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Restriction Mapping and Infectivity of Cloned Geminivirus DNA

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ABSTRACT (100-200 WORDS):

The bipartite geminivirus Squash Leaf Curl Virus (SLCV) has been the subject of several recent studies. Occuring in several strains, the strain known as SLCV-C is the object of this study. Since the virus is familiar to the discipline of plant virology but has not been largely considered in an experimental capacity when present in this isolate, this study endeavored to determine to which of the previously-studied strains SLCV-C appears most similar. Major aspects of the study were concerned with the determination of the physical nature of the strain, including the construction of a restriction endonuclease map and a test of the spectrum of the species to which SLCV-C proves infectious. Plasmids containing the cloned DNA of SLCV-C were isolated and purified from long-term storage, and cleaved with eighteen available restriction endonucleases such that an accurate restriction map could be produced. After a dimeric clone of the virus was produced to facilitate infection of plant tissues by rolling-circle replication, infectivity assays were performed. The results of the restriction map and infectivity assays with subsequent southern analysis show that SLCV-C possesses similar restriction sites and infects tissues in a capacity and range most similar to SLCV-R, a narrow-host-range strain characterized in previous studies.

Key words: DNA cloning, Bipartite geminivirus, Restriction enzyme, Plasmid, Virus.

Introduction: Squash Leaf Curl Virus, as its name implies, is a virus whose symptoms include a noticeable physical distortion or curling of leaf tissue, accompanied by vascular clearing and stunted growth. SLCV has been observed in the field to infect species of squash (Cucurbita spp.) via transmission by whiteflies (Duffus & Stenger, In press). SLCV occurs as at least two well-known strains, differentiated by host range (Ingham & Lazarowitz, 1993). The first of these strains, SLCV-E, possesses a wide host range and has been known to infect species of the Cucurbitae as well as legumes and tobacco (Nicotiana benthamiana) (Duffus & Stenger, In press). The second strain, SLCV-R, possesses a narrow host range (Lazarowitz, 1991). In this study, I am examining a third strain of SLCV (SLCV-C), isolated in by Cohen *et al* (1983) in an effort to determine its degree of similarity to one of the other characterized strains.

A study by Lazarowitz and Lazdins (1991) focused upon both SLCV-E and SLCV-R. This study, with the objective of determining the complete viral nucleotide sequence and range of infectivity, determined that Squash Leaf Curl Virus maintains common ancestry with tomato golden mosaic virus (TGMV), bean golden mosaic virus (BGMV), and African cassava mosaic virus (ACMV).

The virus is comprised of two components, which for the purpose of this study will be designated SLCV-A and SLCV-B. The component known as DNA-B possesses the genes which allow for systemic movement of the virus throughout plant tissues. DNA-A allows for actual infection of the tissues via replication of

the viral genome. It should thus be noted, therefore, that both components must be present for proper infection to occur. The isolate of SLCV I am studying has previously been cloned into plasmids of Escherichia coli (Stenger, unpublished). Both DNA components A and B are of identical size, both consisting of 2650 nucleotide base pairs. The "A" component is incorporated into a plasmid of approximately 3000 nucleotide base pairs known as pGEM-7, and incurs the moniker pSLCV-E3-A1. The "B" component is incorporated into a plasmid of about 2600 nucleotide base pairs known as pUC8, and is known as pSLCV-B4.

Of vital importance in the success of the study is the restriction endonuclease. A bacterial form of defense against viral infection, restriction endonucleases cleave DNA at unique six-nucleotide sequences specifically recognized by that particular endonuclease. A factor that is key to the field of molecular biology is that a restriction endonuclease will cleave DNA regardless of its specific origin (Edberg, 1987) and permits genotypic characterization of a specific DNA molecule.

Over 200 different restriction endonucleases, each derived from a different species of bacteria, have been isolated for use in the fields of molecular biology and, more specifically, genetics and virology. The properties of endonuclease activity relative to DNA cleavage have proven invaluable to the recent advances in these disciplines. For example, cleaving a viral genome with several restriction endonucleases renders the genome into fragments of number and size which are specific to that virus. This allows for

fragments of unknown DNA to be identified as belonging to a particular species, or even to a particular strain. Also, fragments of DNA from two different species, when cleaved with the same endonuclease, will be rendered into fragments whose ends are compatible with one another, thus allowing the fragments to be reattached or *ligated* (using an enzyme known as ligase) to each other. In this way, a chimeric gene has been created: a gene whose constituents are comprised of genetic material from two different sources.

The processes of restriction-endonuclease digestion and subsequent ligation of viral DNA were used in the construction of tandem repeats of the viral genome within its respective plasmid. Earlier experiments postulating that viral DNA was infectious to host plants only as a tandem repeat of the viral genome within plasmid DNA proved conclusive (Stenger *et al.*, 1991). This is due to the tendency of viral DNA to replicate by a process known as "rolling circle," in which the tandem repeat loops upon itself, thus excising one repeat which can subsequently be incorporated into the genome of the host plant (Stenger *et al.*, 1991).
Each viral DNA is incorporated into its respective plasmid by cleavage of both the plasmid and the viral genome with the same endonuclease (which is present on the plasmid in a region known as the MCS or multiple-cloning site), thus rendering the two molecules into fragments with compatible ends such that the viral DNA can be ligated into the circle of plasmid DNA.

Likewise, restriction endonuclease digestion was used to

construct a map of the relative locations of each restriction enzyme on each viral genome component.

Materials and methods: The first order of business in the study was the retrieval and purification of the cloned SLCV-C DNA (components A and B) from cryo-storage (-80°C). This was accomplished by performing a standard large- scale plasmid isolation procedure. After checking to ensure purity, the procedure for constructing the restriction map was initiated. Each viral component was cleaved separately with the eighteen available restriction enzymes at hand. After incubation at the specified temperature for 24 hours, the cleaved DNA was checked for number and size of the fragment(s) into which it had been rendered through standard gel electrophoresis. The number and sizes of the fragments were recorded. The positions of the enzymes which had cleaved the DNA more than once were easily determined using restriction maps of the plasmids into which each viral genome was incorporated. By cleaving the DNA simultaneously with an enzyme which had cleaved only once and with an enzyme whose positions were already known, the position of the former relative to the latter could be determined (Tables 1 and 2, Fig. 1). It was thus possible to determine whether the sites at which the enzyme cleaved the DNA were present within the viral insert, the plasmid, or in the multiple cloning site (MCS).

Next, the procedure for performing the infectivity assay was begun. The first order of business was the construction of dimeric clones of viral DNA. As is visible from Fig. 1, pSLCV-E3-A1 is

ligated into pGem7 at the EcoRI enzyme cleavage site on the MCS. pSLCV-B4 is ligated into pUC8 at the Bam HI site on the MCS. The two viral components were cleaved with these respective enzymes, thereby excising the viral component from the plasmid. The newly-cut DNAs were then immediately religated, under the assumption that some of the viral components would ligate to one another before reincorporation into the plasmid, thereby forming a tandem repeat or dimeric clone.

However, it was necessary to determine whether dimeric clones had truly been produced. This was accomplished by incorporating both components of pSLCV into DH5αF', a strain of Escherichia coli capable of amplifying (large scale growth of the plasmid with incorporated viral insert)either pGem7 or pUC8. After competent cells (cells able to uptake the plasmid DNAs) of DH5αF' were prepared using a standard protocol, each plasmid DNA (containing respective viral component) was transformed into the E. coli cells. The transformed cells were then plated out onto agar media containing antibiotics X-gal and Ampicillin. DNA of both plasmids pGem7 and pUC8 containing no viral insert would contain a gene known as lac-Z, a gene functional in the metabolism of lactose. Colonies of E. coli containing such plasmids would appear blue in color on X-gal media. Plasmids containing a viral insert, either monomeric or dimeric, would have the lac-Z gene disrupted by the viral DNA insert, and, being unable to metabolize lactose, would appear as white colonies of E. coli in the presence of X-gal.

In this way it was possible to identify colonies harbouring a

plasmid containing the viral insert. White colonies were selected, and transferred using asceptic technique to plates which had been gridded, and each square numbered. A sample of each selected colony was transferred to a numbered square on one of four plates, labeled A-D. The selected colonies were thence referred to by plate letter and grid number.

However, in order to ascertain whether or not the insert was dimeric, it was necessary to determine the size in nucleotide base-pairs of the bacterial genome. This was accomplished by performing rapid-lysis screening, a procedure by which a plasmid may be rapidly screened for insert size. By running the recently-lysed DNA in electrophoresis gel, it was possible to determine by size which plasmids contained a dimeric viral insert (those genomes containing such an insert would be considerably larger, and thus readily identifiable by the slower speed with which they moved through the gel) than those containing a monomeric insert). After electrophoresis was complete and a film produced, one colony of each component which appeared to be larger, and thus a possible dimer, was grown up in large-scale and re-purified by large-scale plasmid isolation (it should be noted that several colonies from each type expressed genomes which appeared on film to contain a dimeric insert). After cleavage of the DNA of both components by several separate restriction enzymes, they were run again on electrophoresis gel to compare their sizes with known sizes of the unmodified or "wild-type" genomes of each component. It was thus determined that the selected colony of the A-component did indeed

contain a dimeric viral insert, while the selected B-component colony did not. Another B-component colony was selected, and the procedure repeated until a colony harbouring a plasmid which truly contained a dimeric viral insert was obtained.

Once the genomes of each component containing dimeric inserts were undoubtedly identified by electrophoresis, the DNA was extracted from the gel using the GENECLEAN agarose gel isolation procedure. The plasmids were once again grown up in large-scale and re-purified. After purification, the plasmids were cut with enzymes which would prepare them for transfer into a plasmid known as pMON521 (Stenger *et al.*, 1991), a plasmid which serves as a vector that will allow transfer of the plasmids containing viral inserts from their original E. coli. hosts to a plant-infectious bacteria known as Agrobacterium tumefaciens.

Both pSLCV-E3-A1 (dimer) and pSLCV-B4 (dimer) were cleaved with endonucleases that would (a) excise the insert from the plasmid DNA, and (b) render the ends of the insert DNA compatible with similarly cleaved DNA of pMON521. After cleavage, the newly-cut DNAs were checked on electrophoresis gel, and isolated from the gel using the aforementioned GENECLEAN procedure. After the procedure was complete, the viral inserts were separately ligated into the pMON521 genome yielding pMSLCV-E3-A1 (dimer) and pMSLCV-B4 (dimer).

The newly-ligated DNAs were then transformed into the genome of MM294, a strain of E. coli capable of amplifying the plasmid pMON521. Competent cells of MM294 were produced using a standard

protocol, the viral inserts were transformed into the competent cells, and the mixtures were plated onto agar media. This procedure met with several complications, from lack of growth of colonies of transformed MM294 cells to insufficient yield of GENECLEANED fragments. However, colonies of pMSLCV-E3-A1 (dimer) and pMSLCV-B4 (dimer) in MM294 were eventually obtained, and were transferred to gridded plates for long-term storage.

In order to render the plasmids into a form capable of infecting plant tissue, it was necessary to place them into Agrobacterium tumefaciens, a bacteria renowned for its capabilities of transferring its DNA, along with any viral DNA contained therein, into the genomes of plants, regardless of species origin. This was accomplished by performing a triparental mating according to a standard protocol (Rogers *et al.*, 1986). The first step involved mobilization of both pMSLCV-E3-A1 (dimer) and pMSLCV-B4 (dimer) into Agrobacterium using the transfer functions present on another plasmid known as pRK2013. This plasmid facilitates movement of pMON521 (and other pMON derivatives) from MM294 into Agrobacterium (Lemos & Crossa, 1992).

It was then necessary to induce through the use of antibiotics an inherent selection mechanism to yield only colonies of Agrobacterium which had received plasmid DNA (containing the viral insert). Agrobacterium clones bearing pMSLCV-E3-A1 (dimer) and pMSLCV-B4 (dimer) are by nature resistant to the antibiotic spectinomycin. Likewise, clones of pRK2013 are resistant to the antibiotic kanamycin. Finally, Agrobacterium clones possess

resistance to chloramphenicol. Therefore, Agrobacterium colonies which, after triparental mating, bore the pMON521 derivatives (containing the viral insert) would by nature be resistant to both spectinomycin and chloramphenicol, since the kanamycin-resistant pRK2013 serves only as a mobility vector and is not retained in Agrobacterium. Clones of both components in Agrobacterium were streaked onto plated media containing all three antibiotics. Growth occurred in both cases. One colony of each component was selected and grown up for transfer into liquid media. A sample of each component was transferred to a vial. Liquid samples of Agro were subsequently transferred to each vial, and, after 24 hours incubation at 28°C, were observed to have grown in large quantity. These samples were streaked out onto plates containing no antibiotics. Colonies obtained on these plates were then transferred to liquid media containing all three antibiotics. Growth occurred in these vials. It was therefore evident that the triparental mating procedure had proven successful.

After samples of the triparentally-mated plasmids into Agro were obtained, it was possible to begin the actual infectivity assay. Seeds of tobacco (Nicotiana benthamiana) were obtained, and planted in 4" pots containing a standard humus mixture. Four trays were prepared, each tray containing eight pots. Approximately two weeks were allotted to allow the seedlings to reach a level of maturity sufficient to survive the process of agroinoculation. When two week had elapsed, the trays were designated by the nature of the sample with which they would be inoculated. Plants in tray

#1 were inoculated with a sample containing only the A-component of SLCV. Tray #2 was inoculated with only the B-component. Tray #3 was inoculated with a sample containing both components, while tray #4 was inoculated with a sample containing pMON521 into which no viral DNA had been ligated. Tray #4 served as a positive control.

The procedure of agroinoculation followed as such: Terminal tissue was severed from the top of the plant, exposing the stem and its interior. Two to three drops of the liquid sample were placed on the newly-exposed stem tissue. A pin was subsequently used to wound the tissue in a concentrated area such that a sufficient wound as required by Agrobacterium to properly transfer DNA was produced (Rogers & Klee, 1987).

Once the assay of Nicotiana bethamiana were established, a similar assay of black zucchini (Cucurbita pepo) was performed. Since both components A and B were required for infection to occur at all, only two trays, both containing eight plants in 4" pots, were set up. Individuals in the first tray were inoculated with a media containing Agro cells which had incorporated pMON521 DNA into which both A and B components had been separately inserted. Individuals in the second tray were inoculated with Agro cells which had incorporated into their genome only wild-type pMON521 DNA.

Once the plants were showing symptoms to as great a degree as would evidently transpire (after 14 to 21 days postinoculation, an analysis was performed to determine the extent to which viral DNA had been incorporated into the plant tissue. Known as a Southern

analysis, the technique involves the use of radioactive probes to discern whether and to what degree the viral DNA moved systemically through the plant tissue (Edberg, 1987)

Results: As is evident from Figure 1, a restriction map of both DNA components of SLCV was constructed. The aforementioned lists of the endonucleases used to cleave both pSLCV-E3-A1 and pSLCV-B4, in both single and double digests, are present in tables 1 and 2.

Infectivity assays show that, when both components of the SLCV genome were present, the virus proved to infect 5 of 8 specimens of N. benthamiana or 63% of inoculated specimens. Only 2 of 8 individuals of black zucchini, or 25% of inoculated individuals, showed symptoms of infection. Concurrent southern analysis of DNA extracted from symptomatic plant tissue of both species show DNA of approximately 2600-2700 nucleotide base-pairs, in both single-stranded and double-stranded form. These sizes compare favorably with the sizes of the viral inserts, both A and B components, when present in their original plasmids. We can infer from the data obtained in the southern analysis that the viral insert had indeed been replicated in plants. Bands representative of DNA extracted from symptomatic tissue of Nicotiana benthamiana (Fig. 2, lanes 7 and 9) are markedly more pronounced than the co-migrating bands of symptomatic tissue of black zucchini (Fig 2, lane 11). These results concur with the obtained infectivity efficiencies of SLCV in N. benthamiana and black zucchini. That is to say that N. benthamiana, being a more "hospitable" host to SLCV than black

zucchini, allowed for higher efficiency of infectivity than did black zucchini.

Discussion: The aforementioned studies performed Lazarowitz and Lazdins (1991) endeavored to produce restriction maps and establish host range of the two previously-characterized isolates of SLCV, namely, SLCV-E and SLCV-R. Comparison of the restriction maps of both **A** and B components of these two isolates with the restriction map of SLCV-C constructed in this study provide interesting results. The restriction maps of SLCV-C and SLCV-R show marked similarity, sharing analogous SacI, XbaI, Bam HI and Eco RI sites on the **A** component, and SacI and Bam HI sites on the B component. In contrast, the restriction maps of SLCV-C and SLCV-E are quite dissimilar, sharing no analogous sites on either component. The results apparently indicate a strong similarity between SLCV-C and the narrow-host range isolate.

These results seem to be enforced by comparison of SLCV-C's host range with those of SLCV-R and SLCV-E. The aforementioned infectivity assay of SLCV-E performed by Lazarowitz and Lazdins in 1991 showed infectivity efficiency of 80-100% in all species assays therein, including N. bethamiana as well as Phaseolus vulgaris, a species of the Leguminosae, and squash species Cucurbita pepo and Cucurbita maxima. Infectivity assays performed using the SLCV-R isolate resulted in substantially reduced infectivity efficiencies, with ≤75% efficiency of infectivity in Nicotiana benthamiana, and ≥10% efficiency of infectivity in Phaselus vulgaris (Lazarowitz, 1991. These results compare favorably with those obtained using

the SLCV-C isolate, though Cucurbita pepo was used in place of P. vulgaris. It can thus be inferred from the data at hand that SLCV-C it quite similar to the narrow host range isolate of SLCV.

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Single digests.

Enzyme	Sites in Vector	Sites in Insert	Total Sites	Frag. size
Apa I	1	2	3	3400, 1200 850
Bam HI	1	1	2	5200, 240
Bgl II	0	0	0	--
Csp 45I	1	0	1	linear
Cla I	1	1	2	5100, 310
Dra I	2	1	3	3300,1100
Eco RI	2	0	2	3000,2500
Eco RV	0	0	0	--
HinD III	1	0	1	linear
Kpn I	1	0	1	linear
Nco I	0	2	2	4800, 520
Nsi I	1	0	1	linear
Pvu II	2	0	2	2750,2300
Pst I	0	0	0	--
Sac I	1	1	2	4300, 1150
Sal I	0	1	1	linear
Sau 3AI	many	many	many	--
Sma I	1	0	1	linear
Sna BI	0	0	0	--
Spe I	1	2	2	4300, 800
Ssp I	1	2	3	3500,1750 180
Xba I	1	1	2	3700,1700
Xho I	1	1	2	3100,2300

Double digests.

Enzyme(s)	No. and size of resultant fragments (in bp)
Apa I	3: 3250, 1300, 1050
Sac I	2: 4300, 1350
Apa I & Sac I	4: 2850, 950, 775, 280
Dra I	3: 3400, 1300, 620
HinD III	1: linear (5200)
Dra I & HinD III	4: 2400, 1050, 1200, 650
Nco I	2: 4800, 600
Nco I & HinD III	2: 4800, 600

Table 1. List of respective endonuclease digests (both single and double) of pSLCV-E3-A1.

Single digests.

Enzyme	Sites in Vector	Sites in Insert	Total sites	Frag. size
Apa I	0	0	0	--
Bam HI	2 (in MCS)	0	2	2 @ 2600 bp
Bgl II	0	1	1	linear
Csp 45I	0	0	0	--
Cla I	0	0	0	--
Dra I	3	1	4	1900, 2 @ 1300,690
Eco RI	1	0	1	linear
Eco RV	0	0	0	--
HinD III	1	0	1	linear
Kpn I	0	1	1	linear
Nco I	0	1	1	linear
Nsi I	0	1	1	linear
Pvu II	2	1	3	2 @ 2300,690
Pst I	1 (in MCS)	0	1	linear
Sac I	0	1	0	linear
Sal I	1	3	4	2900, 850, 564, 310
Sau 3AI	many	many	--	--
Sma I	1	1	2	3400, 2000
Sna BI	0	0	0	--
Spe I	0	1	1	linear
Ssp I	1	1	2	3150, 2150
Xba I	0	1	1	linear
Xho I	0	0	0	--

Double digests.

Enzyme(s)	No. and size of resultant fragments (in bp)
HinD III	1: linear (5400)
Bgl II	1: linear (5400)
Bgl II & HinD III	2: 4300, 1100
Kpn I	1: linear (5400)
Kpn I & HinD III	2: 4050, 1350
Nco I	1: linear (5400)
Nco I & HinD III	2: 4420, 980
Nsi I	1: linear (5400)
Nsi I & HinD III	2: 4865, 535
Sac I	1: linear (5400)
Sac I & HinD III	2: 3950, 1450
Spe I	1: linear (5400)
Spe I & HinD III	2: 3640, 1760
Xba I	1: linear (5400)
Xba I & HinD III	2: 3300, 2100

Table 2. Lists of respective endonuclease digests (both single and double) of pSLCV-B4

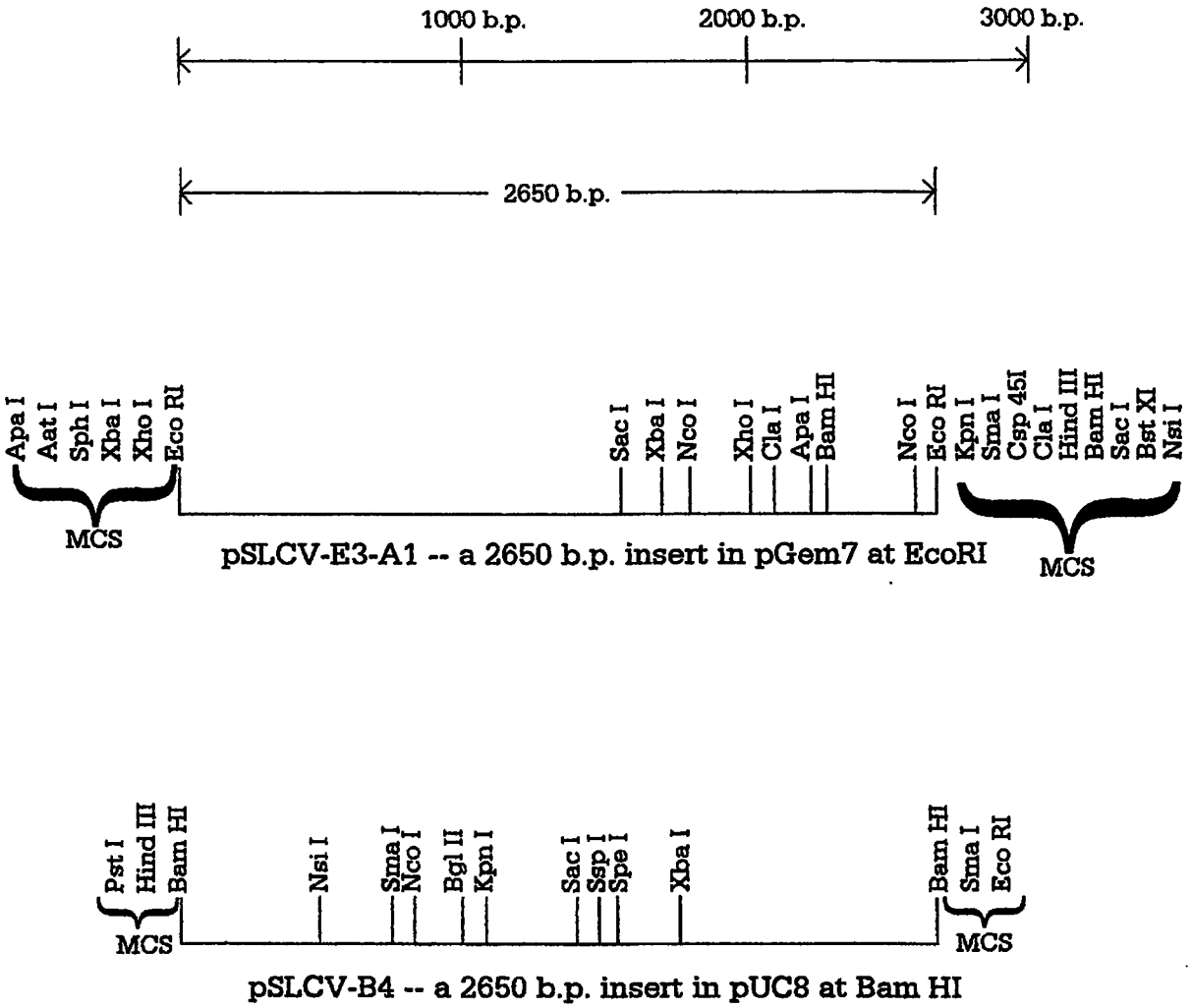

Figure 1 -- Endonuclease restriction maps of unit-length cloned DNA-A (pSLCV-E3-A1) and DNA-B (pSLCV-B4) conponents of SLCV.

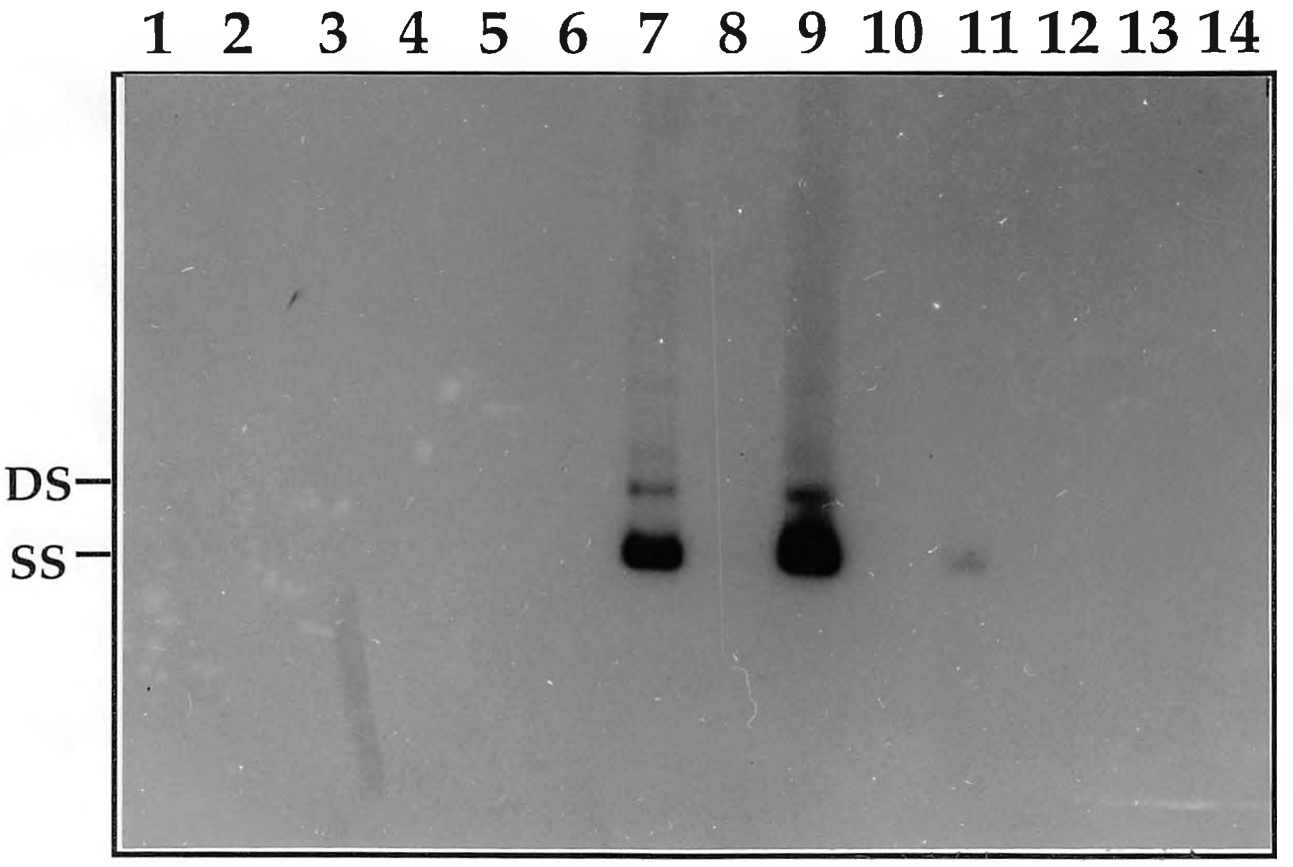

Figure 2. SLCV DNA forms in infected plants. An autoradiograph of a Southern blot of total DNA (1 ug) extracted from Nicotiana benthamiana inoculated with pMon521 (lanes 1 &2), SLCV DNA A (lanes 3 &4), SLCV DNA B (lanes 5 &6), SLCV DNA A + DNA B (;anes 7 & 9); or Black zucchini inoculated with SLCV DNA A + DNA B (lanes 10 & 11), pMON521 (lanes 12 &13). No sample was applied to lane 8. Lane 14 contained Hind III fragments of phage lambda. The blot was probed with a radiolabelled RNA complementary to SLCV DNA A. Single-stranded (SS) and double-stranded (DS) forms of SLCV DNA are indicated at left.