

Structure of Citrus Ringspot-Psorosis-Associated Virus Particles: Implications for Diagnosis and Taxonomy

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ABSTRACT. Negative staining and electron microscopy has revealed particles of similar morphology in preparations of citrus and *Chenopodium quinoa* experimentally infected with several virus isolates associated with citrus ringspot and psorosis symptoms. The morphology is complex; the filamentous particles come in at least two sizes (contour lengths) and occur in an open circular form and a linear form, plus intermediates. The linear (or *L*) form may appear as a sinuous structure about 10 nm in diameter and up to 2,500 nm long; it is in reality a two-stranded structure with loops at the ends, formed from a collapsed circle. The open circular form (*O* form) is a highly kinked filament 3 to 4 nm in diameter. The morphology suggests that this type of virus represents a new genus of viruses similar to the Tenuiviruses and the Bunyaviridae; these possess an ambisense divided ssRNA genome, each RNA being circularized by means of a panhandle structure of complementary base sequences at the 5' and 3' termini. We propose the name 'Ophiovirus' (from the Greek *ophis*, meaning 'serpent') for this new genus, because the particles have a snaky appearance. The finding of morphologically indistinguishable particles in isolates associated with ringspot symptoms or with psorosis symptoms is further evidence that both types of symptom are associated with the same kind of virus.

The commercial not to say emotional rewards brought by the possession of a healthy citrus tree are set out in the following verse:

*Oh that I were an orange tree,
That busy plant!
Then I should ever laden be
And never want . . .*

The date of the poem (16) must have been around 1625, an age of innocence before many of the worst citrus diseases had made their debut.

More recently, but still for almost 100 years, a virus-like disease known as psorosis has been studied by symptomatology, use of indicator plants, cross-protection experiments and mechanical transmission (31). Symptoms of ringspot and psorosis in citrus are both associated with similar viruses, and a breakthrough occurred when Derrick and colleagues (6, 7) partially purified a virus associated with ringspot in Florida, here referred to as citrus ringspot virus (CtRSV). They produced an antiserum to it, and showed that the particles, trapped on antiserum-coated electron microscope grids, were of novel spiral fila-

mentous type, and of at least two length classes. They proposed the name "spirovirus" for this new group of viruses. They further showed that after density gradient centrifugation, infectivity was maximized by combining top and bottom fractions, and that a 48 K protein was associated with the virus.

Further work followed (3, 4, 8, 9, 23, 24, 25, 26, 27) on other psorosis and ringspot isolates from Florida, Spain and Argentina. Use of immunosorbent electron microscopy (ISEM, otherwise known as serologically specific electron microscopy, SSEM) and Western blotting confirmed the widespread presence of viruses similar to the original Florida isolate. Navas-Castillo (23) was the first to suggest that the virus particles resembled those of Tenuiviruses such as rice stripe virus (29). Meanwhile, García, Grau and colleagues (11, 12, 13, 14, 32) obtained results confirming that psorosis in Argentina could be caused by the same kind of virus as CtRSV.

Up until this point, ISEM and positive staining with uranyl acetate (UA), had been found to be the only

system able to reveal the particles in the EM. It was thought that "the high ionic strength involved in negative staining degrades the particles" (6). In continued work, however, no serious difficulty was experienced in seeing CtRSV particles in negative stain, or in seeing them in crude or partially purified preparations, without the aid of ISEM; it was clearly shown that the particles of CtRSV and of a psorosis isolate were indeed similar in morphology to Tenuiviruses (15).

Here, we illustrate this new view of CtRSV particle structure with original micrographs and models, and report detection of such particles in some, but not all, of several other ringspot and psorosis isolates examined. The implications for taxonomy and diagnostics are discussed.

MATERIALS AND METHODS

Viruses. The virus isolates examined are listed in Table 1. Analysis of the particle structure was done with CtRSV-4 from Florida, and citrus psorosis-associated virus (CPsAV) 90-1-1 from Argentina; the other isolates were only examined for presence or absence of the char-

acteristic particles. Among the isolates tested was P-203m, the same as ps203m, reported to contain somewhat flexuous rod-shaped particles with many characteristics of Carlaviruses (1, 18).

Material from young citrus leaves showing symptoms, or *Chenopodium quinoa* local lesions was used to partially purify CtRSV and CPsAV, and fractionate them into top and bottom components on sucrose density gradients (6, 7, 13, 14). The fractions were concentrated by ultracentrifugation and the pellets resuspended in 50 mM Tris-HCl buffer, pH 8.

Electron microscopy. Various dilutions, in the same buffer or in water, were prepared from the partially purified material, and used for electron microscopy. Alternatively, leaf samples about 6 mm² in area were directly homogenized in 50 µl of 0.1 M phosphate buffer pH 7 containing 2% (w/v) polyvinylpyrrolidone (PVP; Bio-Rad, MW 44,000).

For the crude preparations, grids coated with Formvar-backed carbon were used directly. A drop of the sample was placed on the grid for a few seconds, and rinsed with about 30 drops of water followed by 5 drops of 1% (w/v) aqueous uranyl acetate (20). This resulted in negative and,

TABLE 1
RINGSPOT-PSOROSIS ISOLATES EXAMINED FOR PRESENCE OF CtRSV-TYPE PARTICLES IN YOUNG SYMPTOMATIC LEAVES

| Isolate | Symptom | Source | Reference | Host plant | Particles |
|--------------|----------|-------------------------|------------|---------------------------------|-----------|
| CtRSV-4 | Ringspot | Florida | 6 | Madam Vinous sweet orange | + |
| CPsAV 90-1-1 | Psorosis | Argentina | 14 | <i>Chenopodium quinoa</i> | + |
| RS-SR | Ringspot | Spain | 24, 26, 27 | Etrog citron | + |
| RS-SOR | Ringspot | Spain | 24 | Madam Vinous sweet orange | + |
| Sp-1 | Psorosis | Spain ^a | ‡ | Dweet Tangor | - |
| Sp-2 | Psorosis | Spain ^a | ‡ | Madam Vinous sweet orange | + |
| It-1 | Psorosis | Italy ^a | ‡ | Etrog citron | - |
| P-203m | Psorosis | Thailand? ^a | 1, 18 | Sour orange <i>C. quinoa</i> | + + |
| P-205 | Psorosis | California ^a | ‡ | Rough lemon | + |
| P-214 | Psorosis | California ^a | ‡ | Rough lemon | - |

^aCollection of the International Centre for Advanced Mediterranean Agronomic Studies, Valenzano, Bari, Italy.

[‡]No published data available

occasionally, positive staining. We concentrated on the negatively stained areas since virus particle structure in these areas were better conserved and resolved. For a clear demonstration of the advantages of negative over positive staining in a context such as ours, see (38).

Partially purified preparations were treated similarly except they were placed on grids previously treated by glow discharge (20) to increase the proportion of negatively stained areas and the amount of virus absorbed to the grid. The glow discharge, however, did not improve detection from crude preparations. Some crude or partially purified preparations were negatively stained after trapping on antiserum-coated grids (15) using the antiserum of K. S. Derrick (6) diluted 1/1,000. Grids were scanned using 60 kV, a 30 μm objective aperture, a condenser spot size of 2 μm , an instrument magnification of 25,000 X, and a X 10 binocular; this appeared to be the best system for detecting the faintly contrasted particles.

The hypothesis that negative staining degrades the particles was investigated on crude preparations from *C. quinoa* by comparing the morphology and number of virus particles observable on grids negatively stained routinely with UA, or previously fixed for 15 min at 25°C with either 1% glutaraldehyde (GA) or 0.2% osmium tetroxide (Os), both in 0.1 M phosphate buffer, pH 7. This experiment was repeated, negatively staining the grids in 0.5% ammonium molybdate, pH 6 (AM), a stain generally accepted as being the least destructive to virus particles (30).

RESULTS

Particle morphology. Fig. 1A shows top component particles of CtRSV trapped by ISEM. All but two of these correspond to "spirovirus"

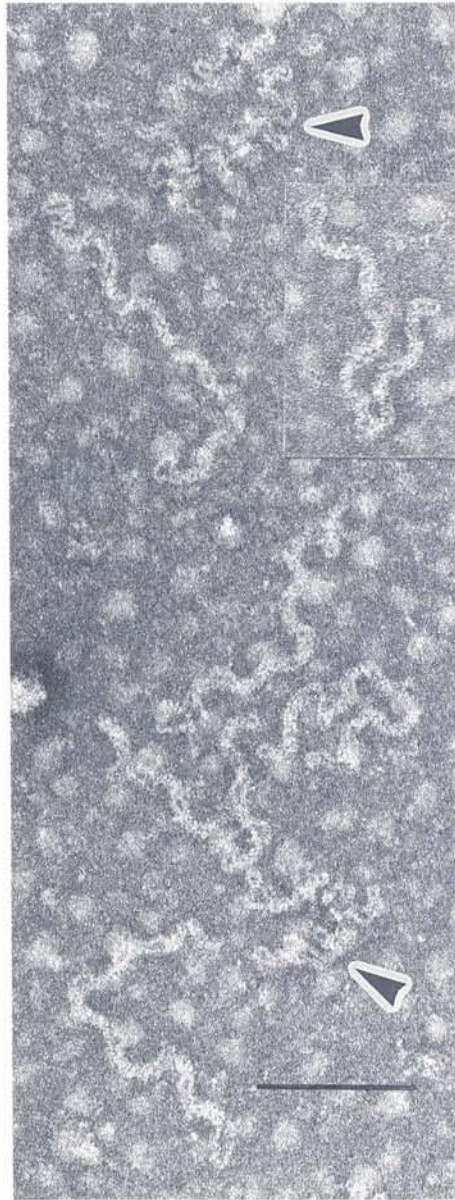


Fig. 1A. Eight particles of CtRSV from a partially purified "top component" preparation, trapped on an antiserum-coated grid. One particle is inset from a different part of the same photographic negative. The arrows indicate particles in the single-stranded open or *O* configuration; the others are in the duplex linear or *L* form similar to that originally described by Derrick et al. (1988) as a "spirovirus". Uranyl acetate negative stain. Bar = 100 nm.

particles (6) and are seen to be composed of two intertwined threads

with a loop at each end, thus, making them essentially circular. We refer to this configuration as the *L* form, as at first sight they appear to represent a linear structure. The component threads had a diameter of about 3 nm. The modal length of the threads composing 62 such particles was estimated as 687 ± 67 nm. To obtain these values, EM negatives taken at 28,500 X were projected at a further 10 X magnification, and the single strands composing the *L* forms were traced on paper; the tracings were then followed with a map measurer. Total contour lengths were slightly more than double the apparent "spirovirus" particle lengths.

The arrows in Fig. 1 (especially the upper arrow) indicate particles composed of the same type of 3-nm thread, highly kinky and probably supercoiled, but in a more open configuration (the *O* form). Where it was possible to estimate the contour length of such threads, it was close to that of the *L* particle thread in the same preparation. We observed that when particles were trapped by incubation on serum-coated grids, a majority took the *L* form, but when particles were adsorbed to grids directly, nearly all were in the *O* form.

Some particles (not shown) were of *L* form for part of their length, and in the *O* form for the rest. Some *L* particles appeared branched (not shown) but in these cases the 3-nm thread composing the branches was seen to be a continuous linear structure looping back upon itself. An interpretation of three of the particles in Fig. 1A is shown in Fig. 1B.

Fig. 2 shows particles, all in the *O* form, from a CtRSV bottom fraction which mostly contained large particles (panels A, and C large arrow); the preparation was contaminated with some top component particles (panels B, C small arrow, and D). The small particles were circular, sometimes relaxed (panel B), more

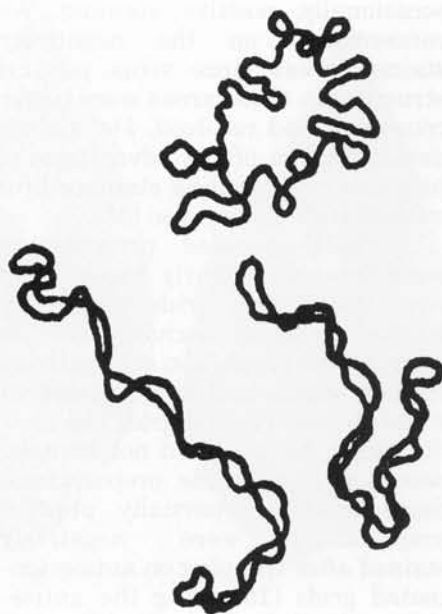


Fig. 1B. An interpretation of the structure of three of the particles in Fig. 1A.

contracted (panel D) or highly contracted and difficult to recognize. The large particles were composed of similar but longer threads, again circular as far as could be judged. We were unable to measure the contour length of such particles.

Fig. 3 presents wire models of bottom (a1) and top (a2) component particles in the *O* form, and a particle (b) in the *L* form.

Negative staining damage to CtRSV particles? The number and condition of virus particles was compared on grids unfixated or fixed with either GA or Os, and negatively stained in either UA or AM. The condition of the particles was found to be similar in all cases (a majority in the *O* form), with no change when preparations were fixed before staining, or with staining in AM. The number of particles detected was highest after simple negative staining in UA. Fixation led to the detection of fewer particles, perhaps because more debris was retained on

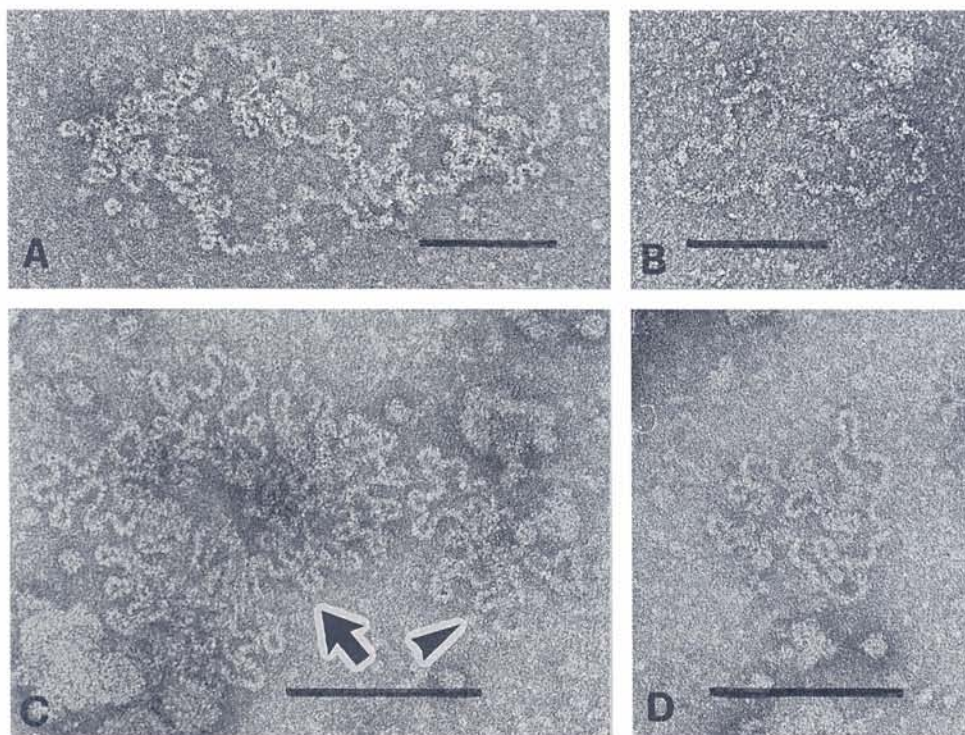


Fig. 2. CtRSV from partially purified "bottom" preparations, showing virus particles in the single-stranded *O* configuration. Uranyl acetate negative stain. Bars = 100 nm. Panels A and C (large arrow) show large "bottom component" particles (a majority in the preparation); panels B and D, and again C (small arrow), show smaller "top component" particles found contaminating the "bottom component" fraction.

the grids. AM gave insufficient contrast for easy particle detection.

Presence of particles in different ringspot-psorosis isolates.

Virus particles, where detected, were similar for all isolates. In crude CtRSV-4 preparations from *C. quinoa* local lesions, we detected about 200 particles per 400-mesh grid square (area approximately 40 μm^2); the corresponding figure for symptomatic citrus leaf tissue was about 40 particles. However, the particles were so characteristic that detection of three or more particles in a preparation was sufficient for a positive result. The majority of particles seen were of the small or top-component type.

Table 1 shows in which isolates such particles were found. Note that similar particles appeared in prepa-

rations of ringspot and psorosis isolates. We draw attention to the fact that typical particles were detected in sour orange leaves infected with P-203m and in *C. quinoa* local lesions derived from them; no carlavirus-like particles (1, 18) were seen. Particles like those of CtRSV were not detected in isolates It-1, Sp-1, and P-214. The first two of these isolates have not been characterized, but C. N. Roistacher (personal communication, 1996) suggests that P-214 is not a typical psorosis isolate since its presence did not protect plants challenged with psorosis B inoculum.

DISCUSSION

Our results may help the study of citrus ringspot-psorosis in several

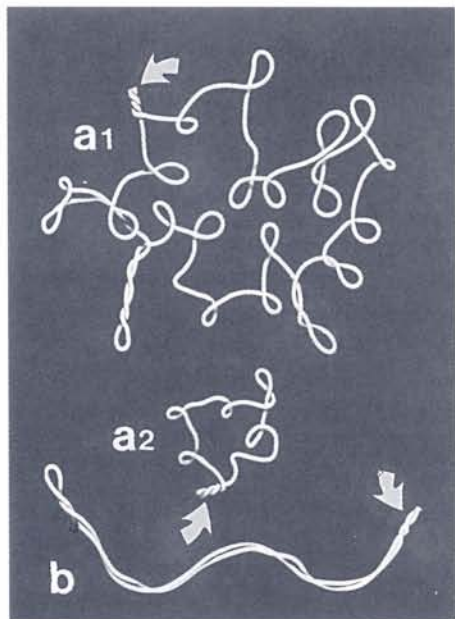


Fig. 3. Wire models of CtRSV particles (not to scale), shown as an aid to interpreting Figs. 1 and 2. Models *a1* and *a2* represent bottom and top component particles, respectively, in the *O* configuration. Model *b* represents a top component particle in the *L* configuration. The "panhandle" (see text), that hypothetically joins the free ends of the linear genomic RNA to make it circular, is indicated by arrows.

ways. First, we can now understand what kind of virus is involved, in a taxonomic sense. This is important because, by analogy with other related but better known viruses, we can suggest many properties this type of virus should possess, from its molecular biology to its behavior in the field.

Second, our ability to recognize the virus particle in its different forms should be an aid in diagnostics using electron microscopy, and help to distinguish this kind of ring-spot-chorioid from other diseases with similar symptoms but with unrelated viruses (1, 2, 18, 24, 31). We can also more easily monitor preparations made in attempts to find better protocols for virus purification.

The description of the CtRSV particle as an elongated flexuous spiral structure about 10 nm in diameter (6, 7) turns out to have been misleading as it brought to mind an essentially rod-shaped virus with no known affinities. It is now clear that the basic structure of the particle is that of an association of ssRNA and one coat protein species such as is contained within the envelope of Rhabdovirus, Tospovirus, and influenza particles (22, 37, 38), and occurs in free unenveloped form in the Tenuiviruses (22, 29, 37) which are viruses with hosts in the Gramineae, and with coat proteins of around 33 K.

In fact, the particles of CtRSV bear a close resemblance to those of Tenuiviruses (10, 17, 34), which consist of highly coiled circular elements about 3 nm in diameter, that can form duplex, apparently linear structures about 10 nm in diameter. Their genomic RNAs, when isolated, are found in double-stranded as well as single-stranded forms (29), a characteristic also noted for CtRSV (9). It appears that Tenuivirus RNA *in vivo* is encapsidated in single-stranded form, but that both (+) and (-) polarities can be encapsidated, so when preparations are deproteinized experimentally, strands of opposite polarity hybridize (29).

With both Tenuiviruses and Tospoviruses, the basically linear genomic RNAs are circularized by a highly conserved so-called panhandle structure of complementary bases at the 5' and 3' end of each RNA (5, 35). If this proves to be correct for ring-spot-chorioid, it would explain why we observed circular particles. The 'panhandles' are modeled in Fig. 3.

Two further points of interest emerge from this comparison. One is that Tospoviruses have three separate RNAs and Tenuiviruses four or, in some cases five. Fractionation experiments have shown that ring-spot-chorioid exists in at least two

components necessary for infectivity, corresponding to longer and shorter particles, but further analysis may show up more than two genomic RNAs. The second point is that plant viruses possessing a thin filamentous nucleocapsid (Rhabdoviruses, Tospoviruses and Tenuiviruses) all have insect vectors in which the viruses also multiply. This suggests that ringspot-psorosis virus is vectored in a similar way, and not, for example, through the soil or by pollen. In practice, ringspot-psorosis is mainly spread through infected budwood, but in some areas, natural spread by a vector has been suggested (28, 31, 33).

Our results with fixation of preparations on the grid before negative staining did not support the idea that the act of negative staining destroys CtRSV particles, or modifies them. On the contrary, more particles in good condition were observed without fixation. There remains the problem of why previous authors did not observe the particles in negative stain, and only observed them after ISEM.

We suggest that the particles were present all along in the preparations; however, in negative stain, although the resolution is better, the contrast is fainter than with positive staining, and the *O* and *L* forms appear as very thin threads, either single or double. With positive staining, the single threads would probably not be detected at all, and only the *L* forms, perhaps trapping extra stain between the two threads, would be large enough to see by this method. We observed that after ISEM, the virus trapped was predominantly in *L* form, but in other cases it was almost all in *O* form. This explains why previous authors only detected the virus following ISEM, and not in preparations adsorbed to grids directly.

Why would *L* forms be observed predominantly in ISEM preparations but less commonly in other

cases? Recent unpublished work by N. Noureddine in our laboratory has shown that when purified preparation of CtRSV are held at room temperature for several hours (as in ISEM incubations), *O* forms are slowly converted to *L* forms. Thus, the presence of *L* forms seems to be associated with long incubation *in vitro* rather than any effect of the antiserum.

A name for the virus under discussion will soon have to be agreed, whether it turns out to be citrus psorosis, citrus ringspot or another. The *type* of virus will also eventually be given generic if not family status, as it is clearly different from the Tenuiviruses at least in coat protein size (48 to 50 K, not 33 K), serological reaction (15), and type of plant host (Dicotyledons, not Gramineae). The name "spirovirus" has been proposed (9) in reference to the spiral rod-shaped particle earlier reported. While this name has clear priority, we have alternatively proposed the name "ophiovirus", from the Greek *ophis*, a serpent, in reference to the snaky appearance of the particles, in both *O* and *L* forms (15). This name may be more appropriate as it is not based on a misconception of the nature of the virus particle; it avoids the problem of the name "spirovirovirus" already used for a genus of bacteriophages (22); and also prevents confusion with the organism, *Spiroplasma citri*, agent of citrus stubborn disease.

CtRSV-CPsAV was the first "Ophiovirus" recognized (15) but other similar viruses have been recently noted. These include a supercoiled filamentous virus in *Ranunculus* from Italy (36) and a very similar virus in tulips from Japan (21), both with circular nucleocapsids; and a somewhat different supercoiled filamentous virus with linear nucleocapsids from papaya in Venezuela (19).

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