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Short communication

Filamentous flexuous particles and serologically related proteins of variable size associated with citrus psorosis and ringspot diseases

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Abstract

Filamentous flexuous particles of unusual morphology, previously associated with several ringspot isolates, were detected also in psorosis A and psorosis B isolates by serologically specific electron microscopy using an antiserum to citrus ringspot. Upon partial purification of six ringspot, six psorosis A, and three psorosis B isolates, a specific protein of 47 kDa was detected in most cases, but two isolates (one psorosis A and one ringspot) had a 46 and a 48 kDa-protein, respectively. These differences in molecular masses were observed when purification was done from different host species or from plants co-inoculated with two isolates differing by their protein size. The three types of protein were serologically related in Western blots. Our results indicate that a common virus with different strains may be involved in psorosis A, psorosis B, and ringspot diseases.

The psorosis group of diseases include some of the most widespread graft-transmissible disorders of citrus [Wallace, 1978; Whiteside *et al.*, 1988], but the etiology of none of these diseases has yet been established. Psorosis A is characterized by causing bark scaling in trunk and limbs of infected field trees, and inducing a shock reaction with leaf shedding and necrosis in the first shoot of young seedlings inoculated in the greenhouse [Roistacher, 1991]. Psorosis B causes in addition rampant scaling of thin branches in field trees, chlorotic blotches in old leaves with gummy pustules in the underside, and blisters in young shoots of inoculated seedlings [Wallace, 1978; Whiteside *et al.*, 1988]. Ringspot, as originally described by Wallace and Drake [1968], was characterized by the presence of chlorotic blotches and rings in the old leaves of inoculated indicator seedlings, whereas infected field trees did not show any specific symptom. Further descriptions of diseases similar to ringspot in different citrus areas included leaf, trunk and fruit symptoms, not observed in the original isolate [Timmer and Beñatena, 1977; Whiteside *et al.*, 1988].

Various isolates of ringspot, psorosis A, and psorosis B have been mechanically transmitted to *Chenopodium quinoa* Willd. [Garnsey and Timmer, 1980, 1988; Da Graça *et al.*, 1991], and infectivity on this host has been associated with two components separable in a sucrose density gradient [Derrick *et al.*, 1988; Da Graça *et al.*, 1991; García *et al.*, 1991; Navas-Castillo *et al.*, 1991, 1993]. Long flexuous particles 300–500 and 1500–2500 nm, respectively, were found in the top and bottom components purified from ringspot CRSV-4 [Derrick *et al.*, 1988], and later from two additional ringspot isolates [Da Graça *et al.*, 1991; Navas-Castillo *et al.*, 1993]. Most of the psorosis and ringspot isolates studied contained in both components a unique protein of 48 kDa, not present in similar extracts from healthy plants and suggested to be the capsid protein of the virus. In contrast, Levy and Gumpf [1991] found rod-shaped particles 660–665 nm long and a 29 kDa

protein associated with a Californian psorosis isolate (Ps 203m).

Here we report detection of filamentous flexuous particles in psorosis A and B isolates, and the presence of serologically related proteins of variable size in isolates of citrus psorosis and ringspot.

The six ringspot (RS-105, RS-ALC, RS-SOR, RS-INV, RS-CV, and RS-SR), six psorosis A (P-121, P-123, P-126, P-137, P-138, and P-ALM), and three psorosis B (PB-102, PB-108, and PB-143) isolates used in this study are part of a virus collection maintained at the Instituto Valenciano de Investigaciones Agrarias (IVIA, Moncada, Valencia, Spain). Data available on biological characteristics of these isolates have been published [Navarro *et al.*, 1980; Duran-Vila *et al.*, 1988; Da Graça *et al.*, 1991; Navas-Castillo, 1991; Navas-Castillo *et al.*, 1991; Navas-Castillo and Moreno, 1993]. All the isolates but P-123 and P-126 were mechanically transmitted to *C. quinoa* under conditions previously described [Navas-Castillo *et al.*, 1993].

Serologically specific electron microscopy (SSEM) was performed with isolates RS-SR, P-121, RS-SOR and PB-108, using the UFI antiserum, raised in rabbit against the CRSV-4 ringspot from Florida [Da Graça *et al.*, 1991], under conditions previously described [Navas-Castillo *et al.*, 1993]. Filmed grids were coated with protein A and the UFl antiserum, and then floated on drops of crude extracts from *C. quinoa* local lesions. The grids were positively stained with 2% (w/v) uranyl acetate in bidest and observed with a JEM 100-S electron microscope. Flexuous particles about 1,000 nm long were found associated with the four isolates. Figure 1 shows some selected particles observed in preparations from P-121. Particles were not observed in similar preparations from uninoculated *C. quinoa* leaves, healthy or infected citrus, or any of the grids prepared with extracts from local lesions using no serum, or pre-immune serum. The observed particles had the same length and morphology as those associated with ringspot RS-SR [Navas-Castillo *et al.*, 1993], which was included as control. This is the first time that flexuous particles have been observed in plants infected with psorosis. P-121, is a previously characterized psorosis A isolate [Navarro *et al.*, 1980; Duran-Vila *et al.*, 1988], and PB-108, is a psorosis B isolate currently used at the IVIA as challenge inoculum for cross protection tests [Roistacher, 1991; Navas-Castillo and Moreno, 1993].

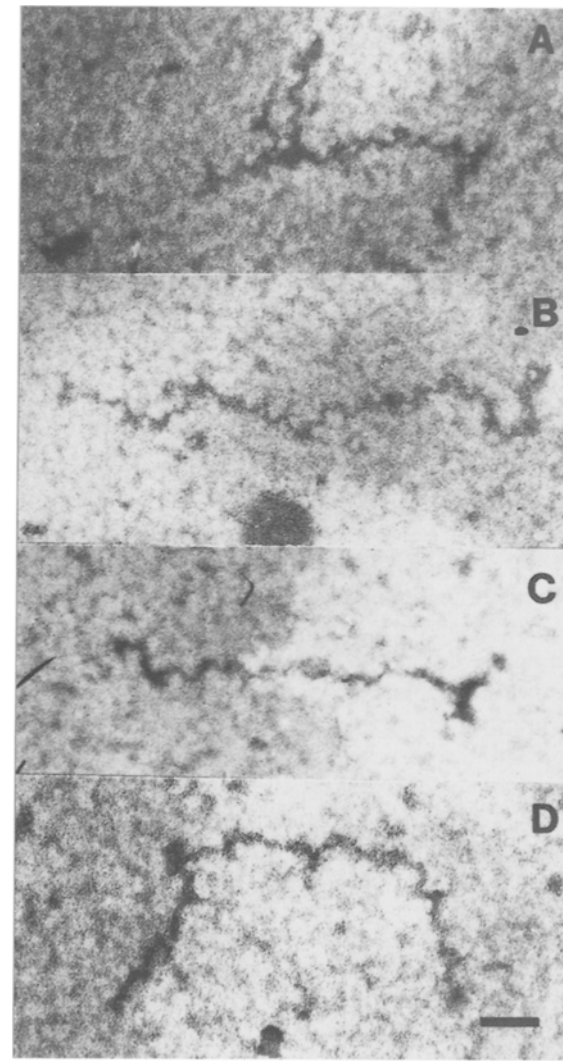

Fig. 1. Selected flexuous particles associated with the psorosis isolate P-121. Particles were observed in crude extracts from *C. quinoa* local lesions by serologically specific electron microscopy using the UFl antiserum and staining with 2% uranyl acetate. The bar represents 100 nm.

Similarity of the symptoms induced by psorosis A, psorosis B, and most ringspot isolates in citrus and in *C. quinoa* [Navas-Castillo and Moreno, 1993], and the unusual morphology of the particles detected by SSEM with the same antiserum in isolates of the three diseases, strongly suggest

that these particles are a common virus causing citrus psorosis and ringspot. The flexuous rod-shaped particles (660–665 nm) observed by Levy and Gumpf [1991], with a 29 kDa putative capsid protein, might be a different virus associated in mixed infection with psorosis isolate Ps 203m. Similar rod-shaped particles have been observed by Garnsey [1975] and by us [unpublished results] in citrus cultivars free of psorosis and ringspot.

Purification experiments were performed starting from young symptomatic leaves and shoots of citrus plants, or *C. quinoa* local lesions, following the procedure of Derrick *et al.* [1988] with minor variations [Navas-Castillo *et al.*, 1993]. Briefly, symptomatic tissue (4–8 g) was extracted with TACM buffer (50 mM Tris-HCl, 0.1% ascorbic acid, 0.1% L-cysteine, 0.5% 2-mercaptoethanol, pH 8.0) and the extracts clarified with trichlorotrifluoroethane (Freon 113) and low speed centrifugation, concentrated by high-speed centrifugation, and then fractionated in a linear sucrose gradient (10–40% sucrose in TACM). The selected fractions were concentrated by high-speed centrifugation and the pellets resuspended in 50 µl of TAE (40 mM Tris-acetate, 2 mM EDTA, pH 7.5) and electrophoresed in 0.5% agarose gels in TAE at 50 V for 4 h. The first 1-cm segments of the agarose gels were analyzed for protein content by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the gels stained with silver nitrate. Molecular weight of the proteins was estimated using the multiplicative method of the Statgraphics program (STSC, Inc., and Statistical Graphics Corporation) and the relative electrophoretic mobilities of the following markers (from Bio-Rad Laboratories, Hercules, CA.): Rabbit muscle phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; hen egg white ovalbumin, 45.0 kDa; bovine carbonic anhydrase, 31.0 kDa; soybean trypsin inhibitor, 21.5 kDa; and hen egg white lysozyme, 14.4 kDa. Serological detection of the specific protein associated with psorosis and ringspot isolates was done by electroblotting proteins separated by SDS-PAGE onto polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore Corporation, Bedford, MA.), and reacting them with UFl or IVIA antisera, as previously described [Navas-Castillo *et al.*, 1993].

When the proteins associated with the 15 isolates studied were compared for electrophoretic mobilities, a molecular mass of 47 kDa was estimated for 13 of them; but 2 isolates, P-126 and RS-SR, had faster (46 kDa) and slower (48 kDa) migrating proteins, respectively (Fig. 2). The difference in electrophoretic mobility between the protein associated with these isolates was consistently observed in many purification experiments using tissue from Duncan grapefruit (*C. paradisi* Macf.), Mexican lime (*C. aurantifolia* (Christm.) Swing.), Washington Navel, Madam Vinous and Pineapple sweet orange (*C. sinensis* (L.) Osb.), sour orange (*C. aurantium* L.), rough lemon (*C. jambhiri* Lush.), Dweet tangor (*C. reticulata* Blanco × *C. sinensis* (L.) Osb.) or Etrog citron (*C. medica* L.) Arizona 861-S-1, indistinctly. The molecular mass of the protein associated with isolates RS-SR (48 kDa) and PB-108 (47 kDa) also remained unchanged when estimated in

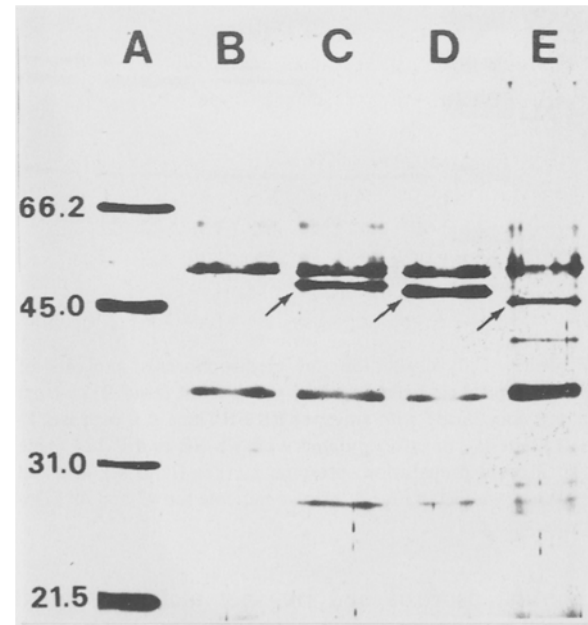

Fig. 2. Polyacrylamide gel electrophoresis (12%) analysis of proteins associated with ringspot RS-SR (lane C) psorosis P-121 (lane D), and psorosis P-126 (lane E) isolates. Concentrated extracts from symptomatic citrus leaves and shoots were fractionated by sucrose density gradient centrifugation and further purified by agarose gel electrophoresis. Proteins contained in the first 1-cm segment of the agarose gel were separated by polyacrylamide gel electrophoresis and stained with silver nitrate. Lane B, healthy control. Lane A, marker proteins: bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhidrase (31,000), and soybean trypsin inhibitor (21,000). Arrows indicate the 48, 47 and 46 kDa-proteins.

preparations obtained from symptomatic *C. quinoa* leaves. The only isolate containing a 46 kDa protein (P-126) could not be transmitted to *C. quinoa*.

The protein associated with isolates RS-SR and P-121 was purified from grapefruit seedlings inoculated with both isolates by either simultaneous co-inoculation, or inoculation with RS-SR or P-121 and, after symptom observation, re-inoculation with P-121 or RS-SR, respectively. All co-inoculated plants contained both the 48 and the 47 kDa-protein (Fig. 3), whereas plants inoculated with a single isolate always contained a unique protein band.

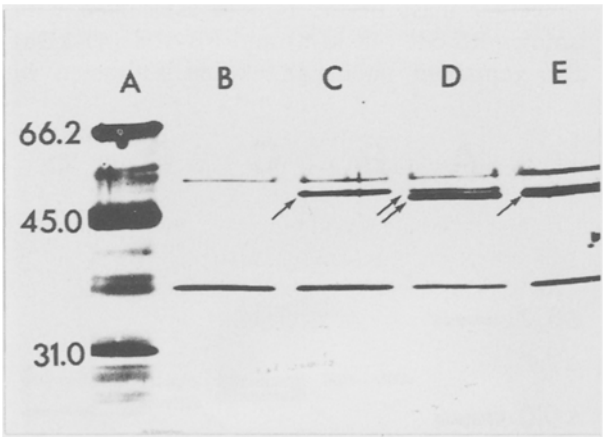

Fig. 3. Polyacrylamide gel electrophoresis analysis of proteins purified from a healthy citrus plant (land B) or from plants inoculated with ringspot RS-SR (lane C), psorosis P-121 (lane E), or co-inoculated with RS-SR and P-121 (lane D). Protein preparation, proteins marker (lane A), and gel staining were as in Fig. 2. Arrows indicate the 47 and 48 kDa-proteins.

Most psorosis and ringspot isolates studied have been reported with a 48 kDa-protein [Derrick *et al.*, 1988; Da Graça *et al.*, 1991], whereas García *et al.* [1991] claimed a 50 kDa protein with an Argentinian psorosis isolate. The size variation between the 47 kDa-protein detected in most of our isolates and the 50 and 48 kDa-protein detected in Argentina [García *et al.*, 1991] and Florida [Derrick *et al.*, 1988; Da Graça *et al.*, 1991], respectively, might be only apparent and due to minor differences in the tissue condition, or in the purification and/or molecular mass estimation procedures used in different laboratories. Indeed when purified extracts from P-121 and RS-SR obtained in our laboratory (with 47 and 48 kDa proteins, respectively, in our estimation) were electrophoresed by Dr K.S. Derrick (University of Florida, C.R.E.C., Lake Alfred) and compared with an extract from CRSV-4 obtained in his laboratory, proteins from P-121 and CRSV-4 had the same migration, whereas protein from RS-SR migrated slower. Variation in the molecular mass of viral proteins depending on the physiological condition of the infected plant or on changes in the purification procedure has been reported [Brakke *et al.*, 1990; Guerri *et al.*, 1990].

RS-SR and P-126 are the first examples of psorosis-ringspot that, upon purification and electrophoresis under the same conditions, have been proven to contain a protein of molecular size differing from other known isolates. The fact that the 46 kDa-protein appeared only in plants infected with P-126, and that purified extracts from plants infected with RS-SR never contained protein 47 or 46 kDa bands, indicates that these are not degradation artifacts. Since differences in molecular mass were observed in extracts purified from several citrus hosts, and in the case of RS-SR also from *C. quinoa*, the variable size of these proteins cannot be considered an artifact due to a host effect. This point is also supported by the co-inoculation experiments with isolates P-121 and RS-SR in which both proteins were detected in the same plant. These experiments also showed that replication of both pathogens apparently do not interfere with each other. Additional experiments on the possible interference between P-126, RS-SR and other isolates are under course.

The 48 kDa-protein associated with RS-SR and the 47 kDa-protein associated with P-121 reacted in Western blot with the IVIA antiserum (obtained to RS-SR) (Fig. 4.1). The 46 KDa-protein associated with psorosis P-126 was discovered after the IVIA antiserum was exhausted and therefore its reactivity with this antiserum could not be assayed. The three proteins reacted in Western blot with the UFl antiserum (Fig. 4.2). The 46 KDa-protein was always in lower concentration than the 47 or 48 kDa-proteins, and its detection by immunostaining of Western blots usually required longer incubation periods. The presence of a background reaction with host proteins made other immunoenzymatic procedures

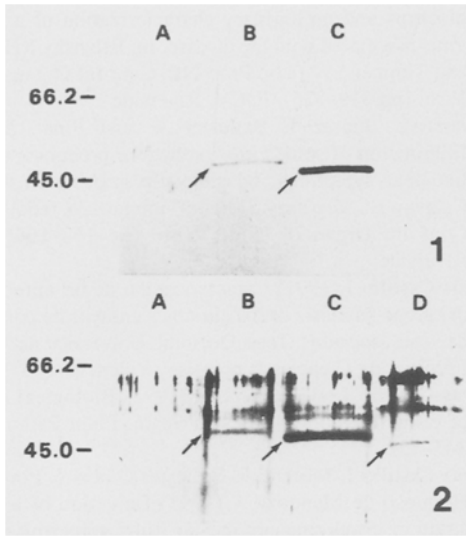

Fig. 4. Western blot analysis of proteins purified from citrus plants healthy (lanes A), or infected with ringspot RS-SR (lanes B), psorosis P-121 (lanes C), or psorosis P-126 (lane D), as indicated in Fig. 2. The proteins separated by polyacrylamide gel electrophoresis were electroblotted to a PVDF membrane, reacted with the IVIA (1) or with the UFl (2) antiserum, and were visualized by the nitro blue tetrazolium method. Arrows indicate the 48, 47 and 46 kDa-proteins.

[Navas-Castillo *et al.*, 1993] not adequate for these studies.

In addition to the three type of proteins detected in this work, the antiserum UFl has been reported to react with a number of psorosis and ringspot isolates of several origins [Da Graça *et al.*, 1991, 1993]. This indicates that the specific protein associated with psorosis and ringspot diseases, independent of variations in molecular size, are serologically related. This relationship supports the idea that such protein must be the capsid protein of a virus involved in both diseases. This idea is further supported by the fact that antiserum UFl attached particles from psorosis A, psorosis B, and ringspot lesion extracts, in SSEM grids, whereas particles could not be detected in the same extracts using grids uncoated or coated with a preimmune serum. Particles were observed in isolates containing the 47 kDa-protein (psorosis A and B, and ringspot) or the 48 kDa-protein (ringspot RS-SR), but not the 46 kDa protein, as the only isolate containing this protein could not be transmitted to *C. quinoa*.

It is remarkable that the antiserum UFl attached numerous particles of the homologous isolate CRSV-4 [Derrick *et al.*, 1988] in SSEM grids, but only scattered particles of the other isolates assayed i.e. CRSV-6 [Da Graça *et al.*, 1991], RS-SR, RS-SOR, P-121, and PB-108. Two reasons might explain this difference. i) CRSV-4 might produce higher concentration of virus particles in infected plants, and/or ii) though some epitopes are shared, serological differences between CRSV-4 and the other isolates assayed enabled attachment of only a limited number of heterologous particles to the grids. The fact that similar amount of protein was usually purified from plants infected with CRSV-4 or with CRSV-6 [Da Graça *et al.*, 1991] and the recent finding [Derrick *et al.*, 1993] that a monoclonal antibody obtained to CRSV-4 does not react with isolate CRSV-6 seems to favor the second hypothesis.

Biological diversity observed among isolates [Navas-Castillo, 1991; Navas-Castillo and Moreno, 1993], differences in the number of particles detected by SSEM, and size variation of the putative coat protein, strongly suggest that different virus strains might be involved in the psorosis-ringspot diseases.

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