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Segregation of Citrus Tristeza Virus Strains Evidenced by Double Stranded RNA (dsRNA) Analysis

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ABSTRACT. A field isolate of citrus tristeza virus (CTV), T-385, was separated from other virus and virus-like diseases by aphid transmission to Mexican lime seedlings. This isolate did not cause decline of sweet orange on sour orange rootstock under field conditions and it induced mild vein clearing and an inconspicuous stem pitting on Mexican lime. Two months after aphid transmission, one of the infected lime seedlings was used as an inoculum source to graft-inoculate 40 citron plants. Two years later, the citron plants were analysed individually for dsRNA content and up to 23 different electrophoretic profiles were found. DsRNA analysis has been repeated three times through the year and the individual patterns remained unchanged. Thirteen of these CTV sub-isolates were graft-transmitted to sweet orange seedlings and the corresponding dsRNA profiles were identical to those previously obtained from citron. All these subisolates induced mild symptoms of Mexican lime similar to those of the original isolate (T-385). These results substantiate that several CTV strains can infect a single citrus plant.
Index words. symptoms, electrophoresis, silver stain.

Citrus Tristeza Virus (CTV) has numerous strains differing in biological properties (12). Severity of symptoms induced in field trees (1, 6) or indicator plants (2, 3, 18), aphid transmissibility (4, 13, 23, 25) and cross-protecting ability (5, 20, 24) have been currently used to characterize CTV strains. In recent years, strain-discriminating monoclonal antibodies (21) and several biochemical methods, including dsRNA analysis (7, 11), peptide map analysis (17) and DNA hybridization (26) have been assayed to characterize virus strains. In particular, dsRNA analysis has proved to be a simple method to distinguish CTV strains (8, 9, 10, 16, 19).

Biological evidence for the presence of different components in some CTV isolates has been presented by several authors (18, 27). Raccah *et al.* (22) showed that individual CTV-infected trees contained several variants of the virus differing in aphid transmissibility. Hermoso de Mendoza *et al.* (13) found seedling yellows (SY) as well as non-SY CTV isolates among the plants inoculated by *A. gossypii* using a CTV-SY source. Roistacher *et al.* (25) found high rates of transmission by *Aphis gossypii* in some CTV isolates that were propagations from others having low aphid transmissibility. Dodds *et al.* (10) and Jarupat *et al.* (15) hypothesized the presence of strain mixtures in some CTV isolates to explain the peculiarities observed in dsRNA patterns obtained from plants infected with those isolates.

In this paper we present evidence for strain segregation from a CTV isolate based on dsRNA analysis of plants infected with this isolate or with different subisolates obtained by graft-transmission.

MATERIALS AND METHODS

CTV isolate T-385 was collected from a symptomless field tree, propagated at the greenhouse on a sweet orange seedling and then transmitted to Mexican lime by *Aphis gossypii*. Two months later, one of the lime seedlings which reacted positively to tristeza by ELISA, was used as an inoculum source to graft-inoculate 40 Etrog citron plants plants propagated on Rough lemon as rootstock.

Two years later, the original Mexican lime plant infected with T-385 was used to graft-inoculate two Navel orange/Troyer citrange plants for the virus collection. At the same time, 13 of the subisolates kept in Etrog citron were graft-transmitted to Mexican lime and to sweet orange seedlings.

Symptom intensity (vein clearing and stem pitting) was visually rated on Etrog citron and Mexican lime using a 0-4 scale in which 0 means no symptoms, and 4 means very severe symptoms.

ELISA was performed using monoclonal antibody 3DF1 in the conditions previously described (28).

DsRNA analysis was carried out on individual Etrog citron and sweet orange plants by phenol extraction of young bark tissue, purification by CF-11 cellulose column chromatography and separation by polyacrylamide gel electrophoresis (PAGE), following the method of Dodds *et al.* (8, 9) with minor modifications. DsRNAs were observed by staining with ethidium bromide, or with silver nitrate (14).

RESULTS

Field symptoms. CTV isolate T-385 was collected from a healthy-looking tree in an 80-yr-old Navel/Cadenera/sour orange planting in Orihuela (Alicante), showing severe decline caused by tristeza. When originally found, this tree reacted positively to CTV by ELISA, but only showed a very mild honeycombing reaction below the budunion. Eight years after the first observation, most trees in the planting have been removed as a result of the severe decline, whereas this tree shows only a slight decline.

Greenhouse symptoms. None of the 40 citron plants graft-inoculated with T-385 from the aphid-inoculated lime, showed vein clearing or stem pitting.

Thirteen of the T-385 subisolates, inducing different dsRNA patterns in citron, were selected and graft-transmitted to Mexican lime. Symptoms induced by these subisolates as well as by the mother isolate were rated as very mild (0 to 1 in the scale). Some of them induced no stem pitting and only an inconspicuous vein clearing in a few leaves.

DsRNA patterns. Fig. 1 shows the dsRNA patterns obtained from one of the Navel plants infected with T-385 and kept at the virus collection, and from the original Navel tree where T-385 was isolated. Though dsRNA recovery from the field tree was very low, silver staining showed that the patterns from both citrus plants were identical.

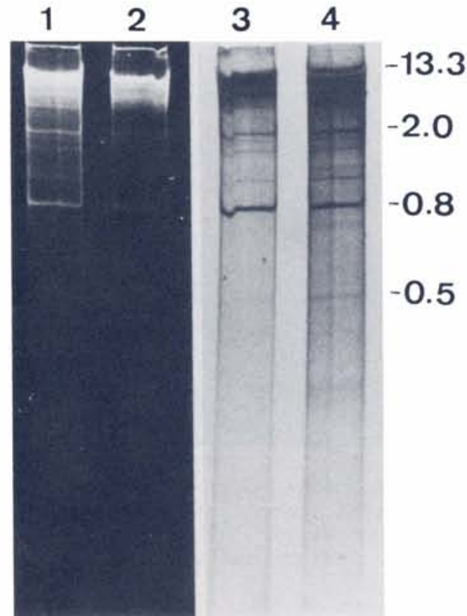

Fig. 1. DsRNA patterns of citrus tristeza virus (CTV) isolate T-385 otained from a Navel orange/Troyer citrange plant kept in the screenhouse (lanes 1 and 3) and from a Navel orange/Cadenera sweet orange/sour orange field tree (lanes 2 and 4). Purified dsRNAs were electrophoresed on 5% polyacrylamide gels and stained with ethidium bromide (lanes 1 and 2) and with silver nitrate (lanes 3 and 4).

Fig. 2 shows a group of the dsRNA profiles obtained from citron plants infected with different subisolates segregated from T-385. The profiles were constant for each subisolate in three successive extractions along the year. In most cases, good dsRNA recovery was obtained by purifying from 2 g of citron bark. The thirteen sub-isolates selected and graft-transmitted to sweet orange induced the same dsRNA pattern in both hosts.

The dsRNA patterns differed in the number and/or position of the subgenomic bands located between the full length replicative form and the 0.8×10^6 Mr. A few subisolates had a

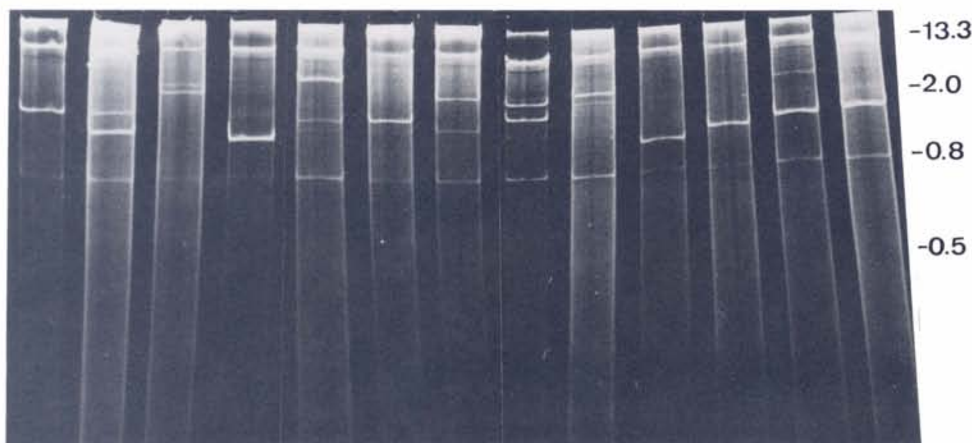

Fig. 2. DsRNA patterns of some citrus tristeza virus (CTV) subisolates segregated from the T-385 isolate. Segregation was obtained when graft-inoculating several citron plants from a single Mexican lime aphid-inoculated with T-385. Purified dsRNAs were electrophoresed on 5% polyacrylamide gels and stained with ethidium bromide.

very weak band in the 0.5 x 10^6 Mr position. Many of the subgenomic bands observed in different subisolates could be detected in the dsRNA profile from the mother isolate.

DISCUSSION

A number of dsRNA patterns was observed among subisolates obtained by graft-transmission from a single plant aphid-inoculated with the isolate T-385 of CTV.

The biological role of dsRNAs in virus-infected plants is not fully understood. Some authors (7) have suggested that minor dsRNAs could be intermediate templates for subgenomic viral ssRNAs, and thus, they could be considered as fingerprints of the virus genome. Regardless of the biological significance of dsRNAs, since usually they are not detected in healthy plants and the profiles obtained from plants infected with different virus strains are consistent, they have diagnostic value (11).

The dsRNA profiles induced by all subisolates assayed had a high molecular weight band in the position expected for the full length replicative form of CTV, but they differed in the number and/or position of the minor bands. This variability in the dsRNA profiles suggest that our subisolates are strains or mixtures of CTV strains. The fact that dsRNA profiles remained constant in successive analyses, and in different hosts (citron and sweet orange) gives support to this suggestion.

These strains should undoubtedly be present in the original T-385 isolate and were separated when graft-inoculating citron plants. A possible cause for the strain segregation obtained would be a different rate of replication and/or diffusion within the plant of the several strains inoculated by the aphids into the lime. Since graft-transmission from lime to citron plants was done early after aphid inoculation, those strains probably were not homogeneously distributed and the inoculum present in bark pieces taken from several parts of the plant contained a different strain composition. The fact that Navel orange plants graft-inoculated two years later from the same Mexican lime had the same dsRNA pattern as the original field tree supports this hypothesis. This period was probably sufficient for all the components in the mixture to become homogeneously distributed within the inoculated lime plant.

Many of the subgenomic bands observed in the subisolates were present in the mother isolate, though with different intensity. This is probably due to the different concentrations reached by the subisolates when being alone or in the mixture. The higher intensity of certain bands in the mother isolate could be due to a higher rate of multiplication of some of the subisolates.

The presence of different virus strains within a single plant could be due to mutations of the original strain arising with time; to successive inoculations by aphids carrying inoculum from different virus sources, or more likely, to both factors.

The possibility of a mixture of strains being present in some CTV isolates was suggested by Dodds *et al.* (10) to explain the presence in those isolates of two dsRNA bands with slightly different mobilities in the position expected for the full length replicative form. A similar explanation was given by Jarupat *et al.* (15) to the fact that certain CTV isolates showed low intensity, or even disappearance of specific dsRNA bands when these isolates were multiplied in grapefruit. Our results show, for the first time, direct evidence of several genetic entities being present in a single CTV isolate, and the possibility of physical separation of these entities.

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