Detection of a Necrotic Strain of Citrus Ringspot in Star Ruby Grapefruit in Spain

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ABSTRACT. A 6-yr-old planting of Star Ruby grapefruit on sour orange rootstock propagated from illegally introduced budwood was inspected for ringspot symptoms. Yellow blotches, chlorotic mottling and ring-like patterns were observed in mature leaves and fruits of some plants. In the spring flush, some young shoots had shock symptoms and young leaves showed different chlorotic patterns. None of the trees showed bark scaling, but a few of them had blisters on some young twigs. Graft-inoculated seedlings of sweet orange, sour orange, Mexican lime, Duncan grapefruit, Dweet tangor and Etrog citron developed a severe shock reaction with leaf abscission and dieback in the first flush. Necrotic pin points or etching appeared in some leaves. Inoculated grapefruit and citron seedlings grew poorly in subsequent flushes and some plants showed dieback, and finally died. This ringspot isolate shows similarities with necrotic strains described in Texas and Florida. The new isolate was readily transmitted to *C. quinoa* inducing chlorotic to necrotic local lesions. Transmission was not obtained using *Aphis gossypii* or *A. citricola* as vectors. Partially purified extracts from infected plants had infectivity associated to two fractions in a sucrose density gradient. A 48-kd protein was present in the two fractions, but not in equivalent extracts from healthy plants. A 38-kd protein was sometimes found associated with infectious fractions, usually when purifying from young shoots at the shock stage. Virus particles were not observed in infectious fractions.

Index words. Purification, protein, double stranded RNA, aphid transmission, immunoelectron microscopy, herbaceous hosts.

A number of graft-transmissible diseases of citrus that induce different chlorotic patterns in young leaves have been currently grouped and named as psorosis diseases (19, 23). The members of this group, which includes psorosis A, psorosis B, ringspot (CRSV), concave gum-blind pocket, impietratura and cristacortis, cause different field symptoms, but they are difficult to identify by indexing on indicator plants.

Mechanical transmissibility to *Chenopodium quinoa* and other herbaceous hosts (20) has been associated to most ringspot isolates, psorosis B and, in general, to the presence of severe bark scaling symptoms in the field trees (8, 18). Citrus plants mechanically inoculated with different local lesion isolates of CRSV reproduced the characteristic leaf and shoot symptoms (8) and, at least with one isolate, bark scaling was also obtained (9).

The etiology of the psorosis disease has not been established as yet but an unusual virus has been associated with infectious fractions of partially purified extracts from CRSV infected citrus plants (5). These infectious fractions contained a 48-kd protein, presumed to be the viral coat protein.

Psorosis diseases are primarily transmitted through budwood, but natural spread of psorosis-ringspot isolates has been observed in Argentina (15, 17) and to a lesser extent in Texas (16, 18).

In recent years, an illegal budwood introduction of Star Ruby grapefruit took place in Spain and some plantings were propagated from this material before release of virus-free budwood by the Citrus Quarantine Station. Some lines of this cultivar in Florida and Texas were known to carry a necrotic strain of CRSV (10, 16). The possibility that trees of Star Ruby grapefruit, propagated from budwood of uncontrolled origin, could be infected with a CRSV strain of this type, prompted us to survey one of these plantings and to test for presence of this pathogen.

MATERIALS AND METHODS

Graft-inoculation tests. Indicator plants were grown in a steam-sterilized artificial potting mix (50% sand and 50% peat moss) and fertilized according

to a standard procedure (1). Inoculated plants were incubated in a temperature-controlled greenhouse kept at 18-26 C, with the only exception of a group of Etrog citron plants (Arizona 861-S-1), used to index for viroids of the exocortis group, that was incubated in a warm greenhouse (26-32 C). A minimum of five plants of each of the following species and cultivars were inoculated to observe symptoms: Madame Vinous sweet orange, Dweet tangor, Mexican lime, Duncan grapefruit, Etrog citron and sour orange.

ELISA. ELISA tests for tristeza were done using the monoclonal antibody 3DF1 in the conditions previously described (21).

Mechanical transmission. Young leaves showing symptoms were selected to use as inoculum source to transmit the pathogen from citrus to herbaceous hosts. Carborundum-dusted leaves were rubbed with leaf extracts prepared in cold TACM buffer (0.05 M Tris-C1, 0.1% ascorbic acid, 0.1% L-cysteine and 0.5% 2-mercaptoethanol, pH 8.0) (5) immediately before inoculation (8). Different fractions during the purification process were also assayed on *C. quinoa*.

Aphid transmission. *Aphis gossypii* and *A. citricola* were tried as potential vectors of the disease following the procedure described by Hermoso de Mendoza *et al.* (11). Madame Vinous sweet orange seedlings were used as donors and as receptor plants. The donor plants were decapitated and the aphids fed in new succulent shoots showing symptoms. The aphid-inoculated shoots on receptor plants were allowed to grow for 6 months and then pruned every three months to force new growth.

Analysis of double-stranded RNA (dsRNA). DsRNAs were purified from several types of tissues and analyzed following the general procedure described by Dodds *et al.* (6). The gels were stained with ethidium bromide and with silver nitrate (12).

Partial purification and characterization. Partial purification of the CRSV isolate used in this study was done following the general procedure described by Derrick *et al.* (5) using young symptomatic leaves of Duncan grapefruit, Mexican lime, Madame Vinous sweet orange or sour orange. Clarified and concentrated extracts were layered on a linear 10-40% sucrose gradient and centrifuged for 3.5 hr at 27,000 rpm in a Sorvall AH-627 rotor using 12 ml tubes. The gradients were fractionated from the top into 0.6 ml fractions using an ISCO gradient fractionator.

A group of fractions, selected for having infectivity on *C. quinoa*, were pooled and precipitated by centrifugation at 300,000 g for 1 hr. The pellet was resuspended and electrophoresed in 0.5% agarose at 50 V for 4 hr as described by Derrick *et al.* (5). The agarose block comprised between 0.2 and 1.2 cm from the top edge of the agarose gel, was sliced, added with 0.4 ml of protein denaturing mixture (0.25 M Tris-Cl pH 8.0, 5.7% SDS, 14.3% (v/v) mercaptoethanol and 28.6% (v/v) glycerol) and heated for 4 min in boiling water. Proteins in this fraction were separated by polyacrylamide slab gel electrophoresis (SDS-PAGE) (3). The gels were stained with Coomassie blue and then with silver nitrate.

Immunoelectron microscopy (IEM). IEM was performed by the methods described by Derrick *et al.* (4, 5). Grids were coated for 30 min with 0.02 mg/ml of protein A and then for 1 hr with an antiserum prepared to the CRSV-4 ringspot isolate from Florida (5) (kindly provided by K. S. Derrick, Univ. Florida, CREC, Lake Alfred). After incubation for 3 hr with the antigen solution, the grids were positively stained with 2% uranyl acetate for 10 min and blotted with filter paper.

RESULTS

Field Symptoms. A 6-yr-old planting of Star Ruby grapefruit on sour orange was repeatedly surveyed for ringspot symptoms in spring and autumn. Trees were vigorous and the general appearance was good, except for a few with decline, general yellow-

ing, and defoliation. The number of declined trees has not increased in 2 yr. None of the trees showed bark scaling, but a few of the declined trees had blisters in some young twigs. In the spring flush, some young shoots had shock symptoms with leaf shedding and necrosis (Fig. 1). Many young leaves showed different chlorotic patterns, this symptom being more noticeable in the spring flush. Some mature leaves showed yellow blotches, chlorotic mottling and ringlike patterns (Fig. 2). Most fruits observed appeared normal, but yellow ringlike patterns could be observed in a few green fruits and light green spots on the pink areas of some ripened fruits.

Symptoms on citrus indicators. In a preliminary test, budwood was taken from different grapefruit trees, including healthy-looking as well as declined trees, and graft-inoculated on sweet orange seedlings. All budwood sources induced a similar CRSV reaction, described below, with no difference between healthy or declined field trees.

Graft-inoculated sweet orange, sour orange, Mexican lime, Duncan grapefruit, Etrog citron and Dweet tangor plants developed a severe shock reaction with leaf abscission and dieback in the first flush. In subsequent flushes, the shock reaction rarely appeared and it affected only very small shoots. Young leaves usually showed chlorotic spots and ringlike patterns,

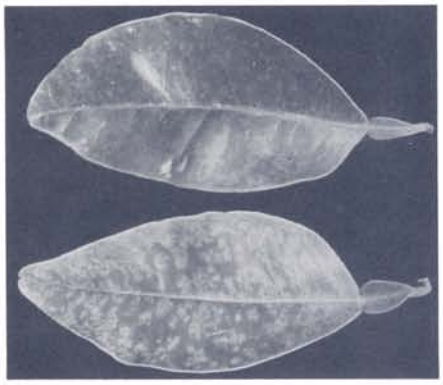

Fig. 2. Chlorotic mottling and ringlike patterns in mature leaves of a Star Ruby grapefruit affected by CRSV.

but some of them had necrotic pin points and etching (Fig. 3). This reaction was more frequent in grapefruit, citron and sour orange. Occasionally, oak leaf pattern was observed in young leaves of sour orange. Sometimes, grapefruit plants showed distortion of young and fully developed leaves. Infected grapefruit and citron plants usually grew poorly and some of them suffered dieback and finally died.

ELISA tests and graft-inoculations on Mexican lime and Etrog citron showed that this CRSV source was free of tristeza, vein enation and citrus variegation viruses, and viroids inducing symptoms on citron.

Symptoms on herbaceous hosts. Crude extracts from young symptomatic citrus leaves were inoculated on *C.*

Fig. 1. Shock reaction induced by CRSV on the spring flush of a Star Ruby grapefruit tree in the field.

Fig. 3. Necrotic etching induced by CRSV (Star Ruby isolate) in young leaves of Etrog citron.

quinoa, *Gomphrena globosa*, *Phaseolus vulgaris* cv. Red Kidney bean, *Vigna unguiculata*, *Nicotiana benthamiana* and *N. megalosiphon*. Only *C. quinoa* was infected. Symptoms consisted of chlorotic to necrotic local lesions that sometimes coalesced producing large necrotic areas (Fig. 4). Extracts from these lesions inoculated on healthy *C. quinoa* plants induced new lesions of similar appearance.

Aphid transmission tests. None of the 20 sweet orange seedlings inoculated with *A. gossypii* showed any symptom in three successive flushes. Bark pieces from the aphid-inoculated plants were graft-inoculated to sweet orange seedlings 4 and 11 months after the aphid inoculation; no symptoms were either observed in graft-inoculated plants. In a second experiment, 25 and 24 sweet orange seedlings inoculated using *A. citricola* and *A. gossypii*, respectively, did not show any symptoms in the first two flushes after aphid inoculation.

Analysis of dsRNA. No dsRNA bands could be detected in extracts prepared from young symptomatic leaves, bark from shoots bearing fully developed leaves, or very young asymptomatic shoots. Similar extracts from plants infected with citrus tristeza virus (CTV) yielded several dsRNA bands typical of CTV infected tissue.

Partial purification and characterization. When clarified extracts from young symptomatic leaves were concentrated and fractionated through a sucrose gradient, infectivity on *C. quinoa* was associated with two components. The top component corresponded to fractions 7-8-9 and was located at ca. 2.5 cm depth in the gradient. The bottom component included fractions 11-12-13 and was located at ca. 3.8 cm depth. The top or the bottom components, separately, did not have significant infectivity on *C. quinoa*, whereas the combination of top and bottom components induced local lesions on *C. quinoa* similar to those induced by crude or concentrated extracts.

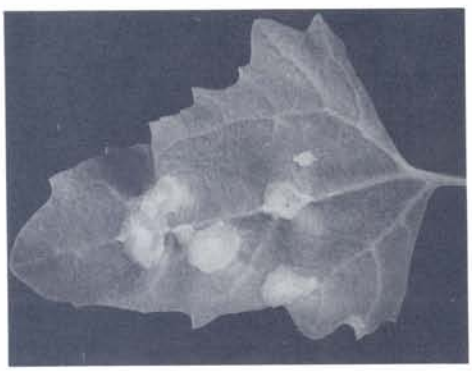

Fig. 4. Necrotic local lesions induced by CRSV (Star Ruby isolate) mechanically inoculated on *C. quinoa*.

Fractions in the top or in the bottom components were pooled, concentrated by centrifugation and further purified by electrophoresis in agarose gel. When proteins in these purified preparations were separated by SDS-PAGE, the top and the bottom components from infected plants had a protein band, about 48-kd molecular weight, that was not present in similar preparations from healthy plants (Fig. 5). This protein band could also be detected by SDS-PAGE in the top and bottom component fractions of the sucrose gradient or in the concentrated extract layered on the top of the gradient, but not in the noninfectious fractions.

A protein band of 38-kd molecular weight was sometimes observed associated to the top and bottom components. This protein was not found as consistently as the 48-kd protein and was mainly associated with young shoots at the shock stage. This protein was detected from the gradient fractions as well as after agarose gel electrophoresis, but it was never observed in similar preparations from healthy controls (Fig. 6).

Immunoelectron microscopy. Viral particles were not detected by IEM in crude extracts, concentrated extracts before sucrose gradient centrifugation, or concentrated top and bottom components, from symptomatic leaves of citrus infected plants. They could not be found in extracts of

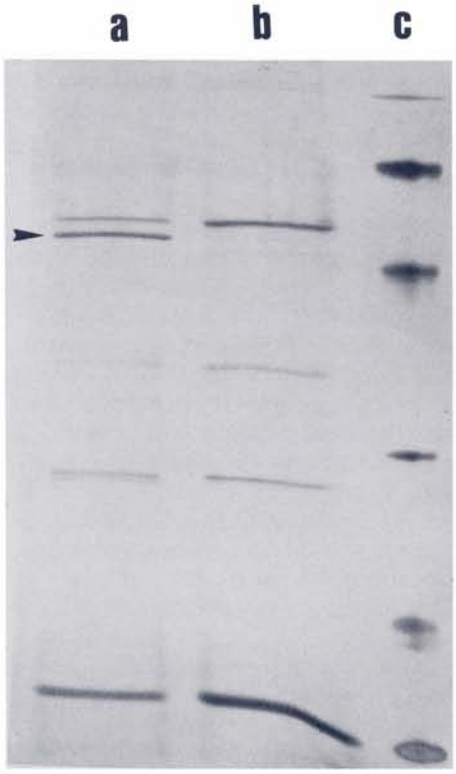

Fig. 5. Discontinuous SDS-PAGE of purified preparations from CRSV-infected (lane a) and healthy (lane b) young citrus leaves. The top component fractions of the sucrose density gradient were concentrated and further purified by agarose gel electrophoresis. A 1 cm agarose block was sliced from the top edge of the gel and the proteins separated by SDS-PAGE. The gel was stained with silver nitrate. Lane c contains molecular weight markers (from Bio-Rad): rabbit muscle phosphorylase b, 97.4 kd; bovine serum albumin, 66.2 kd; hen egg white ovalbumin, 45.0 kd; bovine carbonic anhydrase, 31.0 kd; soybean trypsin inhibitor, 21.5 kd; hen white lysozyme, 14.4 kd. Arrowhead indicates the 48-kd protein detected in extracts from infected plants.

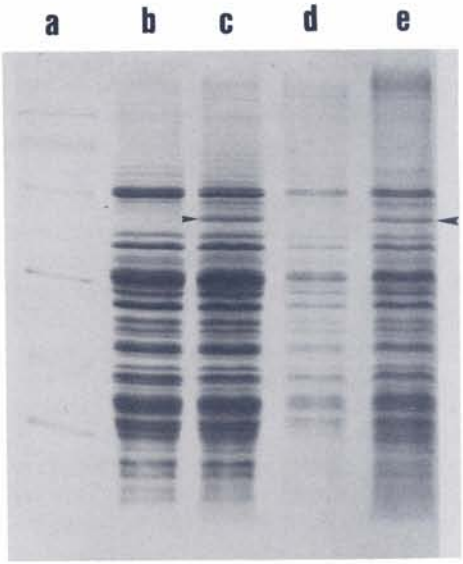

Fig. 6. Discontinuous SDS-PAGE of partially purified preparation from healthy (lanes b and d) and CRSV-infected (lanes c and e) young citrus leaves. The top and bottom component fractions of the sucrose density gradient were concentrated and the proteins separated by SDS-PAGE. The gel was stained with Coomassie blue. Lane a contains molecular weight markers described in Figure 5. Arrowheads indicate the 38-kd protein detected in extracts from infected plants.

local lesion tissue from *C. quinoa* infected leaves either.

DISCUSSION

Ringspot symptoms were observed in different Star Ruby grapefruit trees propagated from illegally introduced budwood. A few trees showed yellowing, defoliation and blisters in some young shoots, but when indexed in the greenhouse, symptoms of indicator plants inoculated from these trees or from vigorous healthy-looking field trees were indistinguishable. All of them seemed to be infected by the same CRSV isolate and decline symptoms in some of the trees were probably due to other causes. This CRSV isolate could have been introduced in this line of Star Ruby in Spain after propagation of the illegal budwood in some infected trees. In fact, viruses of the psorosis group are very common among local varieties (13). Nevertheless, the fact that the Star Ruby trees were free of viroids inducing symptoms on citron, strongly suggests that CRSV was introduced with budwood, since these viroids are present in most field trees in Spain (13).

This CRSV isolate induced severe symptoms including shoot necrosis, leaf shedding and necrotic pinpoints or etching in all indicators assayed. These reactions differ from those described

for other ringspot isolates in Spain (14), Corsica and Greece (22) or Australia (2) in that these isolates did not produce the shock reaction in the hosts assayed here, and are similar to those obtained with the necrotic strains described in Texas and Florida (8, 10, 16, 18, 20). Coincidentally, some of these necrotic strains were found in Star Ruby grapefruit. In contrast with the Texas and Florida strains (8, 16, 20), our CRSV isolate induced a shock reaction on citron and it was not mechanically transmissible to herbaceous hosts other than *C. quinoa*.

Evidence for natural spread of CRSV in Texas were found by Timmer and Garnsey (18), but the failed to experimentally transmit the virus using several species of plant hoppers and leafhoppers, *A. spiraecola* and *Planococcus citri*. In Spain, so far, there is no indication for natural spread of viruses of the psorosis group, and we were unable to transmit the Star Ruby isolate of CRSV by *A. gossypii* or *A. citricola*.

Failure to detect dsRNA in the infected plants might be due to uneven distribution of the virus in infected tissues and/or a low titer of dsRNA.

Trials to purify the causal agent of CRSV showed that infectivity on *C. quinoa* was associated with two fractions in the sucrose density gradient and that these fractions contained a protein, about 48 Kd. This protein was not present in other fractions in the gradient or in similar fractions obtained from healthy plants. Our results are similar to those of Derrick *et al.* (5) with the CRSV-4 ringspot isolate. They found 48-kd protein associated to infectious fractions that they presumed to be the virus coat protein. Garcia *et al.* (7), working with a psorosis isolate from Concordia (Argentina), also found infectivity associated to two components and to the presence of a 50-kd protein.

The presence of a 38-kd protein associated to infectious fractions has not been described previously. The biological significance of this protein is unknown but, since it was detected in different citrus species, it might be directly related to the pathogen. The fact that this protein is detected at the top of the agarose gel after electrophoresis is an indication that it is attached to a large structure, perhaps a nucleoprotein. Thus, the 38-kd protein could be a degradation product of the 48-kd protein, or it coud be a second structural protein of the same virus, or two viruses might be present in our CRSV isolate.

Our results seem to indicate that a new ringspot isolate was introduced into Spain with uncontrolled budwood of Star Ruby grapefruit. The biological characteristics of this CRSV isolate are similar to those of the necrotic strains described in Texas and Florida. Sedimenting characteristics of the infectivity on *C. quinoa* and the association of a protein, about 48-kd, with infectious fractions also indicate similarity between this pathogen and CRSV purified in Florida. Failure to detect viral particles by IEM using an antiserum to the CRSV-4 isolate from Florida may be due to serological differences between both isolates.

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