Emergence and Characterization of Satellite RNAs Associated with Spanish Cucumber Mosaic Virus Isolates

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SUMMARY

Satellite RNAs of CARNA 5s (cucumber mosaic virus-associated RNA 5s) emerged in serial passage lines of Spanish cucumber mosaic virus isolates established in Chenopodium, squash and two tobacco varieties. No satellite was detected in virus obtained from tomato serial passage lines. The satellites were identified by their biological effect on tomato, by their gel electrophoretic mobility and by nucleic acid hybridization, as being very similar in sequence to known CARNA 5s. Interactions among host, virus and CARNA 5 affect the selection of a particular CARNA 5 variant.

INTRODUCTION

Cucumoviruses in general, and cucumber mosaic virus (CMV) in particular, are helper viruses for small satellite-like RNAs, which can modify disease expression. The best known example of such a satellite-like RNA is the 335-nucleotide CMV-associated RNA 5 (CARNA 5). Its presence (and replication) in CMV infections often attenuates virus disease symptoms, except in tomato where CARNA 5 causes a lethal necrotic disease. (For recent reviews on the cucumoviruses, the CARNA 5s and their biological interrelationships, see Kaper & Waterworth, 1981; Kaper, 1983a).

Approximately 30% of the samples from economically important Spanish crops contained CMV. CARNA 5 could not be detected by polyacrylamide gel electrophoretic (PAGE) analysis (Garcia-Luque et al., 1983) from any of these isolates. It was, therefore, of interest to establish whether two of these CMV isolates could support the replication of CARNA 5 or induce its emergence during serial passages in different host species under conditions where other CMV isolates had been shown to do so (Kaper & Waterworth, 1981).

Here, we report on the emergence of two CARNA 5 variants during serial passages of two Spanish CMV isolates in four different host species.

METHODS

Source of virus. Val-CMV-2 was obtained in 1978 from commercially grown melon (Cucumis melo) with mosaic and stunting in Valencia (Eastern region of Spain). The virus was cloned by means of three local lesion transfers in cowpea (Vigna unguiculata L. Walp., Black Eye) and subsequently propagated in N tobacco (Nicotiana tabacum L.) for purification and characterization. N tobacco is an unidentified variety of N. glutinosa N gene [for tobacco mosaic virus (TMV)].

Val-CMV-24 was taken a year later from commercially grown melon in the same area, cloned, propagated and purified as described for Val-CMV-2.

Serial passages. For both CMV isolates, lines of serial transfers were carried out in Chenopodium (Chenopodium quinoa), N tobacco, Xanthi tobacco (Nicotiana tabacum L., cv. xanthi n.c.), tomato (Lycopersicon esculentum Mill., cv. Rutgers) and squash (Cucurbita pepo L., cv. Caserta Bush).

Plants were inoculated with a 20 mm-sodium phosphate pH 7.0 solution containing viral RNA (20 μg/ml), or with infected tissues triturated in the same buffer. Inocula for serial passages consisted of a random sampling of the infected plants produced in the preceding passage. Such samples were representative of inoculated and non-inoculated leaves, age of leaves, and symptoms.
Each individual passage line was totally isolated in growth chambers, the inside walls of which had been washed with 0.5 M NaOH. All utensils for manipulating plant tissues had either never been used before and were sterile (gloves; Q-tips used to apply the inoculum), or were chromic acid-treated, followed by 1 M NaOH treatment and subsequent distilled water rinses (tissue grinders and other glassware). Plant pots were new; soil had been sterilized prior to planting and was discarded after use. For batch purification, groups of plants were removed from the growth chambers. None of these plants or any purified virus-related products obtained from such plants were ever re-introduced into the passage line.

Growth chambers were kept at 25 °C, with a 16 h photoperiod and a light intensity of 11 000 lx. Infected plants were harvested for virus purification and/or serial passage at times when yield of virus was known to be highest. Chenopodium plants were collected on the 4th day, squash and tobacco on the 7th day and tomato on approximately the 14th day.

**Virus purification, RNA isolation and analysis.** Viruses were purified using established methods (Kaper et al., 1976). RNAs were routinely analysed by electrophoresis on 2% polyacrylamide-0.5% agarose gels as described by Piazzolla et al. (1982).

To identify the different CARNA 5(s) generated during serial passages, the RNAs isolated from the purified virus from these passages were analysed with a modified version of the PAGE method previously described for the separation of CARNA 5 sequence variants (Kaper et al., 1981). RNA samples (0.5 to 1 μg) in 75% formamide, 8 mM-EDTA, 0.075% bromphenol blue and xylene cyanol, were boiled for 3 min and quenched in ice-water. The samples were then electrophoresed for 16 h at 10 V/cm on a 9% polyacrylamide (39:1, acrylamide : bisacrylamide) slab gel containing 8 M-urea, 40 mM-Tris-acetate pH 7.8 and 4 mM-EDTA.

RNA visualization and photography were as described previously (Piazzolla et al., 1982).

Purification of CARNA 5 was as in Kaper et al. (1976). When CARNA 5 served as template for cDNA synthesis an additional electrophoretic fractionation step was carried out on 8% polyacrylamide gels (19:1, acrylamide : bisacrylamide), containing 8 M-urea, 100 mM-Tris-borate pH 8.3, 2 mM-EDTA, for 2 h at 20 V/cm. For CARNA 5 elution from the gel, the Maxam & Gilbert (1980) technique was used, except that Mg²⁺ was omitted from the extraction buffer and the gel slurry was shaken overnight at 4 °C.

**cDNA synthesis and hybridization.** Three μg of gel-purified CARNA 5 was polyadenylated in a 25 μl reaction mixture containing 50 mM-Tris HC1 pH 7.9, 10 mM-MgCl₂, 0.25 M-NaCl, 2.5 mM-MnCl₂, 0.5 mM-ATP, 50 μg/ml bovine serum albumin and 1-5 units of *Escherichia coli* poly(A) polymerase (Bethesda Research Laboratories). The mixture was incubated for 25 min at 37 °C. Recovery of polyadenylated CARNA 5 by phenol–chloroform extraction and ethanol precipitation, synthesis and purification of cDNA were as described by Owens & Cress (1980), except that 100 μg/ml actinomycin D was added to the reaction mixture and oligo(dT)₅G (P-L Biochemicals) was used as primer.

³H-labelled cDNA to (n)CARNA 5, from the tomato necrotic variant, was hybridized to 0.5 μg/ml of CARNA 5 in 0.18 M-NaCl, 20 mM-sodium cacodylate pH 7.5, 1 mM-EDTA at 68 °C for the appropriate time intervals. For thermal denaturation, samples were hybridized to Rₜ values of 5.5 x 10⁻³ mol·s⁻¹; 25 μl aliquots were then heated at the appropriate temperatures and quenched in ice-water. Nuclease S1 assay for hybrid formation was performed as described by Owens (1978).

**RESULTS**

**Preliminary experiments**

Dried tissue containing Val-CMV-24 was triturated and inoculated to Xanthi tobacco plants. Tissue from these plants was inoculated to a second batch of Xanthi plants and this serial passage line was continued up to the 5th passage. PAGE analysis of RNA from virus isolated from the 2nd and 5th passages (Fig. 1a) shows that there was no detectable CARNA 5 in the 2nd passage virus but a large proportion of CARNA 5 in the 5th passage virus whose presence is known to vary the relative proportion of viral RNA components (Kaper & Waterworth, 1981). Tomato necrosis assay of the RNA from 5th passage virus and subsequent PAGE analysis in 9% polyacrylamide gels identified the CARNA 5 as the tomato necrotic variant (n)CARNA 5 (Kaper et al., 1981).

A serial passage line initiated with the same Val-CMV-24 inoculum in N tobacco also contained a CARNA 5 from the 3rd passage onwards. However, this CARNA 5 variant was incapable of inducing tomato necrosis. PAGE analysis of the virus preparation from this passage series (Fig. 1b) showed that the CARNA 5 from N tobacco co-electrophoresed with (1)CARNA 5, the non-necrotic CARNA 5 from CMV-1 (Kaper et al., 1981) whose nucleotide sequence was recently determined (Collmer et al., 1983).
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Fig. 1. Electrophoretic analysis on 2% acrylamide, 0.5% agarose (a), or 9% acrylamide, 8 M-urea (b), slab gels of Val-CMV-24 RNA from serial passage lines in Xanthi (a) and N (b) tobaccos using infected dried tissue as starting inocula. Lane numbers indicate the serial passage number; M, marker lane; PARNA 5, peanut stunt virus-associated RNA 5; (1)CARNA 5 and (n)CARNA 5, CMV-associated RNA 5 variants.

The above experiments, in which identical Val-CMV-24 inocula yielded two distinct CARNA 5 sequence variants during serial passage in two different tobacco cultivars, prompted additional serial passage experiments in other hosts.

Serial passage lines from isolated CMV RNA

Val-CMV-24 RNA

A Val-CMV-24 RNA preparation previously isolated in Spain during the cucumovirus survey (Garcia-Luque et al., 1983) infected neither N nor Xanthi tobacco on several attempts. However, it was possible to infect a primary line in Chenopodium and to establish secondary lines in the two tobacco varieties, in tomato and in squash, using 2nd passage infected Chenopodium as inoculum (Fig. 2). In the Chenopodium primary line, a CARNA 5 with PAGE mobility...
identical to that of (1)CARNA 5 and incapable of inducing tomato necrosis was detected in the 4th and subsequent passages (Fig. 3a). Like the CARNA 5 variant isolated from Val-CMV-24 in N tobacco, it was tentatively designated (1)CARNA 5.

The secondary lines in squash and N tobacco also contained large proportions of a CARNA 5 in the 1st and all subsequent passages (Fig. 2). These CARNA 5s co-electrophoresed with the (1)CARNA 5 marker (Fig. 3d, e) and also failed to induce tomato necrosis. They were assumed to be identical to the (1)CARNA 5 identified in the Chenopodium primary line and probably represented its direct progeny.

The secondary line in Xanthi tobacco (Fig. 2) likewise yielded (1)CARNA 5 in virus from the 1st passage onwards. However, the 5th passage produced a trace of a CARNA 5 co-electrophoresing with the (n)CARNA 5. This putative (n)CARNA 5 predominated in the 6th passage and was the only type present in subsequent passages (Fig. 3b). Bioassay in tomato established its necrosis-inducing character. However, the secondary line in tomato (Fig. 2) produced no CARNA 5 in any of six passages (Fig. 3c). This is unlike the behaviour of CMV-1, which in tomato continues to support the replication of (1)CARNA 5 (Kaper, 1983b).

Val-CMV-2 RNA

Val-CMV-2 RNA isolated during the cucumovirus survey in Spain was also introduced in N and Xanthi tobacco and two primary lines were established (Fig. 4a, b). Since the RNA was
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(a) Val-CMV-2 RNA

N tobacco (primary line) → Squash (secondary line) → Tomato (secondary line)

(b) Val-CMV-2 RNA

Xanthi tobacco (primary line) → Squash (secondary line) → Tomato (secondary line)

Fig. 4. Diagram showing the Val-CMV-2 primary passage lines in N (a) and Xanthi (b) tobaccos and subsequent secondary passage lines, using RNA as starting inocula. Legend is otherwise as for Fig. 2.

unable to infect either tomato or squash, secondary lines were initiated by inoculating from the first passage of each tobacco variety (Fig. 4).

In the N tobacco primary line, a CARNA 5 with the PAGE mobility of (1)CARNA 5 and incapable of inducing tomato necrosis, was detected in the 3rd and subsequent passages (Fig. 5a).

The squash secondary line from N tobacco produced both variants, (n)CARNA 5 and (1)CARNA 5, in the 4th passage. Subsequent passages yielded diminishing amounts of (1)CARNA 5 and by the 7th passage only (n)CARNA 5 was detectable (Fig. 5b).

The Xanthi tobacco primary line (Fig. 4b) produced both (n) and (1)CARNA 5s but not until the 5th passage. By the 7th passage only (n)CARNA 5 was detectable (Fig. 5d).

The squash secondary line from Xanthi tobacco contained (1)CARNA 5 in the 4th passage and also (n)CARNA 5 in the 6th passage (Fig. 5e). By the 10th passage only (n)CARNA 5 was present.

In the tomato secondary lines from N or Xanthi tobacco, Val-CMV-2, like the Val-CMV-24, produced no CARNA 5 (Fig. 5c, f).

Tentative identification of the Val-CMV-24 non-necrotic variant CARNA 5

Several serial passage experiments produced a non-necrotic CARNA 5 variant resembling (1)CARNA 5. Therefore a more detailed comparison with (n)CARNA 5 was necessary. Since double-stranded RNAs have characteristic multi-transitional melting profiles (Díaz-Ruiz & Kaper, 1979; Steger et al., 1980) the melting profiles of the two Val-CMV-24 dsCARNA 5s isolated from N and Xanthi tobacco serial passage lines (Fig. 2) were compared with previously described (1) and (n)CARNA 5s (Kaper et al., 1981). The non-necrotic and necrotic Val-CMV-24 dsCARNA 5s isolated from N tobacco and Xanthi tobacco proved indistinguishable from (1)CARNA 5 and (n)CARNA 5, respectively (Fig. 6).

Homologous and heterologous hybridization kinetics of tomato necrotic and non-necrotic Val-CMV-24 CARNA 5 with 3H-labelled cDNA to (n)CARNA 5 are shown in Fig. 7(a). The homologous reactions gave a maximum hybridization of 79% in a single sharp transition with a $R_{\text{m}}$ value of $6.3 \times 10^{-4}$ mol·s/l. Heterologous reactions with non-necrotic Val-CMV-24 CARNA 5 gave maximum hybridization of 67%.

The heterologous hybrids (Fig. 7b) melted with a single sharp transition comparable to that of the homologous hybrids (not shown). This indicates the absence of base-pair mismatching.
Fig. 5. Electrophoretic analysis on 2% acrylamide, 0.5% agarose (c, f), or 9% acrylamide, 8 M-urea (a, b, d, e) slab gels of VaI-CMV-2 RNA from serial passage lines outlined in Fig. 4a (a, b, c) and Fig. 4b (d, e, f). (a) N tobacco primary line; (b) squash secondary line; (c) tomato secondary line; (d) Xanthi tobacco primary line; (e) squash secondary line; (f) tomato secondary line. Legend is otherwise as for Fig. 1.
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Fig. 6. Thermal denaturation profiles of double-stranded CARNA 5 in 0.1 x SSC (SSC = 0.15 M-NaCl, 0.015 M-sodium citrate, pH 7.2). ---, CMV-WT ds (n)CARNA 5; ----, Val-CMV-24 ds necrotic CARNA 5; ..., CMV-1 ds (1)CARNA 5; ..., Val-CMV-24 ds non-necrotic CARNA 5. The temperature was increased at the rate of 0.25 °C/min as described in Diaz-Ruiz & Kaper (1979).

![Thermal denaturation profile graph](image)

Fig. 7. \( R_{O} t \) curves of (a) the hybridization of (n)CARNA 5 \( ^{3} \)H-cDNA to the homologous RNA (●) and heterologous (1)CARNA 5 (▲) and (b) thermal denaturation profile of the heterologous hybrid. Arrow indicates \( R_{O} t_{i} \) in (a).

estimated 85% homology between these two CARNA 5s is consistent with the known 93% homology between (1)CARNA 5 and (n)CARNA 5 sequences (Collmer et al., 1983).

**DISCUSSION**

One of our principal objectives was to see whether two natural Spanish CMV isolates (García-Luque et al., 1983) normally carried CARNA 5 or could induce it after serial transfer under conditions and with experimental hosts that have been previously used for this purpose with other CMV isolates (Kaper & Waterworth, 1981). While virus from field plants or from preliminary transfers in cowpea and N tobacco contained no detectable CARNA 5 (García-Luque et al., 1983), both Val-CMV-2 and Val-CMV-24 induced two dominant types of CARNA 5 after serial transfer in four different host plants. Although only nucleotide sequencing can establish a complete identity of the two dominant variants, they are indistinguishable from the sequence variants (n)CARNA 5 and (1)CARNA 5 that are associated with other CMV infections (Kaper et al., 1981; Kaper, 1983b; Collmer et al., 1983).
This study shows that the emergence of a specific CARNA 5 is probably determined by a selection process in which the specificity of the interaction among the three partners involved, the host plant, the virus and CARNA 5, determines the outcome.

Certain hosts favour the emergence of specific CARNA 5 types. For instance, in Xanthi tobacco, squash and N tobacco, in this order, selectivity for (n)CARNA 5 decreases and selectivity for (1)CARNA 5 increases. On the other hand, Val-CMV-2 and Val-CMV-24, with near identical behaviour in Xanthi tobacco for the emergence of CARNA 5s, show a clear difference in squash.

In all cases where both CARNA 5 types emerge, (n)CARNA 5 predominates over (1)CARNA 5. Secondary structural differences known to occur in the 3' halves of their molecules (Collmer et al., 1983) could account for this.

Chenopodium favours (1)CARNA 5 with Val-CMV-24, but we have not tested it with Val-CMV-2. The failure of tomato to generate detectable amounts of either type of CARNA 5 probably arises from the requirement of a minimal 'threshold' dose of CARNA 5 (Jacquemond & Leroux, 1982) under which its synthesis is unpredictable or at undetectable levels.

In control experiments each of the experimental hosts supported replication of either type of CARNA 5, but the minimal dose is apparently higher for tomato than for the others (results not shown). The 'insensitivity' of tomato has been observed with other CMV strains (I. Garcia-Luque et al., unpublished results).

The initial inoculum probably contains a number of CARNA 5 sequence variants in quantities undetectable by standard physical methods. The emergence during CMV infection of a particular CARNA 5 variant probably depends on specific interactions among viral RNA, CARNA 5 and the RNA replication enzymes. The most efficiently replicating sequence eventually predominates.

We do not know how these CARNA 5s arise initially or even if (n)CARNA 5 and (1)CARNA 5 are the only CARNA 5s in the Spanish isolates. Although at least one other CMV strain contains the same CARNA 5 variants (Kaper, 1983b), there is evidence for many others (Kaper, 1983a, b). This is consistent with the known infidelity of RNA synthesis (Holland et al., 1982).

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REFERENCES


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