Satellite RNA of Cucumber Mosaic Cucumovirus Spreads Epidemically in Natural Populations of Its Helper Virus

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ABSTRACT

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Three hundred thirty-eight isolates of cucumber mosaic cucumovirus (CMV), sampled from natural populations in six areas of Spain between 1989 and 1996, were screened for the presence of satellite RNA (satRNA). The frequency of CMV isolates with satRNA approached 1.00 in Valencia (east Spain) between 1990 and 1994 where a tomato necrosis epidemic induced by CMV+satRNA had started in 1986 and was smaller north and west of this area in 1992 and 1993. After 1994, satRNA almost disappeared from all CMV populations. Genetic typing of satRNA variants

was done by ribonuclease protection assay, and from these data, genetic distances were estimated for any pair of satRNA variants. CMV-satRNA populations were highly diverse, containing 0.07865 nucleotide substitutions per site on average. Data also showed that the whole compared set of 100 satRNA variants form a single population that is not structured according to place, year, host plant, or strain of helper virus (HV). This is in sharp contrast with the metapopulation structure of the Spanish CMV population. Thus, the genetic structure and dynamics of populations of CMV and its satRNA are not coupled. This shows that CMV-satRNA spreads epidemically, as a hyperparasite, in the population of its HV. This conclusion is relevant to the use of CMV-satRNA as a biocontrol agent of CMV.

Additional keywords: population dynamics, population structure.

Cucumber mosaic cucumovirus (CMV), the type member of the genus *Cucumovirus*, is one of the most important viruses worldwide for the economic impact of the diseases it induces in many different crops, particularly ornamentals and vegetables (25). The wide occurrence and economic importance of CMV is, in part, due to its very wide host range (more than 800 species from 85 families of mono- and dicotyledon plants) and its efficient transmission in a nonpersistent way by more than 75 species of aphids (20). Thus, sanitary control strategies, including the control of the vectors, are not efficient. Natural resistance to CMV is often partial or strain-specific, and effective resistance has been incorporated commercially only in cultivars of cucumber. Some strains of CMV, in addition to the tripartite, messenger sense, single-strand RNA genome, encapsidate a satellite RNA (satRNA) (5,22). CMV-satRNA depends on its helper virus (HV), CMV, for replication, movement within the infected plant, encapsidation, and transmission. The presence of CMV-satRNA modulates the symptoms induced by the HV and often depresses HV accumulation in different host species. Thus, CMV-satRNA has some characteristics of a molecular parasite (12).

Many CMV-satRNA strains have attenuative effects on CMV-induced symptoms. It has been proposed that these attenuating satRNA could be used as biocontrol agents of CMV, either in transgenic plants expressing the satRNA or in classical cross-protection programs (24). The efficient and environmentally safe use of satRNA in CMV control would require a good knowledge of the satellite's population genetics and dynamics under natural field conditions. Recent reports on the genetic structure and evolution of CMV-satRNA populations indicate that satRNA may diverge

quickly by sequential accumulation of point mutations (2), that attenuating and aggravating (i.e., necrogenic for tomato) satRNA may follow different evolutionary dynamics (10), and that genetic interchange by RNA recombination may be a frequent event with an important role in mixing up different evolutionary lines (1). It is also important to know whether satRNA would spread epidemically in the HV population or if their spread would be associated with a particular CMV strain. This information is not currently available, and it is important because the symptom modulation ability of a particular satRNA variant may depend on the strain of HV and the species of the host plant (5,22).

We report here an analysis of the dynamics and genetic structure of field populations of CMV-satRNA sampled at different regions of Spain between 1989 and 1996. Our results show that CMV-satRNA in nature behaves indeed as a hyperparasite that spreads epidemically within the CMV population and that this spread is not associated with the type of CMV strain or the host plant.

MATERIALS AND METHODS

Populations and viral isolates. We define a CMV field population as a set of isolates present during one growing season in the same area. Populations were sampled near Valencia (VAL) in June 1989, 1990, 1991, 1994, 1995, and 1996; Barcelona (BAR) in September 1992 or during the summers (June to September) of 1993, 1994, 1995, and 1996; León (LEO) in October 1993; Zaragoza (ZAR) during the summers of 1994 and 1995; Madrid (MAD) during the summers of 1993, 1994, 1995, and 1996; and Badajoz (BAD) during the summer of 1995. The minimum distance between these areas was 250 km (Fig. 1); the distance between sampled fields from one area was less than 30 km. The populations were named with the abbreviated name of the area and year of sample (Table 1). Different host plants, according to CMV outbreaks in each place and year, were sampled for each population,

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as specified in Table 1. Except for weeds (BAR93 population), only symptomatic plants were sampled.

We define a CMV isolate as a virus preparation derived from a single field-infected plant. Isolates were named with the locality name, the first letter from the name of the original host plant (e.g., T = tomato, M = melon, P = pepper, and Z = zucchini squash), the year of the sample, and an ordinal (e.g., BAR.T93/24). CMV virions were purified directly from field-infected material, or sap from the original plant was used to inoculate *Nicotiana tabacum* L. cv. Xanthi-nc plants, from which virions were purified. Virion purification was as in Lot et al. (14), and virion RNA was extracted with phenol and sodium dodecyl sulfate. Virion RNA (6 to 12 µg) from CMV isolates were separated by electrophoresis in semide-naturing polyacrylamide gels (9), which enabled the separation of satRNA variants present in a single CMV isolate. When more than one satRNA variant was present in a CMV isolate, they were identified with a letter after the isolate name. satRNA variants were detected by staining the gel with 0.05% toluidine blue; the bands were then excised, eluted (21), and analyzed (discussed below).

Genetic characterization of satRNA variants. Each satRNA variant was characterized by the pattern of fragments generated by ribonuclease protection assay (RPA) in hybrids with a cRNA probe from a full-length clone of B2-satRNA (4). RPA was done as described by Aranda et al. (2,3).

From RPA data, genetic distances, expressed as nucleotide substitutions per site, were estimated for any pair of satRNA variants as described by Aranda et al. (3). Estimated nucleotide substitution values per site were used to calculate intrapopulational diversity values (18). Also, phylogenetic relationships among variants were inferred from these values by neighbor joining using the PHYLIP package (version 3.57c) of J. Felsenstein (University of Washington, Seattle).

RESULTS

Frequency of satRNA in 19 CMV populations. A collection of 338 isolates of CMV, representing nineteen populations sampled at six areas between 1989 and 1996, was analyzed for the presence of CMV-satRNA. The number of CMV isolates analyzed for each population broadly corresponded to the intensity of the CMV outbreak for each area and year. The presence of CMV-satRNA was detected on polyacrylamide gels followed by RPA. Ninety-one CMV isolates contained a satRNA (Table 1). The distribution of CMV isolates with a satRNA varied largely among populations. satRNA was more frequent in those populations sampled in eastern Spain (VAL and BAR) between 1990 and 1994 when

the frequency of CMV-satRNA in the CMV populations was near 1 in VAL90, VAL91, and VAL94; between 0.3 and 0.5 in BAR92 and BAR93; and around 0.2 in two populations distant from the Mediterranean Coast in 1993 (MAD93 and LEO93). After 1994, satRNA virtually disappeared from CMV populations including those from the Mediterranean Coast (VAL and BAR) (Table 1). Thus, CMV-satRNA, first detected in Valencia in 1986 (11), increased in frequency within the CMV populations around this area (being present in almost all CMV isolates until 1994), expanded northwards to Barcelona and westwards to León and Madrid in 1992 and 1993, and disappeared from all sampled areas after 1994.

Genetic structure of CMV-satRNA populations. Forty electrophoretic variants of CMV-satRNA were characterized by their patterns on RPA (Fig. 2). With the 60 satRNA variants from previously characterized (2) populations, VAL89, VAL90, and VAL91, a total of 100 satRNA variants were analyzed (Table 2). Note that more than one satRNA variant per CMV isolate was sometimes found, particularly in earlier samples from Valencia and Barcelona (Tables 1 and 2).

RPA patterns (i.e., presence or absence of any fragment of a particular size) were used to differentiate satRNA with different nucleotide sequences; thus, a given RPA pattern defined a haplotype. RPA data were then used to analyze the genetic diversity of the different satRNA populations. For all populations, the number of haplotypes approached the number of analyzed satRNA isolates except for VAL94 and BAR92, in which the ratio types/isolates was about 0.7 (Table 2). RPA data also were used to estimate genetic distances, as nucleotide substitutions per site, for any pair of satRNA, and from these data, population diversities were estimated. Except for BAR92, population diversities were of the same order of magnitude and, in some cases, approached the estimated diversity for the whole set of analyzed satRNA (0.07865). Diversity values were not dependent on the number of satRNA variants analyzed for each population (Table 2). The two populations with smaller diversity values were those with a lower ratio of haplotypes/isolates (VAL94 and BAR92). Still, and in spite of the higher number of isolates analyzed, populational diversity was much

TABLE 1. Details of cucumber mosaic cucumovirus (CMV) populations analyzed and the frequency of satellite RNA (satRNA)

Population ^a	Host plants ^b	No. of CMV isolates	No. of CMV isolates with satRNA	Frequency of satRNA
VAL89	Tomato	24	12	0.50
VAL90	Tomato	22	21	0.95
VAL91	Tomato	11	9	0.85
VAL94	Tomato	7	7	1.00
VAL95	Tomato, melon	20	0	0.00
VAL96	Melon	22	1	0.04
BAR92	Tomato	36	17	0.47
BAR93	Tomato, weeds ^c	39	12	0.31
BAR94	Tomato	16	1	0.06
BAR95	Melon	5	0	0.00
BAR96	Melon	15	1	0.06
LEO93	Pepper	24	4	0.17
MAD93	Tomato, squash, melon	25	5	0.20
MAD94	Squash, pepper, lettuce, bean, melon	17	0	0.00
MAD95	Melon	7	0	0.00
MAD96	Melon	14	1	0.07
ZAR94	Melon	10	0	0.00
ZAR95	Melon	10	0	0.00
BAD95	Melon	14	0	0.00

^a Data for VAL89, VAL90, and VAL91 are from Aranda et al. (2). The genetic composition of CMV populations VAL89, VAL90, VAL91, VAL94, VAL95, BAR92, BAR93, BAR94, MAD93, MAD94, and LEO93 was analyzed by Fraile et al. (6).

^b Sampled host plants reflect CMV outbreaks in each area and each year.

^c Weeds are *Parietaria officinalis*, *Convolvulus arvensis*, and *Portulaca oleracea*.

Fig. 1. Map of Spain showing the areas where the different cucumber mosaic cucumovirus populations were sampled.

Fig. 2. Autoradiography of ribonuclease protection assay patterns for cucumber mosaic cucumovirus-satellite RNA variants LEO.P93/10 (lane 3), MAD.Z93/11 (lane 4), BAR.T93/9a (lane 5), BAR.T93/8 (lane 6), BAR.T93/24b (lane 7), VAL.T94/31 (lane 8), VAL.T90/10 (lane 9), BAR.T92/4 (lane 10), BAR.T92/12 (lane 11), VAL.T89/12 (lane 12), VAL.T89/20a (lane 13), VAL.T89/20b (lane 14), MAD.M94/4 (lane 15), VAL.T89/19 (lane 16), VAL.T94/35 (lane 17), VAL.T90/17a (lane 18), VAL.T90/12 (lane 19), VAL.T91/2 (lane 20), VAL.T91/3 (lane 21), and VAL.T91/8 (lane 22). Lane 1, undigested probe; Lane 2, digested, unhybridized probe.

lower for BAR92 than for VAL94, indicating a higher similarity among the different haplotypes of this population.

Estimated genetic distances between satRNA also were used to infer phylogenetic relationships by the neighbor-joining method. Figure 3 shows a phylogenetic tree in which a random sample of satRNA variants from VAL89, VAL90, VAL91, and BAR92, plus most satRNA variants found in other populations, were included. In addition to information on place, year, and host (in the name of the satRNA), information on the genetic type of HV also is presented. This tree shows that satRNA variants from the same population did sometimes cluster together (e.g., LEO93, VAL94, and most of BAR93), but this was not always the case (e.g., MAD93, VAL89, VAL90, and VAL91). No consistent clustering according to the host plant from which the CMV isolate was obtained, nor according to the type of CMV isolate, was found. Also, no node in the tree in Figure 3 had a significance above 19% in a bootstrap analysis with 1,000 random replicates. Thus, data in Figure 3 indicate that the CMV-satRNA population present in Spain between

TABLE 2. Genetic diversities of cucumber mosaic cucumovirus (CMV)-satellite RNA (satRNA) populations

Population	No. of haplotypes/ No. of analyzed variants (per one)	Nucleotide diversities ^a
VAL89 ^b	18/18 (1.00)	0.05670
VAL90 ^b	30/32 (0.94)	0.06252
VAL91 ^b	12/12 (1.00)	0.05367
VAL94	4/6 (0.67)	0.02382
BAR92	11/15 (0.73)	0.00853
BAR93	8/9 (0.89)	0.07262
LEO93	4/4 (1.00)	0.04545
MAD93	4/4 (1.00)	0.06688

^a Nucleotide diversities, D_k , were estimated as in Nei (18): $D_k = (n_k/n_{k-1}) \sum x_i x_j d_{ij}$, in which n_k is the number of analyzed satRNA variants in population k ; x_i and x_j are the frequency of haplotypes i and j in population k ; and d_{ij} is the estimated number of nucleotide substitutions per site between haplotypes i and j .

^b Data from Aranda et al. (2).

1989 and 1994 is not genetically structured into subpopulations, but forms a unique, unstructured population.

DISCUSSION

CMV is endemic in most horticultural areas of Spain, where every year it causes outbreaks of different intensities (11,15). Spanish CMV isolates from horticultural crops were mostly free of CMV-satRNA until the appearance of a necrotic syndrome in tomato near Valencia in 1986 (11). This syndrome was shown to be due to CMV isolates supporting satRNA variants necrogenic for tomato, and it spread northwards and southwards on the Mediterranean coast of Spain until 1992, disappearing afterwards. CMV isolates from this epidemic outbreak were found to support satRNA variants that were necrogenic for tomato, as well as variants that induced a curl stunt syndrome in tomato or that did not modify the HV-induced symptoms (11). The analysis of CMV populations from VAL89, VAL90, and VAL91 showed that the frequency of CMV isolates with satRNA increased from 1989 to 1990 to almost 1.00 (2). The data we report now show that this high frequency of satRNA in CMV isolates was maintained in Valencia until 1994, was lesser in those years in northeastern parts of Spain where the tomato necrosis had spread from Valencia, and was lesser still in areas of inland Spain, which were not affected by the tomato necrosis epidemics. After 1994, CMV-satRNA were not found or were present with very low frequency in five different regions surveyed. Thus, the data in Table 1 indicate that CMV-satRNA had spread epidemically from Valencia to other areas during the period of 1989 to 1994, and disappeared afterwards.

The genetic structure of the satRNA from populations VAL89, VAL90, and VAL91 has been reported; populations were highly diverse, and CMV-satRNA seemed to diverge quickly by the accumulation of mutations within different evolutionary lines (2). A similar situation was reported in Italy (10). Recombinants between different evolutionary types were frequent in the populations from Valencia, and RNA recombination may be an important mechanism to generate new variants and bridge the divergent evolutionary lines above (1). Interestingly, a high proportion of the satRNA variants present one year would be found, be parentals for new variants, or both the next year (2). The data we report here extend and support these previous conclusions. All the analyzed CMV-satRNA populations were highly diverse including those in which CMV-satRNA was found at low frequency and, thus, few isolates were available for analysis, as LEO93 or MAD93. The exceptionally low diversity of BAR92, which is not explained by the lower ratio of satRNA haplotype/satRNA isolate, may reflect a founder effect. Phylogenetic analysis shows that the whole set of analyzed CMV-satRNA variants represents a single, undifferentiated population not structured by host plant or by HV strain. For some populations, satRNA showed a tendency to cluster together. This clustering

Fig. 3. Neighbor-joining tree for 33 cucumber mosaic cucumovirus (CMV)-satellite RNA (satRNA) variants from eight CMV populations. In addition to information on area, year, and host plant (in the name of the satRNA variant), the genetic type (subgroup I, types 1, 2, or 3 as defined by Fraile et al. [6], or subgroup II) of the helper virus isolate is indicated (HV type); nd = not determined. Bar represents nucleotide diversity value of 0.1.

was not significant and did not occur for all analyzed populations; thus, the satRNA population is structured neither according to the year nor the place where the sample was obtained. This population structure is in sharp contrast with that of the HV. We have recently reported the analysis of CMV isolates in 11 out of the 19 populations in Table 1, including all those with a significant frequency of satRNA. The results showed that the genetic composition of these CMV populations differed significantly according to place and year; the CMV population from Spain was shown to have a metapopulation structure with local extinctions and random recolonizations by different or similar genetic types (6). Thus, the population dynamics and genetics of CMV and CMV-satRNA are not coupled, and all the results presented here are consistent with CMV-satRNA behaving as a hyperparasite that causes epidemics on an endemic CMV population. Results are not consistent with the alternative hypothesis of CMV-satRNA epidemics being due to the dispersion of particular CMV strains with associated satRNA.

CMV-satRNA have been proposed as biocontrol agents for CMV, and encouraging results have been obtained in different countries (8,17,23,24). Symptom modulation by CMV-satRNA depends on the strain of CMV, the host plant species, and the satRNA variant itself (5,22); and it has been shown that a single-point mutation may be sufficient for the generation of deleterious variants out of benign ones (19). Thus, data indicating fast genetic divergence in field populations of CMV-satRNA (2,10, this work) and lack of significant association between CMV-satRNA variants and strains of CMV or host plant species (10, this work) are a warning against the widespread use of CMV-satRNA in biocontrol programs. CMV-satRNA are infrequent in natural CMV populations (11,13), and in the few reported instances of epidemics of CMV+satRNA (always associated with tomato necrosis), have disappeared after a few years (7,11,16). However, the dynamics of the satRNA populations were not analyzed. Such an analysis is re-

ported here, and an important conclusion from our current results is that CMV-satRNA causes epidemics on the CMV population as would any other hyperparasite. These results are encouraging to the targeted use of attenuative satRNA as biocontrol agents, which might spread on the CMV population. For this, the factors that determine the parameters of satRNA epidemics and evolution should be better known.

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