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Double-stranded RNAs were isolated from plants infected with five different isolates of apple chlorotic leaf spot virus (ACLSV). Analysis by PAGE and by Northern blot hybridization showed that six major species of viral dsRNA of approximately 7·5, 6·4, 5·4, 2·2, 1·1 and 1·0 kbp can be detected in infected plants, irrespective of the ACLSV isolate used. In addition to the dsRNA of 7·5 kbp corresponding to the full-length genome, the size and position on the genome of the 2·2 and 1·1 kbp species indicate that these are very probably double-stranded forms of subgenomic RNAs allowing the expression of the internal open reading frames coding respectively for the ACLSV 50K and coat proteins. The subgenomic messenger for the coat protein was indeed detected in total RNA preparations from infected plants. Surprisingly, the two most abundant dsRNA species, of 6·4 and 5·4 kbp, were found to be 5′-coterminal with the genomic RNA. A model for the expression of the genome of ACLSV and for the production of the molecules 5′-coterminal with the genomic RNA is presented.

Introduction

Apple chlorotic leaf spot virus (ACLSV) has so far been classified as a member of the closterovirus group, which also contains citrus tristeza virus (CTV), beet yellows virus, carnation necrotic fleck virus and other viruses with similar flexuous, elongated particles (Bar-Joseph & Murant, 1982). The heterogeneity of this taxonomic group has resulted in its subdivision into three subgroups (A, B and C), subgroup A being typified by ACLSV.

ACLSV is known to infect most rosaceous fruit tree species and is responsible for serious diseases in stone fruits, including peach dark green sunken mottle, false plum pox and plum bark split. It also induces severe graft incompatibilities in some *Prunus* combinations (Dunez & Delbos, 1988). ACLSV has a particle size of 720 × 12 nm containing a single coat protein species with an M_r of about 22K (Yoshikawa & Takahashi, 1988) encapsidating a single-stranded positive sense genomic RNA of M_r $2{\cdot}5 \times 10^6$ (Lister & Bar-Joseph, 1981). We have recently determined the nucleotide (nt) sequence of the genomic RNA of the P863 isolate of ACLSV (German *et al.*, 1990). The ACLSV genomic RNA is 7555 nt in length excluding the poly(A) tail and possesses three overlapping open reading frames (ORFs) coding for proteins of 216·5K, 50K and 28K. The 28K ORF contains, in frame, a 21·5K ORF encoding the capsid protein (T. Candresse *et al.*, unpublished results).

The dsRNAs produced in plants upon infection by ACLSV have previously been analysed (Dodds & Bar-Joseph, 1983; Yoshikawa & Takahashi, 1988). However, different results were obtained by these two groups and the observation of several (two or three) high M_r dsRNA species cannot easily be interpreted in the light of the nt sequence data. As different isolates have been used in these studies, these conflicting results could indicate some level of variability between isolates of ACLSV. Differences between isolates of CTV involving the presence of specific dsRNA segments have already been demonstrated, and it was suggested that the dsRNA electrophoretic patterns could be used as markers to identify specific CTV isolates (Dodds *et al.*, 1987).

Therefore, one aim of this study was to see whether dsRNA analysis could be used to detect differences between isolates of ACLSV, depending on their original host, geographical origin and symptomatology. Our results show that the five isolates analysed produce the same pattern of six virus-specific dsRNAs. The three largest dsRNAs with estimated sizes of 7·5 (double-stranded form of the genomic RNA), 6·4 and 5·4 kbp were directly detected following PAGE. Three smaller, not previously detected, dsRNA species which probably correspond to the double-stranded forms of the subgenomic messenger RNAs for the 50K and coat protein ORFs and to an additional internal form were identified in Northern blot hybridization experiments.

No subgenomic RNAs were detected in RNAs purified from viral particles but an ssRNA of a size corresponding to the putative subgenomic RNA for the capsid protein was found in total RNAs extracted from infected plants. Surprisingly, the Northern blot experiments also demonstrated that the two most prevalent

dsRNA species (6·4 and 5·4 kbp) map in the 5′ part of the genome. From these results, we propose a model for the expression of the ACLSV genome and for the generation of the 5′-coterminal and internal dsRNAs.

Methods

Isolates of ACLSV. The P863 isolate of ACLSV, isolated from *Prunus domestica* and responsible for the bark split disease (Dunez *et al.*, 1973) was inoculated mechanically and propagated in *Chenopodium quinoa*. Isolates P1710 and Balaton 1 were kindly supplied by J. C. Desvignes (Centre Technique Interprofessionnel des Fruits et Légumes, Centre de Lanxade, France), transmitted on GF305 peach seedlings by chip budding, and then propagated in *C. quinoa*. Isolate P1710, collected in France from a mirabelle plum, is responsible for more severe bark split symptoms than the P863 isolate. Balaton 1 was originally isolated from a wild cherry tree in Hungary and causes severe symptoms of mottling and pitting on cherry and peach leaves and fruits. Isolates M17 and P1, isolated in Japan from *Malus prunifolia* and *M. pumila* respectively, cause symptoms of dark green mottle on peach leaves and were obtained from the American Type Culture Collection.

Purification of virus and viral ss- or dsRNAs. All plants used were inoculated and grown under greenhouse conditions (16 h day, 24 °C). Virus and viral genomic RNA were purified as previously described by Dunez *et al.* (1973). The dsRNA extraction procedure was basically as described by Dodds & Bar-Joseph (1983). Frozen leaves (200 g) were homogenized in the presence of 400 ml of 2 × STE buffer, (1 × STE is 0·1 M-sodium chloride, 0·05 M-Tris–HCl, 1 mM-EDTA pH 8·0), with 56 ml of 10% SDS, 320 mg of bentonite and 570 ml of Tris–HCl pH 8·0 saturated phenol. The mixture was stirred for 30 min at room temperature and then centrifuged at 10000 *g* for 20 min. The aqueous phase obtained by centrifugation was adjusted to 16% ethanol and gently mixed with 15 g of Whatman CF-11 cellulose powder for at least 20 min at 4 °C. The cellulose, with bound dsRNAs, was then collected through a Pharmacia chromatography column, washed with several volumes of 1 × STE containing 16% ethanol to remove unbound impurities prior to the release of the dsRNAs in a small volume of ethanol-free 1 × STE buffer. The dsRNAs contained in the eluate were then precipitated by the addition of 2·5 volumes of 96% ethanol and 0·1 volume of 3 M-sodium acetate pH 5·5. The dsRNAs were collected by centrifugation and resuspended in diethylpyrocarbonate-treated water.

The dsRNA samples (equivalent to 2 g of starting plant material) were loaded on 6% polyacrylamide gels (acrylamide : bisacrylamide, 40 : 1 v/v) and subjected to electrophoresis for 15 h at 70 V. The gels were then stained with silver nitrate (Bio-Rad silver staining kit) according to the supplier.

Extraction of total RNAs from infected plants. Total ssRNAs were obtained from 10 g of young leaves of healthy or ACLSV-infected *C. quinoa*, following the method described by Verwoerd *et al.* (1989).

Preparation of radioactively labelled probes. Complementary DNA clones of ACLSV genomic RNA obtained previously in plasmid pBluescript (Stratagene) (German *et al.*, 1990) were labelled by nick translation, using a nick translation system (BRL) with [α-^{32}P]dCTP (3000 Ci/mmol, Amersham) as radioactive precursor. The position on the genome of the various cDNA fragments is precisely known from previous sequencing (German *et al.*, 1990); probe 1 (nt 38 to 305), probe 2 (nt 2304 to 4248), probe 3 (nt 5502 to 6088), probe 4 [nt 6518 to the poly(A) tail] and probe 5 [nt 7303 to the poly(A) tail]. RNA transcripts complementary to the viral genomic RNA were synthesized from the same cDNA clones, using the Riboprobe Gemini System (Promega) and phage T3 or T7 RNA polymerase, according to the supplier. The radioactive precursor used was [α-^{32}P]CTP (800 Ci/mmol, Amersham).

Northern blot analysis. Total RNAs and dsRNAs were analysed by electrophoresis under denaturing conditions in 1% agarose gels containing MOPS–EDTA (50 mM-MOPS, 1 mM-disodium EDTA pH 7·0) and 6% (v/v) formaldehyde (Miller, 1987). The dsRNAs in MOPS–EDTA, 50% (v/v) formamide, 6% (v/v) formaldehyde (final concentration 2·2 M), were incubated at 70 °C for 10 min before loading on the gel. The electrophoresis buffer was MOPS–EDTA with no formaldehyde.

Electrophoresis was carried out at 100 V for 2 h, followed by toluidine blue staining or by transfer onto nylon (Zeta-Probe, Bio-Rad) or nitrocellulose membranes (BA45, Schleicher & Schuell) using 20 × SSC pH 7·0 as the transfer buffer. All transfer, prehybridization, hybridization and washing steps were done as described in Ausubel *et al.* (1987). Dried membranes were exposed to Kodak X-AR film at −80 °C for 2 to 3 days using intensifying screens.

Results

Detection of ACLSV dsRNAs by PAGE

In plants inoculated with the P863 isolate of ACLSV, viral dsRNAs are first detected 6 days after inoculation. They can still be detected in the plant extract 16 days post-inoculation when the symptoms are well developed. During this period, no variations in the pattern of bands or quantity of dsRNAs are observed (data not shown). The youngest elongated apical leaves of *C. quinoa* plants harvested 12 to 14 days post-inoculation were used throughout this study. Since, as already noticed in previous studies (Dodds & Bar-Joseph, 1983), ACLSV induces the synthesis of only small amounts of dsRNAs, the phenol extraction procedure of Dodds & Bar-Joseph (1983) was used. Compared to aqueous extraction procedures, this technique allows a better recovery of viral dsRNAs but also results in a higher background of plant dsRNA material (see for example Fig. 1, lane 2).

The dsRNAs extracted from tissue infected with each of five isolates of ACLSV differing by their original host, their symptomatology or their geographical origin were analysed by non-denaturing PAGE, together with the corresponding material from healthy plants. Each gel lane in Fig. 1 and subsequent experiments and figures, was loaded with dsRNAs recovered from 2 g of plant tissue.

As can be seen from Fig. 1, all the ACLSV isolates used induce the production of three major bands of virus-specific dsRNAs, designated I, II and III in order of increasing mobility, dsRNAs II and III being clearly more abundant than dsRNA I. These molecules were not detected in the uninfected control. According to the size standards used in the non-denaturing gel of Fig. 1 (1 kb DNA ladder from BRL), these molecules have apparent sizes of 6·9 kbp (I), 6·1 kbp (II) and 5 kbp (III) differing

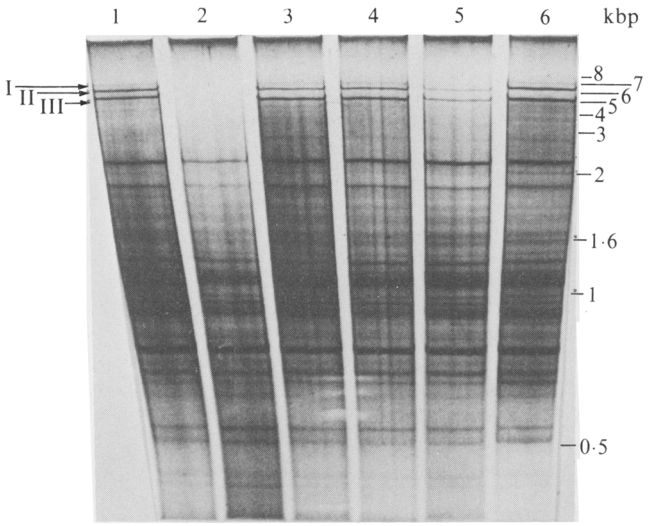

Fig. 1. PAGE of dsRNAs purified from leaves of *C. quinoa* infected with various isolates of ACLSV. Lane 1, dsRNAs from plant tissue infected with the P863 isolate of ACLSV; lane 2, dsRNA fraction from healthy plant tissue; lanes 3 to 6, respectively, dsRNAs from plant tissue infected with P1710, Balaton 1, M17 and P1. The arrows indicate the three virus-specific dsRNA bands I, II and III. The position of the dsDNA fragments of the 1 kb DNA ladder (BRL) are indicated on the right of the figure.

from the apparent sizes of 7·5 kbp (I), 6·4 kbp (II) and 5·4 kbp (III) estimated on a denaturing agarose gel, using either the same DNA standard (not shown) or the BRL single-stranded RNA ladder (Fig. 2). This discrepancy is probably explained by the difficulty in estimating the size of large nucleic acid molecules by PAGE. The double-stranded nature of these three species was confirmed by their resistance to DNase I and to RNase A in 2 × SSC buffer but not in 0·1 × SSC (data not shown). The size of dsRNA I estimated by denaturing agarose gel electrophoresis (7·5 kb) is compatible with its interpretation as the double-stranded form of ACLSV genomic RNA. Besides the double-stranded form of the genomic RNA, the detection, for each of the five isolates tested, of two major, high M_r ACLSV-specific dsRNAs, confirms and extends the results of Yoshikawa & Takahashi (1988).

Analysis of the ACLSV dsRNAs by Northern blot hybridization

To demonstrate that dsRNAs I, II and III are derived from the genome of ACLSV and to try to identify other ACLSV dsRNAs which might have been hidden by plant materials in the experiments described above, dsRNA extracts were subjected to Northern blot analysis (Fig. 2). No non-specific reactions with healthy plant extracts were observed with a mixture of all the ACLSV-specific probes used in this study (Fig. 2*a*, lane 2).

Probe 5, which corresponds to the last 250 nt at the 3′ end of the genome, was first used to detect double-stranded forms of putative subgenomic mRNAs, since these should be 3′-coterminal with the genomic RNA. As can be seen in Fig. 2(*a*) (lane 7), this probe specifically hybridizes to dsRNA I but also to two smaller dsRNAs of 2·2 and 1·1 kbp, which were therefore named dsRNA IV and dsRNA V respectively. Since dsRNAs IV and V have a size similar to the bulk of the dsRNA material from healthy plants, it is not surprising that they could not be observed upon staining of the polyacrylamide gels and were not detected in previous studies. The double-stranded nature of dsRNAs IV and V was confirmed by blotting experiments demonstrating their resistance to DNase I and to RNase A in 2 × SSC but not in 0·1 × SSC (data not shown). It must be noted that this probe, specific for the P863 isolate, also detects these two small dsRNAs among the dsRNAs induced by the four other isolates of ACLSV used in this study (data not shown).

Surprisingly, probe 5 does not hybridize with dsRNAs II and III, which indicates either that they are of plant origin and induced upon infection or that they are of viral origin but lack at least the 3′-terminal part of the genome present in probe 5. To try to settle this issue we used another probe, probe 2, which contains sequences internal to the genome. Probe 2 hybridizes to dsRNAs I, II and III (Fig. 2*a*, lanes 4 and 4′), demonstrating that, despite the fact that they do not contain the virus 3′-terminal sequences, dsRNAs II and III are indeed of viral origin. Probe 2 also reacts with a smear of lower M_r material but, as expected from their size and 3′-terminal location, dsRNAs IV and V do not appear to cross-hybridize with this probe (Fig. 2*a*, lane 4′).

Probe 1, complementary to nt 38 to 305 at the 5′ end of the genome, also hybridizes only with the three largest dsRNAs (Fig. 2, lane 3). Although the hybridization signal with dsRNA I is weak in the experiment shown in Fig. 2(*a*), lanes 3 to 7, it was visible on the original autoradiogram as well as in other similar experiments such as the one shown in lane 8 of Fig. 2(*a*). These results indicate that, as would be expected for the double-stranded form of the genomic RNA, dsRNA I contains both the 5′ and 3′ regions of the genome. They also show that dsRNAs II and III are molecules that are 5′-coterminal with the genomic RNA and that they do not contain the 3′-terminal part of the genome.

Results of hybridizations with probe 4 (Fig. 2*a*, lanes 6 and 9) are essentially the same as those obtained with probe 5 (Fig. 2*a*, lane 7) except that, in some experiments such as the one shown in lane 9, a faint hybridization signal with dsRNA II can be observed.

Probe 3 hybridizes with dsRNAs I, II and IV and, in

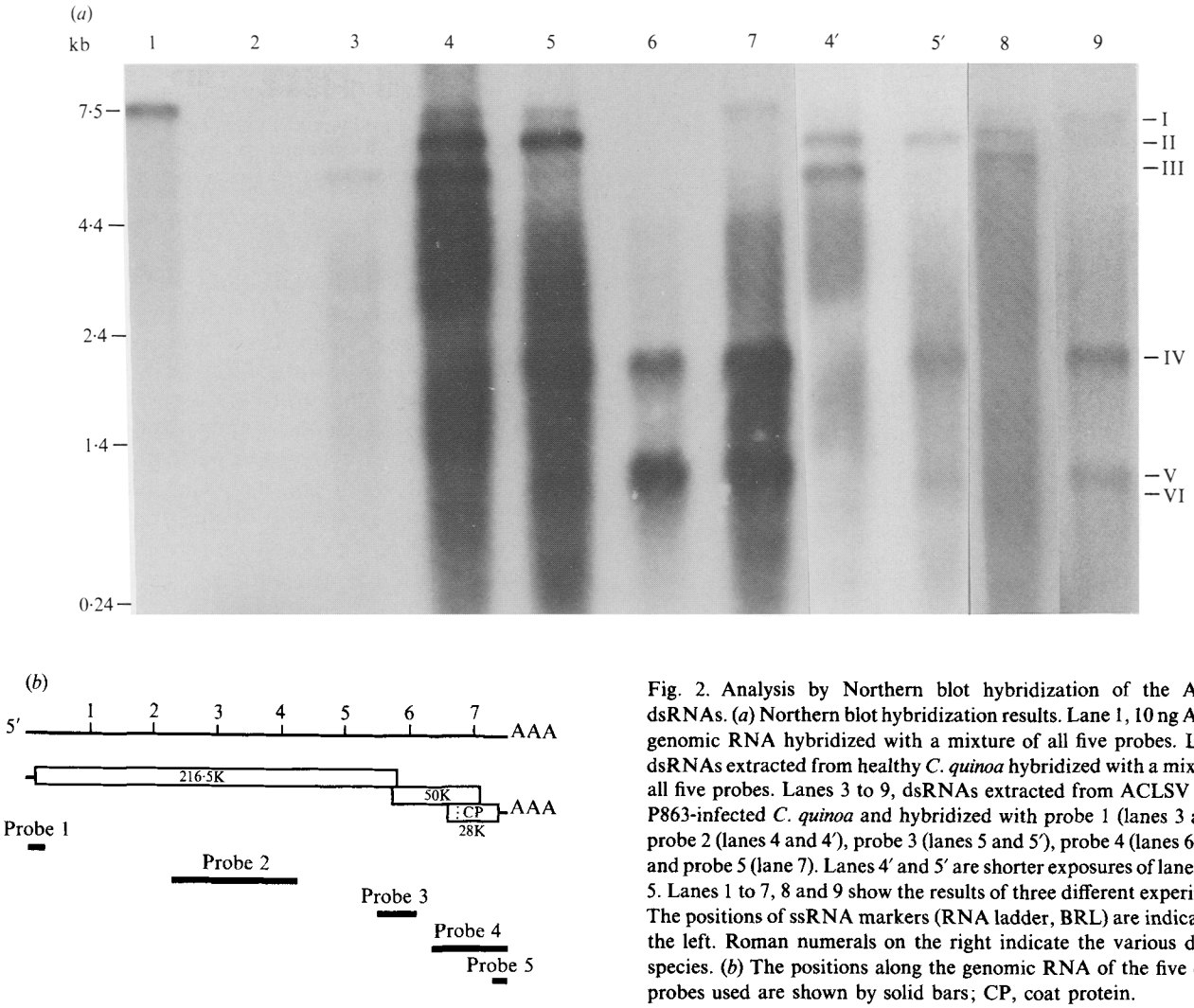

Fig. 2. Analysis by Northern blot hybridization of the ACLSV dsRNAs. (*a*) Northern blot hybridization results. Lane 1, 10 ng ACLSV genomic RNA hybridized with a mixture of all five probes. Lane 2, dsRNAs extracted from healthy *C. quinoa* hybridized with a mixture of all five probes. Lanes 3 to 9, dsRNAs extracted from ACLSV isolate P863-infected *C. quinoa* and hybridized with probe 1 (lanes 3 and 8), probe 2 (lanes 4 and 4′), probe 3 (lanes 5 and 5′), probe 4 (lanes 6 and 9) and probe 5 (lane 7). Lanes 4′ and 5′ are shorter exposures of lanes 4 and 5. Lanes 1 to 7, 8 and 9 show the results of three different experiments. The positions of ssRNA markers (RNA ladder, BRL) are indicated on the left. Roman numerals on the right indicate the various dsRNA species. (*b*) The positions along the genomic RNA of the five cDNA probes used are shown by solid bars; CP, coat protein.

addition, to a previously undetected form, dsRNA VI, migrating slightly faster than dsRNA V (Fig. 2, lanes 5 and 5′).

Taken together, these results allow us to propose a model for the location of the six dsRNAs along the ACLSV genomic RNA: dsRNA I corresponds to the double-stranded form of the genomic RNA, dsRNAs II and III are 5′ coterminal with the genomic RNA, dsRNA VI is internal to the genome and dsRNAs IV and V, 3′ coterminal with ACLSV genomic RNA, could correspond to the double-stranded forms of putative subgenomic mRNAs for the 50K and capsid proteins.

Detection of the putative coat protein subgenomic mRNA

Although subgenomic RNAs can be encapsidated, in many cases they are not found in virions but only by extraction of RNA from infected tissues (Palukaitis, 1984). Purified ACLSV particles, when disrupted, yielded only one readily resolved toluidine blue-stained band of 7·5 kb in agarose gels. Even if a smear of decreasing size and stain intensity was present, no other discrete band was apparent, and no encapsidated subgenomic RNA could be detected, even by Northern blot hybridization using a mixture of probes 1 to 5 (Fig. 2*a*, lane 1).

We therefore analysed total RNAs from infected plants by Northern blot hybridization with the capsid gene probe (probe 4). Fig. 3 shows that in addition to the band of 7·5 kb corresponding to the genomic RNA, a band of 1·1 kb can be seen, which could correspond to the single-stranded form of the subgenomic mRNA coding for the capsid. No band corresponding to a candidate 50K subgenomic RNA could be detected.

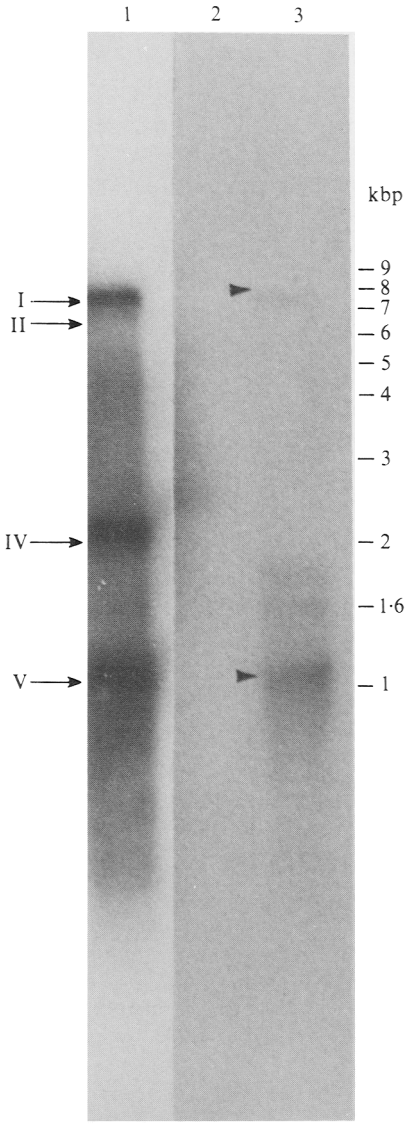

Fig. 3. Northern blot analysis using probe 4 (see Fig. 2) of dsRNAs and total ssRNAs extracted from ACLSV P863-infected leaves. Lane 1, dsRNAs from plant tissue infected with the P863 isolate of ACLSV, after RNase A treatment in 2 × SSC buffer. Lane 2, dsRNAs from healthy plant tissue treated similarly. Lane 3, total RNAs purified from plant tissue infected with the P863 isolate of ACLSV. Arrows and roman numerals indicate the position of the dsRNA species. The arrowheads indicate hybridization signals of the 7·5 kb genomic RNA and the 1·1 kb single-stranded subgenomic RNA for the coat protein.

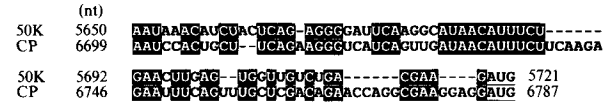

Fig. 4. Alignment of the sequences of the putative promoters for the 50K and the coat protein subgenomic RNAs. The numbering refers to the nt immediately outside the sequence shown. Identical nt are boxed.

Discussion

The five ACLSV isolates analysed in this report produce three dsRNAs directly detected on polyacrylamide gels, dsRNA I (7·5 kbp), II (6·4 kbp) and III (5·4 kbp). Three smaller dsRNAs, IV (2·2 kbp), V (1·1 kbp) and VI (1 kbp) are detected following Northern blot hybridization experiments. The dsRNA patterns on polyacrylamide gels are similar for all the isolates and the mobility of each of the dsRNAs I, II and III is similar to the mobility of the three major dsRNAs obtained with the Japanese P-205 isolate of ACLSV (Yoshiakwa & Takahashi, 1988). Therefore, our results show that dsRNA analysis cannot be used to detect differences between the isolates of ACLSV analysed in this report.

A model for the expression of the genome of ACLSV can be derived from our results. In this model, only the 5′-proximal ORF (216·5K) coding for the putative viral replicase is translated directly. The two other ORFs encoding the 50K and coat proteins are translationally silent and expressed through two subgenomic RNAs. To test this hypothesis, we tried to identify sequences on the genomic RNA of ACLSV that could correspond to the two subgenomic RNA promoters, one each for the 50K and coat protein mRNA. A conserved region with respect to nt sequence as well as distance from the initiation codons can be found on the RNA of ACLSV. It starts 84 nt from the coat protein initiation codon and 67 nt from the 50K initiation codon (Fig. 4) and could be the sequence recognized by the replicase to initiate synthesis of the subgenomic RNAs. This conserved sequence has, however, no similarity to other known subgenomic RNA promoter sequences.

The putative subgenomic mRNA for the capsid protein is detected among the total RNAs extracted from infected plants but not the putative 50K subgenomic RNA. As the 50K protein is suspected to mediate cell-to-cell movement of the virus (German *et al.*, 1990), which probably corresponds to an early function, the timing of expression of the 50K gene can be such that at 14 days post-inoculation the 50K subgenomic RNA may be present at a level too low to be detected. Kinetic studies may help to analyse the expression of this gene.

It has been shown that tobamovirus genes expressed via subgenomic mRNAs appear to be expressed in increasing amounts when positioned closer to the 3′-terminus (Lehto & Dawson, 1990). This could also explain why the coat protein subgenomic mRNA is expressed at a higher level than the subgenomic mRNA for the 50K protein.

The dsRNAs II and III, 5′-coterminal with the genomic RNA, were produced by all the isolates analysed. Although not specifically emphasized in the

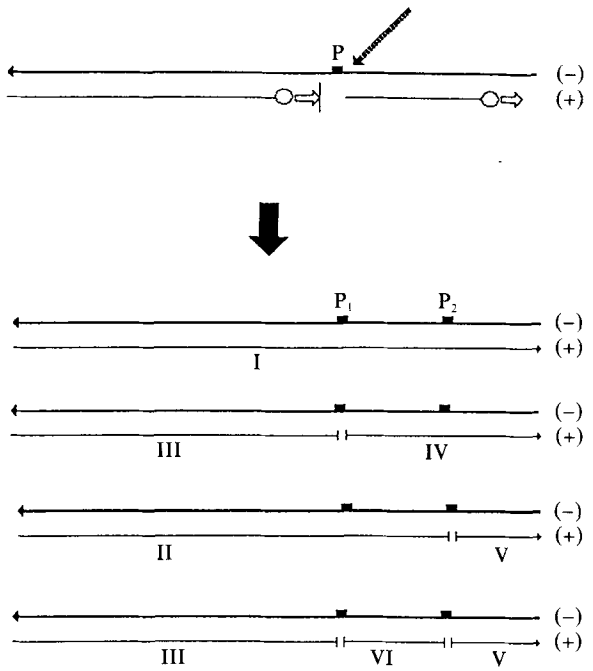

Fig. 5. Model for the production of ACLSV dsRNAs. O, Polymerase. The arrow with a bar in front indicates a stalled polymerase. P, subgenomic RNA promoter. Large striped arrow, nuclease attack. P_1, promoter for the subgenomic mRNA for the 50K protein; P_2, promoter for the subgenomic mRNA for the capsid protein. Roman numerals indicate the various dsRNA species observed. For more details see the text.

original reports, probably similar dsRNAs can be seen on polyacrylamide gel photographs shown in studies concerning the purification of dsRNAs from plants infected with carlaviruses and potexviruses (Valverde *et al.*, 1986). In our case, these molecules are found in a greater amount than dsRNA I, the double-stranded form of the genomic RNA. The origin and the role of dsRNAs II and III are unknown. The status of dsRNA VI which our data shows to be internal is similarly puzzling.

It can be noted that the length of dsRNA I (about 7·5 kbp) approximately corresponds to the sum of the lengths of dsRNA II (6·4 kbp) plus dsRNA V (1·1 kbp) and also to the total lengths of dsRNA III (5·4 kbp) plus either dsRNA IV (2·2 kbp) or dsRNAs VI plus V (1 plus 1·1 kbp). From this observation, we propose the model for the production of these molecules illustrated in Fig. 5. During the replication of the ACLSV RNA, the viral replicase copies the positive-sense genomic RNA to produce a complementary negative-sense strand. Then the replicase initiates the synthesis of a new positive strand at the 3′ extremity of the negative-sense strand. Internal initiation on the negative strand can also occur at the level of the subgenomic promoter P_1 or P_2 (promoters for the subgenomic RNAs coding for the 50K and the capsid proteins, respectively), leading to the synthesis of subgenomic messengers.

We hypothesize that during the synthesis of new positive strands, a fraction of the replicase molecules could arrest synthesis prematurely, close to or at the subgenomic RNA promoters P_1 or P_2, leaving a region of the negative strand template in a single-stranded form. A possible reason for this premature termination might be steric hindrance due to the presence in these regions of replicase molecules initiating the synthesis of subgenomic RNAs. Nuclease attack of these single-stranded regions, either in the cell or during dsRNA extraction, would yield dsRNAs II and III. Similarly, dsRNA VI would be generated by premature termination at P_2 of subgenomic RNA synthesis initiated at P_1. Since molecules possibly similar to dsRNAs II and III appear to be present among the dsRNAs produced by other viruses such as tobamo-, potex- and carlaviruses (see for example Valverde *et al.*, 1986), the model we suggest might apply generally to viruses expressing part of their genome by producing subgenomic RNAs.

Experiments are under way to study the fine structure of dsRNAs II, III and VI and to determine whether these molecules are mere replication artefacts or play a role during the viral infection cycle.

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