved independently, distant from SPLCV-CN and SPLCV-GA, or SPLCV may be the result of recombination among other sweepoviruses. In Fig. 4, the phylogeny of each protein coding region among the sweepoviruses shows that the SPLCV-KR genome might have originated from several SPLCV genomes, and recombination within those SPLCV genomes would involve frequent recombination across the AC1 to AC4 coding region. Therefore, evidence of recombination between SPLCV and the other geminiviruses is required for further analyses of the evolutionary history of the sweepoviruses.

In our phylogenetic analysis using the full genome sequence of 61 geminiviruses (data not shown), the sweepoviruses were distinctly branched with the New World begomoviruses, and diverged from the Old World begomoviruses, as previously reported. On the neighbor joining tree (Saitou and Nei, 1987), the bootstrap value (90%) on the branch node between the New and Old World begomoviruses was highly significant. However, interestingly, a group of SPLCVs recently diverged from the Old World begomoviruses with a monopartite genome, and the bootstrap value (68%) on the branch node

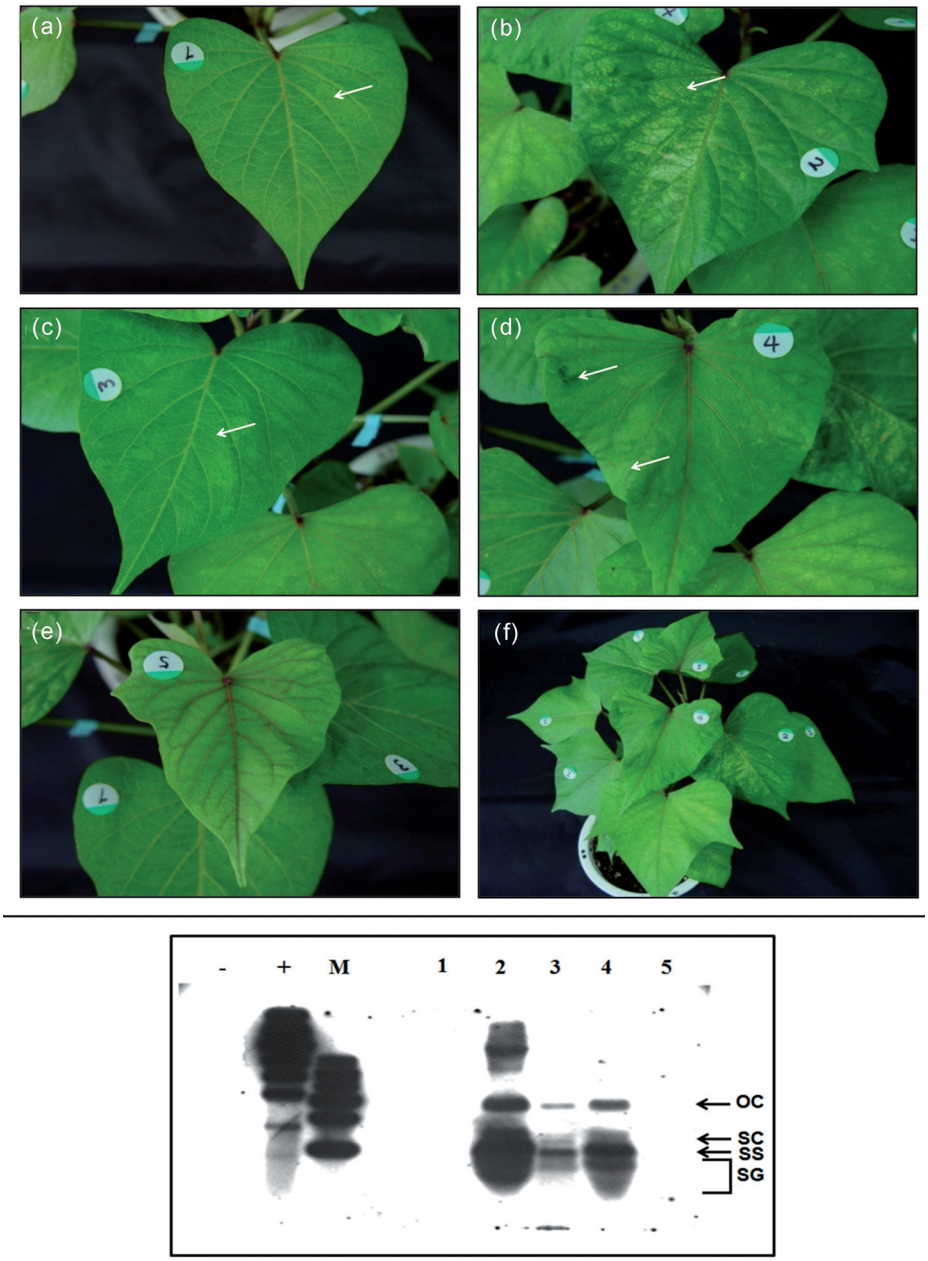


Fig. 5

Symptomatic presentation of *I. batatas* inoculated by particle bombardment and Southern blot detection of SPLCV virus recovered from an infected clone

(a-f) Symptom development (arrow) in five new leaves. (a) vein thickening, (b) crumpling and early vein yellowing, (c) vein clearing, (d) crumpling, slight curling and early vein yellowing, (e) unclear symptoms (newest leaf), and (f) the whole tested plant. Lower panel) Southern blot analysis of symptomatic leaves [negative (-): uninfected (micropropagated) sweet potato; positive (+): pSPLCV2.0; M: size marker (1 kbp ladder); lanes 1-5: new leaves (a–e); OC: open circular; SC: supercoiled; SS: single-stranded; and SG: subgenomic form]. The bands of size marker indicate from bottom 3, 4, 5, 6, 8, and 10 kbp in size.

between Old World begomoviruses and the sweepoviruses was less significant than the former. This low level of significance may provide us with some insights into the evolutionary relationship between curtoviruses and Old World begomoviruses involving the sweepoviruses. Curtoviruses, which include BCTV, and the Old World begomoviruses have monopartite genomes, whereas the New World begomoviruses are principally bipartite as they possess two covalently closed-circular

single stranded DNA genomes (Timmermans *et al.*, 1994). Many reports have interpreted this interesting fact in the evolution of geminiviruses, although the movement through evolutionary niches from monopartite to bipartite in geminivirus evolution remains unclear (Briddon *et al.*, 1992; Lotrakul and Valverde, 1999; Lotrakul *et al.*, 1998, 2002).

In the phylogenetic analysis of the amino acid sequences of AC4 (Fig. 4), the tree showed that SPLCV-GA was more initially branched in the sweepoviruses, which is different from results for other proteins, and compared to the other viral proteins, the AC4 of BCTV-CA also evolved differently. This finding supports the ambiguity inherent to the evolutionary niches of the sweepoviruses and might be correlated with recombination among viruses. However, unfortunately, the full complement of relevant data is still not available because reports of SPLCV sequence data were not fully investigated from pathological and ecological perspectives. To evaluate the pathological effects of SPLCV in this study, we introduced an infectious clone to the micropropagated sweet potatoes via particle bombardment, but the resulting disease symptoms were quite mild in the newly generated leaves (Fig. 5), as described in a previous report (Zhang and Ling, 2011). Although the double-stranded replicative form (RFI and RFII) in the infectious clone-bombarded plants exhibited a pattern similar to that of the SPLCV-infected sweet potatoes in the field on Southern blot (Fig. 1 and Fig. 5), the symptoms induced by the infectious clones were critically different from those of the SPLCV-infected samples obtained in the field. This symptomatic difference might be a result of differences in the inoculation methods utilized because whitefly-mediated inoculation in fields can deliver the virus more efficiently to phloem cells than bombardment methods, thereby resulting in more critical symptoms (Osaki and Inouye, 1991). To confirm this hypothesis, a simple passage by whitefly-re-inoculation would be followed as the future step of our study.

Interestingly, our Southern blot analysis consistently demonstrated that greater amounts of viral DNA had accumulated in the root tissues of SPLCV-infected sweet potatoes than in the other tissues (Fig. 1b). This observation is consistent with results from previous studies involving the localization of GFP-tagged viruses and Southern blot analyses in *Arabidopsis*-geminivirus interactions (Hur *et al.*, 2008). Morilla *et al.* (Morilla *et al.*, 2006) reported that accumulated viruses can actively replicate in root tissue and Tuttle *et al.* (Tuttle *et al.*, 2008) also showed that the cotton leaf crumple virus was maintained for a longer time in the root than in other tissues. In the interactions between BCTV and *Arabidopsis thaliana,* more viral DNA was accumulated in the actively dividing tissues of the root and shoot tip than in fully matured rosette leaves (Park *et al.*, 2002). Specially, BCTV was more accumulated in roots than in shoots which showed more disease symptoms. In this study, we also found that more SPLCV DNA was accumulated in roots than leaves even though roots did not show any typical disease symptoms (Fig. 1).

We noted that the SPLCV infectious clone remained biologically functional as described in the Southern blot analysis (Fig. 5, lower panel). Thus, we expect that research on SPLCD can be accelerated with the use of this infectious clone, which can be used to survey susceptible hosts and generate mutant viruses. We also attempted to gain some insight into the evolutionary history of SPLCV in geminiviruses by comparing its sequences with those of other geminiviruses, since SPLCV occupies an important position in the evolutionary pathway from curtoviruses to begomoviruses. To enhance our current knowledge regarding this evolutionary niche, further phylogenetic analysis focusing on the assessment of recombination among the sweepoviruses is needed.

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