Comparison of Isolates of Citrus Ringspot, Psorosis, and Other Viruslike Agents of Citrus

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ABSTRACT

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A collection of graft-transmissible psorosis and psorosislike diseases of citrus were compared with a partially characterized isolate of citrus ringspot virus (CRSV-4) from Florida. Fourteen isolates of citrus ringspot and psorosis from Florida, Spain, and Argentina were mechanically transmissible to *Chenopodium quinoa* and induced local lesions. The infectivity associated with several isolates was separated into top and bottom components by sucrose gradient centrifugation, which is characteristic of CRSV-4. A 48-kilo dalton (kDa) protein, which is assumed to be the CRSV capsid protein, was detected in preparations of top and bottom components from 13 of the isolates. The 48-kDa protein was not detected in preparations from healthy plants or plants with symptoms of concave gum, cristacortis, or impietratura. Thus, there was a good correlation between the detection of the 48-kDa protein, indicative of a CRSV-type virus, and the designation of diseases as either ringspot or psorosis based on symptoms. An antiserum, produced to purified CRSV-4, was used to detect the 48-kDa protein in western blots and virus particles by serologically specific electron microscopy (SSEM). This is further evidence that the 48-kDa protein is the capsid protein of the virus.

A disease characterized by bark scaling of citrus that had been observed in Florida and California was named psorosis by Swingle and Webber in 1896 (7). In 1933, Fawcett (4) observed flecking in young leaves of trees with psorosis bark lesions and suggested the disease was caused by a virus. Subsequently, a number of graft-transmissible pathogens of citrus that cause bark scaling and/or chlorotic patterns on young leaves were placed in a loosely defined group referred to as psorosis diseases (12). Currently, this includes psorosis A, psorosis B, ringspot, concave gum, cristacortis, and impietratura (14). Using the flecking symptoms produced in young leaves as an indicator, psorosis bark scaling symptoms have been eliminated from many citrus areas by budwood certification programs (12). Two other diseases that were at one time considered to be psorosis diseases, crinkly leaf and infectious variegation, have been shown to be caused by distinct ilarviruses (14). The agent or agents that cause psorosis diseases have not been characterized but are presumed to be viruslike.

Citrus ringspot disease, which is characterized by spots, ring spots, and blotches on leaves, was described in 1968 (13) as being caused by citrus ringspot virus (CRSV). CRSV has proven difficult to characterize, but an unusual virus was recently associated with Florida isolate CRSV-4 (2). Because this virus is associated with ringspot, it is herein referred to as CRSV. The virus is mechanically transmitted to a wide range of herbaceous plants and produces lesions on inoculated leaves of *Chenopodium quinoa* Willd. (9). The infectivity associated with the virus, as measured on *C. quinoa*, is readily separated into top and bottom components by sucrose density gradient centrifugation. Short and long extremely flexible, filamentous particles are associated with the top and bottom components, respectively. In addition, a 48-kDa protein, which is associated with the short and long filamentous particles and may function as a capsid protein, is readily detected in partially purified preparations (2).

The purpose of this study was to compare other psorosis diseases with Florida isolates of ringspot. A collection of graft-transmissible diseases from Argentina, Spain, and Florida that are maintained under quarantine in Beltsville, MD, and had been designated psorosis diseases based on symptomatology were examined for their ability to induce local lesions on *C. quinoa*, resolution of infectivity into two components by sucrose density gradient centrifugation, and the presence of the 48-kDa protein. A preliminary report has been published (1).

MATERIALS AND METHODS

The virus isolates used in this study are listed in Table 1. Isolate CRSV-4, which is considered a subisolate of Texas citrus necrotic ringspot (10,11), was collected from a Star Ruby grapefruit (*Citrus* × *paradisi* Macfady.). Isolate CRSV-6 was collected from a navel orange (*C. sinensis* (L.) Osbeck) with extensive bark scaling and leaf flecking and oak leaf patterns in young leaves (5). Both Florida isolates have been transmitted mechanically from citrus to citrus and from citrus to herbaceous hosts, including single lesions of *C. quinoa*, and back to citrus. These local-lesion isolates have been maintained in citrus for several years. Symptoms of bark scaling have never been observed with CRSV-4, but CRSV-6 readily induces bark scaling (5).

Isolates B84 and B75 are from Argentina. B84 is from a trifoliate orange (*Poncirus trifoliata* (L.) Raf.) with bark scaling that was designated as psorosis or ringspot when collected. B75 was collected from a trifoliate orange as a possible source of citrus tristeza virus (CTV). Psorosislike flecking was observed in graft-inoculated sweet orange (*C. sinensis*), which subsequently tested negative for CTV.

The following isolates are from Spain. B86 and B87 were detected in two shoot-tip grafted plants from Oroval Clementine (*C. reticulata* Blanco). They differ in that B86 induced a shock reaction on sweet orange, whereas B87 did not. The original tree had bark scaling and induced a shock reaction when budded

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on sweet orange (3). B99 is from an Oroval Clementine that had ring spots on fruit but not bark scaling. B89 is from a declining navel orange tree with severe bark scaling on the trunk, limbs, and small twigs and chlorotic patches and ring spots on leaves. Isolate B95 is from a shoot-tip grafted plant from a Clauselina satsuma (*C. unshui* Markovich) that did not have bark scaling. This isolate induces a shock reaction when budded to sweet orange. Isolate B96 is from a shoot-tip grafted plant from a Kara mandarin (*C. reticulata*) that had bark scaling. B93 is from a plant of Wilking mandarin showing severe concave gum symptoms, including trunk and limb concavities and distortions and gumming. The specific source tree of this isolate is not known, but some trees in the planting are now showing severe bark scaling as well as symptoms of concave gum. B101 was collected in Corsica and described as a typical cristacortis isolate. B94 is from a shoot-tip grafted plant from a navel orange. The donor tree did not have trunk symptoms, but fruit on the tree has symptoms of impietratura. In a cross protection test, this isolate protected against concave gum and is considered to be a concave gum isolate (6). B90 is from an old-line Clementine tree showing bark scaling and chlorotic flecks on young leaves and chlorotic patches and ring spots on mature leaves. B98 is from a Clementine that did not show bark scaling. This isolate was selected because it induced chlorotic patches and ring spot when budded on Mexican lime. B97 is from a Clementine showing unusually severe bark scaling. B88 is from a young Clementine plant showing chlorotic patches and ring spots. At the time of collection, the tree was too young to have bark symptoms. B103 is from a sweet orange with severe impietratura fruit symptoms but no bark scaling. In a cross protection test, it protected against concave gum but not psorosis.

The isolates were graft-transmitted to Duncan grapefruit seedlings under quarantine in Beltsville, MD. One month later, young symptomatic leaves were harvested for assay. Leaves of *C. quinoa* with local lesions were collected for assay 7 days after inoculation with Florida isolates CRSV-4 and CRSV-6. Leaves from healthy grapefruit and symptomless leaves from plants infected with CRSV-4 were used as controls.

Infectivity assays on *C. quinoa*, partial purification of virus, infectivity assays of sucrose density gradient fractions, agarose gel electrophoresis of partially purified virus preparations, and analysis of proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were done as previously described (2). The partial purifications and infectivity assays were done in the quarantine facilities in Maryland.

An antiserum was prepared to CRSV-4 by COCALICO Biologicals, Inc. (Reamstown, PA) in rabbits with virus particles purified by electroelution from agarose gels as the inject antigen. Fractions containing infectious virus components from sucrose density gradient centrifugation were subjected to electrophoresis at 80 V for 4 hr at 4 C on 0.5% agarose gels in 0.04 M Tris-acetate, 0.002 M EDTA (TAE). The area of the gel containing virus, previously determined to be 0.5–1.0 cm from the sample wells (2), was cut out and electroeluted in dialysis tubing for 4 hr at 75 V in TAE. The eluted virus was concentrated by centrifugation at 250,000 *g* for 1 hr. Serologically specific electron microscopy (SSEM) of CRSV was done as previously described (2). Extracts for western blot analysis were concentrated by one cycle of differential centrifugation (2) and subjected to SDS-PAGE. The gels were electroblotted onto nitrocellulose with the Semi-Dry Electroblotter (JKA-BIOTECH, Denmark) following instructions provided by the manufacturer. Immunostaining of nitrocellulose blots was done with the ProtoBlot Western Blot AP System following instructions provided by the manufacturer (PROMEGA, Madison, WI).

Table 1. Tests for the presence of 48-kilo dalton (kDa) protein and transmission to *Chenopodium quinoa* from citrus ringspot, psorosis, concave gum, cristacortis, and impietratura

Sample no.	Virus isolate[a]	48-kDa protein[b]		Infectivity[c]			
		T	B	T	B	T+B	Extract
1	Florida ringspot-4 in *C. quinoa*	+	+	−	−	++	+++
2	Florida ringspot-6	+	+	−	−	++	++
3	Spain psorosis P-121 (B86)	+	+	−	−	++	++
4	Spain psorosis P-123 (B87)	+	+	−	−	−	+++
5	Argentina psorosis (B84)	+	+	−	−	+	+++
6	Argentina psorosis (B75)	+	+	−	−	+	++++
7	Spain ringspot RS-105 (B99)	+	+	−	−	+	++
8	Spain ringspot (B89)	+	+	−	−	+	+
9	Spain psorosis P-138 (B95)	+	+	−	−	−	+++
10	Spain psorosis P-137 (B96)	+	+	−	−	−	++
11	Spain concave gum CG-212 (B93)	+	+	−	−	−	++++
12	Spain cristacortis C-601 (B101)	−	−	−	−	−	−
13	Florida ringspot-4	+	+	−	−	++	+++
14	Florida ringspot-4 symptomless	−	−	−	−	−	−
15	Healthy grapefruit	−	−	−	−	−	−
16	Spain concave gum CG-214 (B94)	−	−	−	−	−	+
17	Spain ringspot (B90)	+	+	−	+	++	++
18	Spain ringspot RS-101 (B98)	−	−	−	+	+	+
19	Spain psorosis P-126 (B97)	+	+	−	+	+	+
20	Spain ringspot (B88)	+	+	−	−	+++	++
21	Florida ringspot-4	+	+	−	+	+++	+++
22	Spain impietratura I-501 (B103)	−	−	−	−	−	−

[a] Beltsville accession numbers in parentheses.
[b] As detected by SDS-PAGE analysis of top (T) and bottom (B) components purified by sucrose density gradient centrifugation and agarose gel electrophoresis.
[c] Number of local lesions per leaf inoculated with sucrose density gradient centrifugation fractions containing top (T) and bottom (B) components, a mixture of T and B, or a crude extract. − = No lesions, + = 1–4 lesions, ++ = 5–14 lesions, +++ = 15–29 lesions, and ++++ = more than 30 lesions per leaf.

RESULTS AND DISCUSSION

Psorosis, ringspot, concave gum, cristacortis, and impietratura of citrus are caused by graft-transmissible agents that have not been characterized, and the identification of these diseases has been based solely on symptoms (8,14). The disease isolates used in this study were collected and named on the basis of symptoms and propagated by graft transmission. Except for the two Florida CRSV isolates (which had been subjected to single-lesion transfers on *C. quinoa* and passed through *Gomphrena globosa* L. and back to citrus) (5), no attempt was made to biologically purify the infectious agents associated with the disease symptoms. What is known about the biological characteristics of the various isolates is given in the Material and Methods section. Other than testing for mechanical transmission to *C. quinoa*, no further biological characterizations of the isolates were done in this study.

All 14 psorosis and ringspot isolates produced local lesions on *C. quinoa* (Table 1). There were differences in lesion numbers produced by different isolates that may be attributable to strain and titer differences. Recovery of infectivity by mixing top and bottom components after sucrose density gradient centrifugation, which is characteristic of CRSV-4 (2), was demonstrated for several of the psorosis and ringspot isolates (Table 1). The partial purifications reported here were done with ice bath temperatures when possible, but it was necessary to collect the gradient fractions at room temperature. The infectivity of CRSV is not stable, and better recovery of infec-

tivity after gradient centrifugation would probably have been obtained if the experiments could have been done in a cold (4 C) room, as was the original work with CRSV-4. The low levels of infectivity associated with bottom components (samples 17, 18, 19, and 21) are probably attributable to incomplete separation of the infectious components. Because the gradient fractions were collected by pushing from the bottom of the tube, the bottom component fractions tend to be contaminated with top components.

The 48-kDa protein, previously associated with CRSV-4 (2), was readily detected in the top and bottom components of all of the psorosis and ringspot isolates except sample 18 (Table 1). Typical SDS-PAGE assays are shown in Figure 1. Ringspot sample 18 induced very few lesions on *C. quinoa*, suggesting the failure to detect the 48-kDa protein could be attributable to low virus titer, but additional examinations of this isolate must be done before any conclusions can be drawn. One isolate, designated as concave gum when collected (sample 11), was very infectious on *C. quinoa* and contained the 48-kDa protein. In subsequent indexing, the isolate was positive for psorosis and is considered to be a mixed infection. The other isolate designated as concave gum (sample 16) produced a few lesions on *C. quinoa* but was negative for the 48-kDa protein. Obviously, additional isolates of concave gum need to be examined to determine if the lesions we observed are attributable to a mixed infection or if the agent causing concave gum is mechanically transmitted to *C. quinoa*. Healthy grapefruit (sample 15) and isolates of cristacortis (sample 12) and impietratura (sample 22) did not cause any local lesions on *C. quinoa* and did not contain the 48-kDa protein. Infectivity was not recovered from symptomless young leaves from plants infected with CRSV-4 (sample 14), which were also negative for the 48-kDa protein. This is consistent with the report that transmission of CRSV from symptomless portions of infected plants by mechanical means or by grafting has commonly been unsuccessful (9).

The filamentous particles associated with CRSV were first observed by SSEM with an antiserum prepared to a partially purified preparation (2). This serum has a strong reaction to host components, which restricts its use to SSEM assays. The antiserum produced in this study has a limited host reaction and readily detects the 48-kDa protein in western blots (Fig. 2) and filamentous particles by SSEM (Fig. 3). We take this as further evidence that the 48-kDa protein is a capsid protein.

In direct comparisons of the two Florida isolates, CRSV-6 always produced fewer and slower developing lesions on *C. quinoa* than CRSV-4. As shown here, both isolates contain the 48-kDa protein, and the unusual virus particles associated with CRSV-4 (Fig. 3) are observed in SSEM assays of CRSV-6 (*data not shown*). Comparable preparations of CRSV-4 and CRSV-6 appear to contain approximately equivalent amounts of the 48-kDa protein, but in SSEM assays (using antiserum to CRSV-4), more particles are seen with isolate CRSV-4 than with CRSV-6. This may indicate that the two isolates are not serologically identical. They also differ in that CRSV-4 has never induced significant bark scaling (5). In contrast, CRSV-6 produces all of the symptoms associated with psorosis or ringspot, including bark scaling and leaf flecking, blotches, and ring spots (5). Therefore, CRSV-6 is the first isolate that produces all of the classical symptoms of psorosis to be associated with a specific virus. Because CRSV-6 was biologically purified through local lesions (5), the possibility that it is a mixture of two or more pathogens is significantly decreased.

Considering that different workers have identified and collected the diseases used in this study, there is good agreement in the designation of a disease as psorosis or ringspot, the production of lesions on *C. quinoa*, and the presence of the 48-kDa protein. In addition, the infectivity associated with a number of the psorosis and ringspot isolates was separated into two components by sucrose density gradient centrifugation, which is characteristic of CRSV-4. This suggests that psorosis and ringspot found in various parts of the world are caused by a virus similar or identical to CRSV-4 and is consistent with previous suggestions that psorosis and ringspot are similar (14). There are obviously differ-

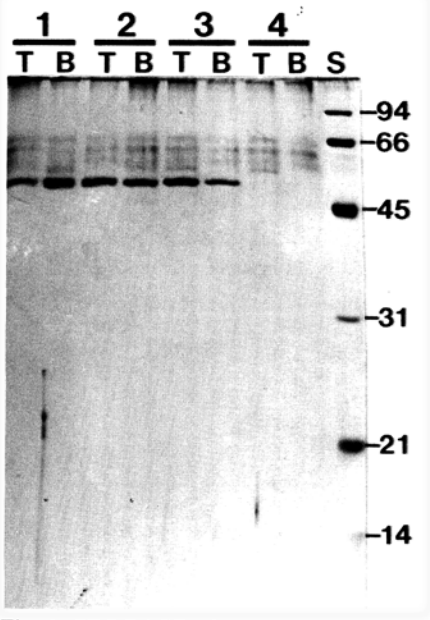

Fig. 1. SDS-PAGE of top (T) and bottom (B) components of citrus ringspot virus (CRSV) purified by sucrose density gradient centrifugation and agarose gel electrophoresis. Lanes 1–4 are of virus isolates B95, B96, B93, and B101, respectively. Lane 5 is marker proteins (molecular masses in parenthesis) phosphorylase B (94,000), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), and soybean trypsin inhibitor (21,000).

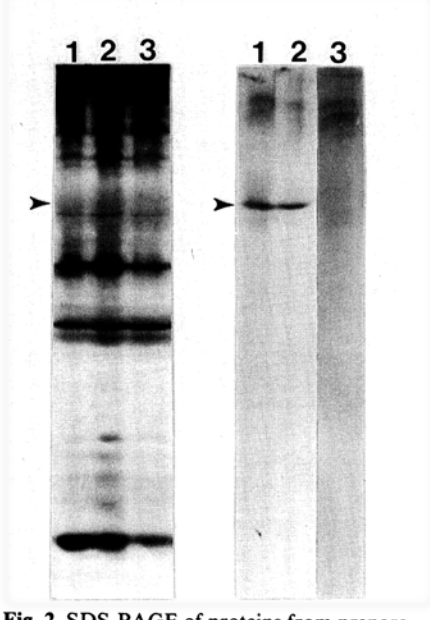

Fig. 2. SDS-PAGE of proteins from preparations from Florida CRSV-4 (lane 1) and Spain R5-1O5 (lane 2) infected and healthy (lane 3) leaves. Left, stained with silver nitrate. The 48-kDa CRSV protein is not visible. Right, western blot immunostained with antiserum to CRSV. The position of the 48-kDa CRSV protein is marked with arrows.

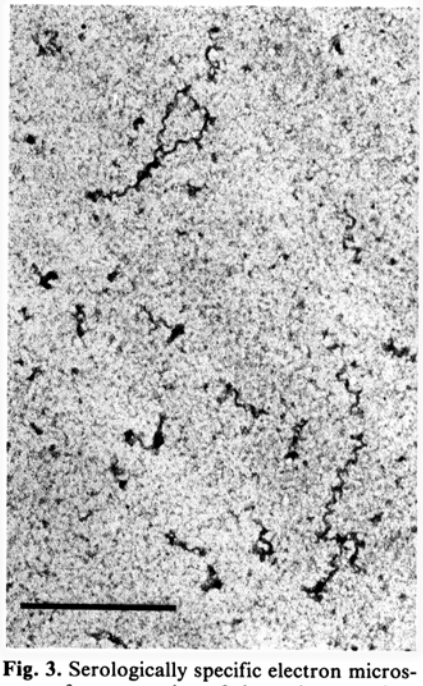

Fig. 3. Serologically specific electron microscopy of a preparation of citrus ringspot virus (CRSV-4) using antiserum to CRSV-4. Scale bar = 500 nm.

ences in the severity of some of the isolates that could be attributable to the existence of severe and mild strains of the virus.

The failure to detect the 48-kDa protein in isolates of impietratura, concave gum (when not obviously coinfected with psorosis), and cristacortis suggests these diseases are not caused by a virus related to CRSV-4. This is consistent with observations that these three diseases are readily differentiated from psorosis and ringspot by symptoms (14).

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LITERATURE CITED

1. da Graca, J. V., Derrick, K. S., Lee, R. F., Moreno, P., and Civerolo, E. L. 1988. Comparative study on several citrus psorosis and ringspot isolates. (Abstr.) Phytophylactica 20:101.
2. Derrick, K. S., Brlansky, R. H., da Graca, J. V., Lee, R. F., Timmer, L. W., and Nguyen, T. K. 1988. Partial characterization of a virus associated with citrus ringspot. Phytopathology 78:1298-1301.
3. Duran-Vila, N., Cambra, M., Pina, J. A., Ballester, J. F., and Navarro, L. 1988. Virus content and growth patterns of callus cultured in vitro from healthy and virus-infected citrus species. Pages 310-321 in: Proc. Conf. Int. Organ. Citrus Virol., 10th. L. W. Timmer, S. M. Garnsey, and L. Navarro, eds.
4. Fawcett, H. S. 1933. New symptoms of psorosis, indicating a virus disease of citrus. (Abstr.) Phytopathology 23:930.
5. Garnsey, S. M., and Timmer, L. W. 1988. Local lesion isolate of citrus ringspot virus induces psorosis bark scaling. Pages 334-339 in: Proc. Conf. Int. Organ. Citrus Virol., 10th. L. W. Timmer, S. M. Garnsey, and L. Navarro, eds.
6. Navarro, L., Juarez, J., Ballester, J. F., and Pina, J. A. 1980. Elimination of some citrus pathogens producing psorosis-like leaf symptoms by shoot-tip grafting in vitro. Pages 162-165 in: Proc. Conf. Int. Organ. Citrus Virol., 8th. E. C. Calavan, S. M. Garnsey, and L. W. Timmer, eds.
7. Swingle, W. T., and Webber, H. J. 1896. The principle diseases of citrus fruits in Florida. U.S. Dep. Agric. Div. Veg. Physiol. Pathol. Bull. 8. 42 pp.
8. Timmer, L. W., and Benatena, H. N. 1977. Comparison of psorosis and other viruses causing leaf flecking in citrus. Proc. Int. Soc. Citric. 3:930-935.
9. Timmer, L. W., and Garnsey, S. M. 1979. Variation in the distribution of citrus ringspot and psorosis viruses within citrus hosts. Phytopathology 69:200-203.
10. Timmer, L. W., and Garnsey, S. M. 1979. Natural spread of citrus ringspot virus in Texas and its association with psorosis-like diseases in Florida and Texas. Pages 167-171 in: Proc. Conf. Int. Organ. Citrus Virol., 8th. E. C. Calavan, S. M. Garnsey, and L. W. Timmer, eds.
11. Timmer, L. W., Garnsey, S. M., and McKitchie, J. J. 1978. Comparative symptomatology of Florida and Texas isolates of citrus ringspot virus on citrus and herbaceous plants. Plant Dis. Rep. 62:1054-1058.
12. Wallace, J. M. 1978. Virus and viruslike diseases. Pages 67-184 in: The Citrus Industry, Vol IV. W. Reuther, E. C. Calavan, and G. E. Carman, eds. University of California, Berkeley.
13. Wallace, J. M., and Drake, R. J. 1968. Citrange stunt and ringspot, two previously undescribed virus diseases of citrus. Pages 177-183 in: Proc. Conf. Int. Organ. Citrus Virol., 4th. J. F. L. Childs, ed.
14. Whiteside, J. O., Garnsey, S. M., and Timmer, L. W. 1988. Compendium of Citrus Diseases. American Phytopathological Society, St. Paul, MN. 88 pp.