Epidemic of Cucumber Mosaic Virus Plus Satellite RNA in Tomatoes in Eastern Spain

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ABSTRACT

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In eastern Spain, an epidemic of tomato necrosis has been progressing since 1986, with dramatic effects on tomato production. We show that the systemic necrosis syndrome is induced by a cucumber mosaic virus (CMV) that supports a satellite RNA (satRNA) of a necrogenic phenotype. In addition to necrogenic isolates of CMV satRNAs in tomato plants infected with CMV, other satRNAs were found that do not modify the symptoms induced by the helper virus, or that induce a leaf-curl, shoot-stunt syndrome, also with severe effects on the production. A subepidermal necrosis of the fruits also present at epidemic levels was induced by isolates of CMV without satRNA.

Tomato is the most important horticultural crop in eastern Spain, with an acreage of about 20,000 ha and with a yearly production of about 1 million T. This represents 30% of the acreage and 40% of the total tomato production for Spain. The crop is directed mostly to the fresh market and has a high economic value; 40% corresponds to out-of-season production. In 1986, growers drew our attention to a new disease, characterized by necrosis in the stems, leaves, and fruits, that had a dramatic effect on productivity, even destroying whole tomato fields.

Tomato necrosis was first detected north of Valencia in 1986 and has spread since then northward and southward, affecting in 1990 most areas of tomato crops in eastern Spain (Fig. 1).

In the newly affected areas tomato necrosis was found in 60–80% of the tomato fields. For individual fields 50–100% of the plants showed the necrotic syndrome, and fields often had to be replanted with other crops. All the cultivars in the area developed the syndrome, which was especially severe in some highly prized local cultivars. It has also been found in the Canary Islands, but not in Extremadura or La Rioja (central Spain), important areas of tomato for industrial processing.

Typical necrotic symptoms appeared during the spring and the beginning of the summer, at any stage of the plant's growth. Necrosis started as purple streaks in young stems, soon evolving to necrotic streaks up to several centimeters in length and some millimeters wide (Fig. 2A); stem necrosis might affect only cortical tissues or, more frequently, vascular and pith tissues as well, giving rise to dry, depressed areas. Necrotic streaks extended to leaf petioles that became twisted, and necrotic areas developed in the leaf lamina, followed by its complete necrosis. Young shoots were stunted and developed a systemic apical necrosis (Fig. 2B). Fruits showed necrotic depressions (Fig. 2C). Some fruits developed an acorn-shaped profile by diminished growth of the epidermis and the basal outer part of the parenchyma and the five carpels protruding at the fruit's distal end. The necrotic syndrome would affect the upper parts, or the whole, of the plants, according to their development at the onset of the disease. With the sudden rise of temperatures at the end of June, necrotic development would cease, and plants with mild symptoms sometimes recovered. In September the syndrome would reappear in fall crops. Another commonly found syndrome consisted in internal browning of the fruit due to a subepidermic necrosis (Fig. 2D,E). Fruits with internal browning appeared in plants otherwise showing the mosaic and filimorphism induced typically by common CMV strains.

Since 1989 a second syndrome has been found in the area around Valencia, where the epidemic started. The shoots of the plants affected by it are stunted by a shortening of the internodes, and leaf laminae show a strong curling, but are less reduced than in the typical symptom induced by CMV (Fig. 2F). Fruits show no symptoms but plants grow and produce poorly. Hereafter, we will call this syndrome curl-stunt. Curl-stunt was found in the same fields as necrosis, apparently out-competing it: In 1989 only 15% of the plants in some fields showed this syndrome, whereas in 1990 more than 50% of the plants showed curl-stunt and only about 20% of the plants were necrotic.

A viral etiology for the primary tomato necrosis was investigated; this paper presents data showing that the causal agent is cucumber mosaic virus (CMV) carrying a satellite RNA (satRNA) of a necrogenic phenotype, as has been reported for two other instances of epidemics of tomato necrosis (2,22). Nevertheless, and at odds with these previous reports, we found for the Spanish epidemic a variety of types of satRNA inducing these later, different symptoms in addition to necrosis, also with severe effects on productivity. Moreover, a subepidermic necrosis of the fruits also present at epidemic levels was found to be induced by a strain of CMV without an satRNA.

MATERIALS AND METHODS

Viral isolates and virus purification. Tomato plants showing disease symptoms were collected from commercial fields in the area indicated in Figure 1. From sap of these plants, or from sap of tomato cv. Rutgers infected from them, virions were purified following the procedure of Lot et al (12), and the encapsidated RNA was obtained by SDS/phenol extraction and ethanol precipitation.

Serology and electron microscopy. Samples were examined under the electron microscope after negative staining with the sodium salt of phosphotungstic acid at 2%, w/v, at pH 7.2. Immunological assays were performed by van Slogteren microprecipitins and ELISA double sandwich tests. The CMV antisera were ATCC30-PAVs and ATCC242-PAVs, commercial sera from Sanofi S.A., and a serum of our own made against a field isolate from a necrotic tomato plant.

Nucleic acids analyses. Total nucleic acid preparations from field-infected tomato plants were obtained and analyzed electrophoretically in semidenaturing polyacrylamide gels according to the procedure of White and Kaper (26). Virion-encapsidated RNA was analyzed in either semidenaturing polyacrylamide electrophoresis gels (4) or in 1.2% agarose gels (23). From agarose gels a bi-

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directional transfer to nitrocellulose was made. Blots were probed (19) with ^{32}P-labeled cRNA trancribed from clones with inserts representing sequences of

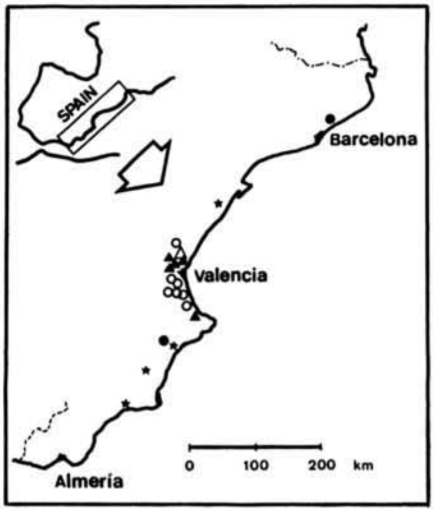

Fig. 1. Map showing the spread of tomato necrosis in eastern Spain. Sites where CMV plus satRNA-induced necrosis has been found since 1986 (△), 1987 (▲), 1988 (○), 1989 (●) or 1990 (★) are indicated.

Fny-CMV, a fast-multiplying strain belonging to subgroup I of CMV strains (18,21) or of the attenuating CMV-satRNA, B2-satRNA (3).

Biological assays. Virion-encapsidated satRNAs from field-infected or sap-inoculated plants were purified from 7 M urea, 6% polyacrylamide gels (20). For biological assays virion-encapsidated RNAs, or a combination of the purified satRNAs plus Fny-CMV RNAs, were inoculated into the expanded cotyledons of Rutgers seedlings; inoculum consisted in 10 μl per plant of an RNA suspension in 0.1 M Na_2HPO_4 (200 μg of CMV-RNA plus or minus 25 μg of satRNA per milliliter). The symptoms shown by inoculated plants were recorded for 1–4 wk, when virions were extracted and the encapsidated RNAs analyzed.

RESULTS

Etiology of the necrosis, curl-stunt, and internal browning syndromes. Electron microscopy of negatively stained sap from necrotic plants consistently showed spherical particles, 30 nm in diameter, as those of CMV. By electron microscopy, a potyvirus was found sporadically but not in consistent association with the syndrome, and no tomato mosaic virus, by itself or associated with potato virus X, was ever found in necrotic plants. Also, plants showing necrosis or the curl-stunt syndrome were consistently positive in immunological assays with antisera to CMV (not shown).

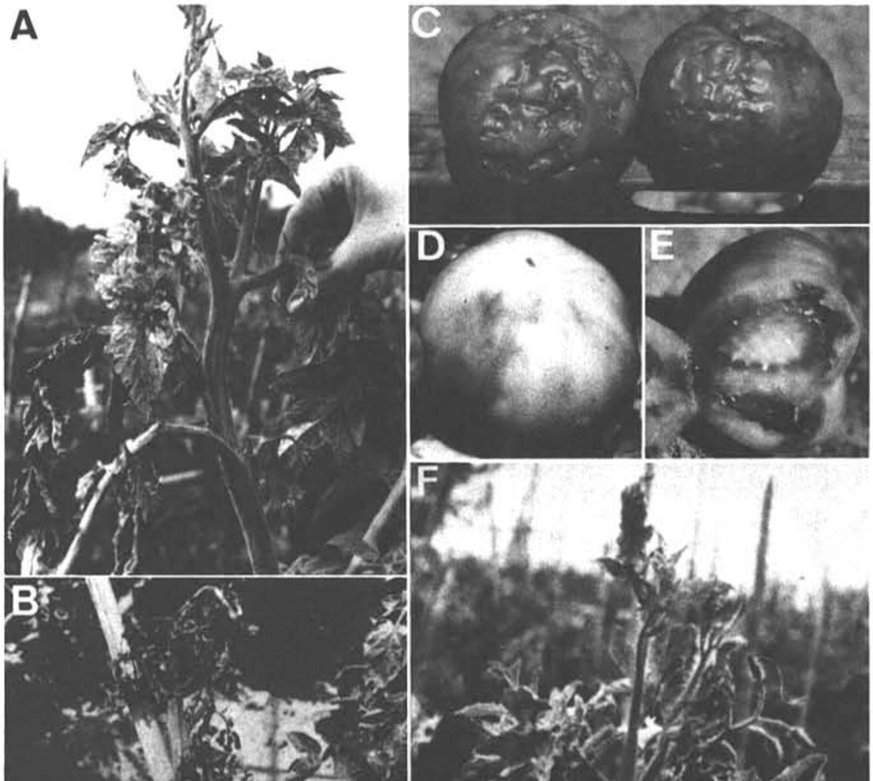

Fig. 2. Field-infected tomato plants with necrosis syndrome. **(A)** Necrotic streaks in stems and petiole, necrosis of leaf laminae; **(B)** apical necrosis of a young stem; **(C)** necrotic depressions of fruits; **(D)** and **(E)** external view and section of fruit showing subepidermic necrosis (internal browning); and **(F)** curl-stunt syndrome.

Nucleic acids from 40 field-infected necrotic plants collected from all the areas shown in Figure 1 were analyzed. When total nucleic acids extracts from leaves were analyzed in semidenaturing polyacrylamide electrophoresis gels (26), the presence of dsRNA bands corresponding in size to dimer or monomer CMV satRNA were always found (Fig. 3A). In some cases, however, the corresponding ssRNA bands were not detected, a previously described phenomenon (26). Agarose gel electrophoresis of virion-encapsidated RNA from necrotic plants showed four bands, corresponding to CMV RNAs 1–4, plus a fifth RNA band (Fig. 3B). Northern blot analysis showed the fifth RNA to be homologous to B2-satRNA (Fig. 3C), thus being an satRNA instead of a genome-related RNA5. Hybridization analysis showed, in all instances, CMV isolates to belong to subgroup I of CMV strains (not shown) (18,21), the more prevalent one in Spain (14).

Curiously, satRNA bands were found associated not only with the necrosis syndrome, but also consistently (15 plants tested from the restricted area where the syndrome was found) with plants showing the curl-stunt syndrome (Fig. 3B,C, lanes 4) and, in 30% (25 plants tested) of plants showing only the typical fern-leaf symptoms of CMV. Partially denaturing gel electrophoresis (Fig. 3D) revealed differences in mobility and, thus, in nucleotide sequence, among satRNAs from different field isolates, suggesting that satRNAs with different biology could coexist in the field. Fifty-eight percent of the 40 analyzed samples showed more than one satRNA band, indicating mixed infections. Virion encapsidated total RNA from samples showing only one satRNA band, or the purified satRNA plus Fny-CMV RNA as a helper virus, were inoculated to tomato seedlings. Samples from six necrotic plants, four plants with curl-stunt syndrome and two plants with typical CMV symptoms were assayed onto 10 or 20 tomato plants, depending on how much sample RNA was available. Three types of satRNA were found: type 1 satRNAs did not modify the stunt, mild mosaic and shoestring symptoms induced by Fny-CMV; type 2 sat RNAs modified Fny-CMV-induced symptoms to a leaf-curl, shoot-stunt syndrome; type 3 satRNAs induced a typical lethal necrosis as described for other necrogenic satRNAs (9) (Fig. 4). The symptoms induced by the assayed satRNAs corresponded to those found in inoculations with the parent preparation of virion-encapsidated RNA from field-infected plants (not shown) and with the symptoms shown by the mother field-infected plant.

Internal browning of the fruit was consistently associated with the presence of CMV as identified by serology and electron microscopy, but only sporadically with the presence of CMV-satRNAs. No other virus was found associated consistently with this syndrome. Infection of tomato cv. Rutgers plants with satRNA-free CMV from these plants reproduced the fruit symptoms (not shown).

DISCUSSION

An epidemic of tomato necrosis has been progressing in eastern Spain since 1986, with a dramatic economic impact on the important tomato crop, to the point that production has even been abandoned in some traditional areas. Our analysis of the etiology of the necrosis syndrome showed it to be induced by CMV plus necrogenic satRNAs, and not by coinfection of different tomato viruses, as other reported tomato necrosis (15) because: 1) there was a consistent association of satRNA and necrosis in field samples, 2) there was no consistent association of another virus with necrosis, and 3) isolated satRNA from necrotic plants was able to induce necrosis when coinoculated with a purified helper CMV. This is not the first reported case of CMV plus satRNA-induced epidemics of tomato necrosis of devastating effects: A tomato necrosis epidemic in Alsace, France, in the early 1970s lead to the first description of a necrogenic CMV-satRNA (8), and concurrently with the epidemic described here another one has been progressing in southern Italy (2). Differing from these two other reported epidemics, a variety of satRNAs and satRNA-mediated symptoms were found in the Spanish one, with three main types of satRNA occurring: satRNAs that induce necrosis, satRNAs that induce a leaf-curl, shoot-stunt syndrome, and satRNAs that do not modify the symptoms induced by the helper virus. No truly attenuating satRNAs were found. This heterogeneity of the satRNA population could be ascribed to natural evolution of the satRNAs, the high potential to vary of RNA genomes being well documented, e.g., reviewed by Domingo and Holland (1), and CMV satRNAs have been shown to be highly variable under experimental conditions (11). Alternatively, the heterogeneity found might be related to different origins for the different types of satRNAs described.

The origin of these satRNAs remains a matter of speculation. For many years CMV has been a virus commonly found in horticultural crops in eastern Spain (13), but prior to 1986, CMV satRNAs were not detected (unpublished data), a situation also found for other geographical areas (10). The satellites may have originated in alternative hosts in which satRNAs that are necrogenic to tomato have no, or an attenuating effect on the symptoms induced by CMV (6,25) and thus would have gone unnoticed. A possible, but not exclusive, candidate would be the perennial weed *Nicotiana glauca* Grah., common in eastern Spain, where it is frequently infected by CMV plus satRNAs (F. García-Arenal, *unpublished*), as has also been reported for Israel (24), and in which satRNA accumulated to high levels, as in other Solanaceae.

A second question relates to the occurrence of necrogenic satRNAs in the tomato crop of 1986 and not in previous crops. The depression of CMV accumulation due to the presence of CMV-satRNAs (7) is well known, which could confer a disadvantage to satRNA-supporting isolates vs. satRNA-free ones,

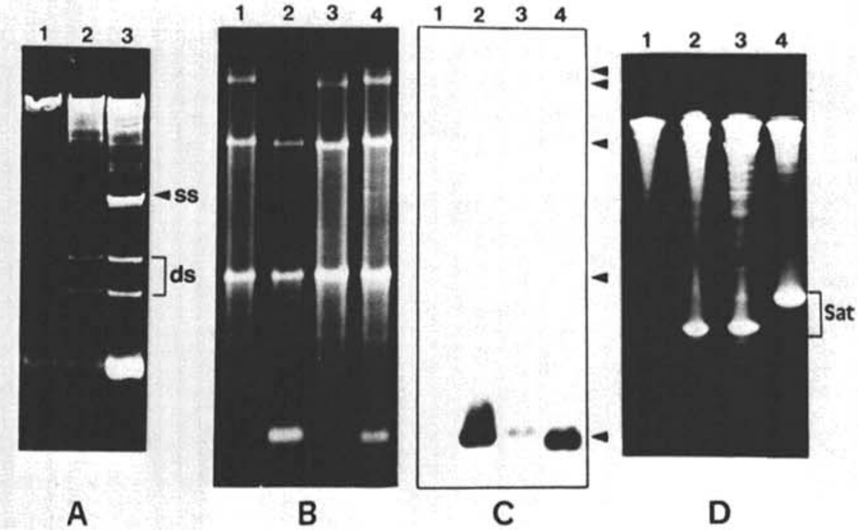

Fig. 3. Nucleic acid analyses from field-infected tomato plants. **(A)** Ethidium bromide-stained semidenaturing polyacrylamide electrophoresis gel of total nucleic acid extracts from tomato plants showing typical CMV symptoms (lane 1) or necrosis (lanes 2 and 3); mobilities of double stranded monomer or dimer satRNA (ds) and of single-stranded satRNA (ss) are indicated. **(B) and (C)** Ethidium bromide-stained agarose electrophoresis gel (B) of virion encapsidated RNAs from tomato plants showing typical CMV symptoms (lane 1), necrosis (lanes 2 and 3) or curl-stunt (lane 4) and autoradiograph (C) of its RNAs transferred to nitrocelullose and probed with cRNA from cloned B2-satRNA sequence. Mobilities of CMV RNAs 1-4 and of satRNA are indicated. **(D)** Ethidium bromide-stained semidenaturing polyacrylamide electrophoresis gel of virion encapsidated RNAs from tomato plants showing typical CMV symptoms (lane 1), necrosis (lanes 2 and 3) or curl stunt (lane 4). Mobilities of satRNAs are indicated.

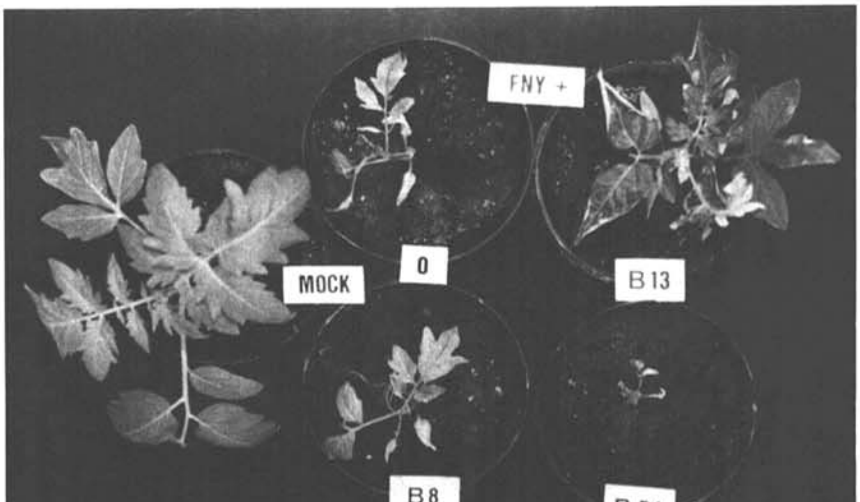

Fig. 4. Tomato cv. Rutgers plants mock-inoculated (mock) or inoculated with Fny-CMV RNA minus (0) or plus satRNAs from field-infected tomato plants with typical CMV symptoms (B8), necrosis (B5A) or curl-stunt (B13), showing reproduction of the mother plant's symptoms by satRNAs.

except under conditions highly favorable for the transmission of CMV. Once CMV plus satRNA isolates start to spread, satRNA-free isolates could easily acquire the satRNA by overinfection of plants with satRNA supporting isolates (17). Mild winters since 1985 have resulted in anomalously high spring populations of alate aphids in eastern Spain since 1986, and the well-known vector of CMV, *Aphis gossypii* Glover has been the prevalent species since then (5,16). Very possibly this change in the size and composition of aphid populations has created conditions extremely favorable to CMV transmission and has made possible the infection of tomatoes with CMV plus satRNA from other hosts and its spread to epidemic proportions.

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