The closely related citrus ringspot and citrus psorosis viruses have particles of novel filamentous morphology

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Some properties of the particles of citrus ringspot virus (CtRSV) and the related citrus psorosis-associated virus (CPsAV) are described. The particles of CtRSV have been reported to be sinuous linear structures about 10 nm in diameter and of two lengths, 300 to 500 nm and 1500 to 2500 nm representing ‘top’ and ‘bottom’ sedimentation components. We show that these particles are collapsed double-stranded forms of nucleocapsid-like, highly flexuous open circles formed of filaments 3 to 4 nm in diameter. Top-component filaments had contour lengths of 600 to 1000 nm, i.e. twice that reported for the corresponding collapsed form. Bottom-component filaments had contour lengths about four times longer than those of top-component filaments. The structures suggest that CtRSV represents a new genus (possibly family) related to the tenuiviruses. However, we failed to demonstrate any serological relationship between CtRSV and several tenuiviruses; moreover, the capsid protein sizes and host ranges are quite different. We offer the name Ophiovirus for the proposed new genus.

Symptoms of ringspot and psorosis in citrus have been described worldwide (Whiteside et al., 1988), and may be associated with the presence of citrus ringspot virus (CtRSV) (Navas-Castillo & Moreno, 1993). The virus is reported to have highly sinuous particles about 10 nm in diameter and of two lengths, 300 to 500 nm and 1500 to 2500 nm (Derrick et al., 1988, 1991; da Graca et al., 1991; Navas-Castillo et al., 1993). In sucrose density gradients CtRSV particles sediment as at least two components, ‘top’ and ‘bottom’, (the short and long particles respectively); top and bottom components, not infective separately, become infective when mixed (Derrick et al., 1988, 1991; da Graca et al., 1991; Navas-Castillo et al., 1993). In sucrose density gradients CtRSV particles sediment as at least two components, ‘top’ and ‘bottom’, (the short and long particles respectively); top and bottom components, not infective separately, become infective when mixed (Derrick et al., 1988, 1991). Partially purified preparations of CtRSV contain ssRNA and also dsRNA, and a 48K protein common to both components, probably the coat protein (Derrick et al., 1991).

Here we show that the sinuous particles previously described are one morphological form of circular filamentous particles 3 to 4 nm in thickness, and of different lengths, similar to those of tenuiviruses (Espinoza et al., 1992; Francki et al., 1985; Gingery, 1988; Ramírez & Haenni, 1994).

The virus isolates used were CtRSV-4 (Garnsey & Timmer, 1980; courtesy of K.S. Derrick and L.W. Timmer, University of Florida, Lake Alfred, Fla., U.S.A.), and the related citrus psorosis-associated virus (CpsAV), isolate 90-1-1, from Argentina (García et al., 1991a). Both viruses were mechanically inoculated from Citrus limon, C. jambhiri or C. sinensis to Chenopodium quinoa using young leaves homogenized in TACM buffer (50 mM-Tris, 0·1% ascorbic acid, 0·1% L-cysteine, 0·5% 2-mercaptoethanol, adjusted to pH 8 with HCl; Derrick et al., 1988). The local lesions obtained, and also young systemically infected citrus leaves, were used as sources of virus, and non-inoculated leaves were used as healthy controls.

Both viruses were partially purified from C. quinoa or citrus, followed the protocol of García et al. (1991b), modified from Derrick et al. (1988). Briefly, tissue was homogenized in TACM buffer and, after differential centrifugation, resuspended pellet material was centrifuged into a sucrose density gradient. Top and bottom fractions were collected, and each was concentrated by ultracentrifugation. The pellets were resuspended in 50 mM-Tris–HCl, pH 8·0, and samples examined on glow-discharged grids by negative staining in 1% uranyl acetate, and electron microscopy (Milne, 1993a).

The only structures (here called type A) that distinguished infected from healthy preparations were those illustrated in Fig. 1 (a, b), except that occasionally, on some grids, type B structures (Fig. 1c and see below) were also seen. Small type A structures were found in the top fraction, and large ones plus some small ones in the bottom fraction. The same results were obtained whether the starting material was citrus or C. quinoa and whether the virus was CtRSV or CpsAV, except that much lower numbers of the structures were found in CpsAV extracts. Similar results were obtained when pellets were resuspen-
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Fig. 1. CtRSV particles from partially purified preparations, negatively stained in uranyl acetate. (a) 'Small' and (b) 'large' type A particles from top and bottom sucrose density gradient fractions respectively. Arrows indicate subunits repeating every 3 nm along the nucleocapsid. (c) Type B particles from an unfractionated partially purified CtRSV preparation; note the double filamentous structure, and a looped end (arrowhead). Bars, 100 nm.

Both small and large structures were composed of kinked, apparently highly twisted filaments about 3 nm in diameter, with beadlike units repeating along their length every 3 nm (Fig. 1b); (artifactual background grains caused by defocus were about 1 nm in diameter in these micrographs). A likely interpretation of these units is that they represented monomers of the 48K coat protein. Most of the filaments appeared to have continuous contours with no free ends, and this was especially clear on some of the small filaments. When the contour lengths of 20 typical small filaments were measured, the mean was 760 ± 100 nm. We were not able to measure contour lengths of the large filaments, but these were estimated to be approximately four times longer.

When pellets from CtRSV bottom fractions were resuspended in TACM buffer and negatively stained, kinked 3 nm filaments were sometimes replaced by type B structures (Fig. 1c). These particles, about 9 nm wide, appeared to be homologous with those, positively stained, described by Derrick et al. (1988; and later papers cited above). However, in negative stain it became clear that the particles were composed of pairs of filaments each about 3 nm wide (Fig. 1c), homologous with the open circular type A filaments. On some type B particles, the ends were seen to be formed of small loops (Fig. 1c) and sometimes we observed branching, also indicating a finer filament looping back on itself.

Type A and occasionally type B particles were seen when crude preparations of citrus or C. quinoa leaves infected with CtRSV or CPsAV were examined by simple negative staining. When the immunotrapping protocol of Derrick et al. (1988) was followed (except that the grids were negatively stained) using crude preparations of CtRSV from citrus or C. quinoa leaves in 50 mM-Tris–HCl, pH 7.2, and a CtRSV antiserum (Derrick et al., 1988; courtesy of K. S. Derrick), we saw type B structures and also forms approaching type A. When grids coated for 20 min at room temperature with CtRSV antiserum diluted 1/1000 (without use of protein A) were incubated for 2 h with crude preparations of CtRSV or
CPsAV, particles were also successfully trapped, and were of type B or showed transition to type A. In all these experiments we saw that the A and B structures were alternative forms of the same basic structure; however, we were not able, by manipulating buffer type, molarity and pH, or EDTA, Mg$^{2+}$ or Ca$^{2+}$ concentrations, to convert one into the other consistently.

Partially purified CtRSV preparations were further purified by electrophoresis on agarose gels (Derrick et al., 1988; Garcia et al., 1991b). Consecutive 5 mm slices of the gels (from the slot toward the anode) were then tested by electron microscopy and by SDS–PAGE.

With top component preparations, the second fraction was seen to contain only purified small type A structures (in abundance), and with bottom component the first fraction appeared to contain only purified large type A structures (comparatively few). Other fractions contained no structures visible by negative staining.

Equivalent gel slices were melted at 100 °C in 60 mm-Tris–HCl, pH 6.8, containing 2% SDS, 10% glycerol, 5% mercaptoethanol and 0.001% bromophenol blue, and samples were electrophoresed on 14% SDS–polyacrylamide mini-slab gels as described by Conejero & Semancik (1977), then silver stained. Unstained gels

Fig. 2. (a) SDS–PAGE of proteins present in the second 5 mm slice of agarose gels after electrophoresis of top components of CPsAV and CtRSV, and parallel healthy preparations. Silver stain. Lanes are: P, CPsAV; CR, CtRSV contaminated with rubisco; C, CtRSV; H, healthy material; PR, CPsAV contaminated with rubisco. Sizes of the molecular mass markers are indicated. Arrows indicate the rubisco (large subunit) bands, identified as below. (b) Western blots of gels equivalent to those in (a), probed using anti-CPsAV, anti-CtRSV and anti-rubisco antisera. Lanes are labelled as in (a). Note that in the left-hand and middle panels, CPsAV and CtRSV proteins both react but each reacts more strongly with its homologous antiserum. In the right-hand panel, the band migrating just above the CtRSV protein is identified as rubisco (large subunit).
were electroblotted onto nitrocellulose membranes using Bio-Rad mini-slab gel equipment following the manufacturer's instructions. The blots were probed with a rabbit antiserum against CtRSV (Derrick et al., 1988), a mouse antiserum prepared in the La Plata laboratory against CPsAV, a rabbit antiserum against Pisum sativum ribulose bisphosphate carboxylase (rubisco) large subunit (courtesy of M. Cambra, IVIA, Moncada, Valencia, Spain), and pre-immune rabbit and mouse sera. The sera were diluted 1/500.

With top-component fractions of CtRSV or CPsAV, bands were seen only in material from the second 5 mm slice; these were estimated as 48K in size for CtRSV and 50K for CPsAV; some preparations gave an extra band of about 52K (Fig. 2a). In Western blots, each of the virus antisera recognized both the homologous and heterologous virus proteins, although the homologous reactions were stronger, indicating that the antigens were related but not identical; the anti-rubisco serum reacted with the 52K band (Fig. 2b, third panel, right-hand lane).

With bottom-component fractions, similar but faint bands were seen in material from the first 5 mm slice. The bands may have been faint because the relatively large bottom-component structures did not migrate into the agarose gel in sufficient numbers.

The above results provided evidence that the type A structures observed in the gel slices were associated with proteins (48K and 50K) of appropriate size to be the coat proteins of CtRSV and CPsAV respectively (Derrick et al., 1991; García et al., 1991b), and that the additional band sometimes observed at 52K was formed by the large subunit of host-plant rubisco. The reaction of the 48K and 50K bands with their appropriate antisera is strong evidence that the type A structures seen were indeed virus particles, although they did not possess the morphology described in the literature.

As an additional proof that the type A particles were related to those of type B, we attempted to immunolabel partially purified bottom-component preparations of CtRSV, containing type A particles, using the CtRSV antiserum known to react with particles of type B (Derrick et al., 1988). After adsorption of the virus samples directly to grids, these were rinsed and blocked for 30 min with buffered 1% BSA and 0-1% Tween 20. After rinsing, grids were incubated with diluted antisera for 60 min, rinsed, and labelled for 20 min with goat anti-rabbit IgG conjugated to 5 nm gold (Amer sham); the grids were negatively stained with uranyl acetate (Milne, 1993b).

We observed that type A particles were indeed labelled with CtRSV antiserum but that host-derived structures were not labelled above the low background level; particles exposed to normal serum were unlabelled (Fig. 3). The optimal dilution of the antisera for maximal labelling with minimal background was 1/800.

CtRSV thus appears to be a multicomponent virus with circularized nucleocapsid-like particles. No envelope such as that of tospoviruses has been reported for CtRSV. The only group of viruses with unenveloped particles about 9 nm or alternatively 3 nm wide resembling the A and B particles of CtRSV is the tenuiviruses (Gingery, 1988; Ishakawa et al., 1989; Francki et al., 1991; Ramírez & Haenni, 1994). We therefore tested whether the capsid proteins of CtRSV and tenuiviruses might be serologically related.

The antigens used were partially purified top and bottom fractions of CtRSV combined, material from healthy citrus, and purified rice stripe virus (RSV) (courtesy of C. Toriyama, National Institute of Environmental Sciences, Tsukuba, Japan). The antisera used were to CtRSV, to RSV (courtesy of C. Toriyama), to rice hoja blanca virus (RHBV) and maize stripe virus (MSV) (courtesy of B. Falk, Department of Plant Pathology, University of California, Davis, Calif., U.S.A.), to RHBV (courtesy of F. Morales, CIAT, Colombia, and supplied by N. Nguyen and A.-L. Haenni, Institut Jacques Monod, Paris, France) and to MSV (courtesy P. Jones, Rothamsted Experimental Station, Harpenden, U.K.). In addition, we used a monoclonal antibody to CtRSV (courtesy of K. S. Derrick). The method used was PAGE followed by Western blotting; this appeared to be the most appropriate technique, as it is known to be able to detect distant relationships (Koenig & Burgermeister, 1986). The antibody preparations were tested at dilutions from 1/40 to 1/10000.

To summarize the results, the CtRSV 48K capsid protein band reacted with its homologous polyclonal and monoclonal antibodies but did not react with any of the tenuivirus antibodies. Likewise, the 32K RSV capsid protein band reacted strongly with its homologous antiserum, more weakly with the MSV and RHBV antisera, and gave no reaction with the CtRSV polyclonal and monoclonal antibodies. We conclude that, at least in these limited tests, there was no evidence of serological relationship between the capsid proteins of CtRSV and tenuiviruses.

These latter have hosts only within the Gramineae, are not mechanically transmissible, and possess coat proteins in the range of 31K to 34K. This suggests that CtRSV and related viruses could be classified near the tenuiviruses but separated from them at least at genus level. Evidence is accumulating (see, for example, Huiet et al., 1993; Ward, 1993; Ramírez & Haenni, 1994) that tenuiviruses are related to the Arenaviridae and Bunyaviridae, enveloped multisegmented negative-strand or ambisense RNA viruses that possess circularized nucleo-
capsids. The RNAs of the tenuiviruses, Bunyaviridae and Arenaviridae are circularized by means of complementary 5' and 3' terminal sequences that form a 'panhandle', and this suggests that upon analysis, the RNAs of CtRSV will be found to possess similar panhandle structures, and will prove to be of negative or ambisense polarity. The vectors, if any, of CtRSV and CPsAV are unknown but a further prediction is that the vectors will turn out to be arthropods, in which the viruses also multiply. This is suggested because the plant-infecting bunyaviruses and the tenuiviruses all have vectors of this type.

Derrick et al. (1992) have already suggested that CtRSV belongs to a new virus group, for which they have proposed the name 'Spirovirus'. However, this name refers to a particle assumed to be a linear structure, which we have shown to be only one somewhat misleading form of a more fundamental particle. Further, there already exists a genus of phages (family Microviridae) with the similar name 'Spiromicrovirus' (Francki et al., 1991). We therefore, alternatively, put forward the name 'Ophiovirus' for a proposed new genus with CtRSV as the type species. The Greek noun ophis means 'serpent', and here refers to the snake-like appearance of both the A and B particles.

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