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State of the art on the agent of citrus psorosis disease

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SUMMARY - Recent results and insights on the probable causal virus of citrus psorosis disease are summarized. Symptoms can include ringspots, and the best characterized isolate is known as citrus ringspot virus; however, we use the alternative name citrus psorosis-associated virus (CPSaV) to avoid confusion with other ringspot virus diseases of citrus. CPSaV is a multicomponent ssRNA virus with a coat protein (CP) of 48-50 K. The particles are fine kinky filaments of different contour lengths and about 3 nm in diameter, with the ends joined to form circles. The rodshaped "spirovirus" particles described earlier are collapsed duplex forms of the circles. Two other viruses with some similar properties have recently been described: ranunculus white mottle from Italy and tulip mild mottle mosaic from Japan, with CPs of 43 and 46 K respectively. A new genus Ophiovirus has been proposed for the three viruses. The particles of "Ophioviruses" and also some of their molecular properties resemble those of Tenuiviruses (phloem-restricted viruses of cereals) and the nucleocapsid components of viruses in the family Bunyaviridae, which includes the Tospoviruses; however, the CPs of Tenuiviruses and Tospoviruses have sizes of about 31 and 29 K respectively. Regarding diagnostics, partial sequences of an isolate of CPSaV have been reported, together with the use of PCR primers based on them. A DAS-ELISA using a polyclonal antiserum has also been reported and is currently being employed to detect psorosis in some citrus orchards and nurseries in southern Italy

Key words: citrus, ophiovirus, psorosis, ELISA, diagnosis

RESUME *- Un aperçu est donné des résultats et des informations récemment acquises sur le probable virus, agent causal de la psorose des agrumes. Les symptômes induits peuvent inclure des taches annulaires et l'isolat le mieux caracterisé est le virus des taches annulaires des agrumes. Cependant, il est préférable d'employer la dénomination de virus associé à la psorose des agrumes (CPSaV) pour éviter toute confusion avec d'autres viroses des agrumes induisant des taches annulaires . Le CPSaV est un virus multicomponentiel à RNA monocaténaire avec une protéine capsidique de 48-50K. Les particules sont de fins filaments de diverse longueur et d'environ 3nm de diamètre, présentant les extrémités unies à former des cercles. Les particules en bâtonnet des 'Spirovirus' décrites précédemment sont en fait les filaments des formes circulaires repliés sur eux mêmes. Deux autres virus ont été décrits dernièrement montrant des propriétés similaires: Le ranunculus white mottle en Italie et le Tulip mild mottle mosaic au Japon, avec des protéines capsidiques respectives de 43K et de 46K. Un nouveau genre 'ophiovirus' a été proposé pour les trois virus.*
Les particules du genre 'ophiovirus' et quelques-unes de leurs propriétés moléculaires ressemblent à celles des tenuivirus (virus à localisation phloémique des céréales) et des similitudes avec les composantes nucléocapsidiques des virus de la famille des bunyaviridae qui incluent les tospovirus. Toutefois, les protéines capsidiques des tenuivirus et des tospovirus ont

une taille respective de 31 et 29K. En ce qui concerne le diagnostic, il a été rapporté l'obtention des séquences partielles d'un isolat de CPSaV et leur utilisation pour la mise au point d'amorces pour la PCR. Parallèlement un test DAS-ELISA utilisant un antisérum polyclonal a été décrit et cette technique est actuellement employée pour détecter la psorose dans des vergers d'agrumes et des pépinières dans le sud de l'Italie.

***Mots clés**: agrumes, ophiovirus, psorose, ELISA, diagnostic*

For nearly one hundred years, the virus-like disease known as psorosis has been widespread and damaging in many parts of the world including the Americas and the Mediterranean basin; it has been studied by symptomatology, use of citrus indicator plants, cross-protection experiments and mechanical transmission to species such as *Chenopodium quinoa* (Roistacher, 1993). Recently this rather messy scene has been transformed by detection of the virus particle in the electron microscope (EM), partial purification of the particles, antiserum production, and partial sequencing of the viral nucleic acid. This revolution, especially that part concerning electron microscopy, is the subject of my short review.

First, a note on nomenclature. As well as the classical bark-scaling that gave rise to the name "psorosis", chlorotic flecks and ringspots may also be produced in the leaves of infected trees, and for this reason the name citrus ringspot has been used for some isolates, e.g. citrus ringspot-4 (CtRSV-4; Garnsey & Timmer, 1980). It now seems that ringspot and psorosis are usually caused by the same virus (Navas-Castillo *et al.*, 1993; Roistacher, 1993) but a single name for this virus has not yet been officially agreed. Here, the name citrus psorosis-associated virus (CPSaV) will be used; the cumbersome term "associated" is still advisable because in only one case (CtRSV-4) has an approach to fulfilling Koch's postulates been made (Garnsey & Timmer, 1988). The postulates, as adapted to viruses, would require isolation of the virus in a purified form, and demonstration that such a preparation can be inoculated directly or indirectly back into citrus, provoking the original symptoms, and enabling re-isolation of the virus once more. These are severe tests for the virus, unstable and present in low concentration, and for the citrus host plant, which is not easy to inoculate successfully with purified preparations.

A decisive advance in understanding the virus came when Derrick *et al.* (1988a, b) partially purified CtRSV-4. They produced a preliminary antiserum to it and published evidence that the particles were of a novel spiral filamentous type, coming in at least two length classes. They further showed that after density gradient centrifugation, infectivity in *C. quinoa* was maximized by combining top and bottom fractions, and that a 48 K protein was associated with the virus.

Further work followed (da Graça *et al.*, 1991, 1992; Derrick *et al.*, 1991, 1993; Garcia *et al.*, 1991a,b, 1992; Navas-Castillo, 1991; Navas Castillo & Moreno, 1993, 1995; Navas-Castillo *et al.*, 1991, 1993) on psorosis and ringspot isolates from Florida, Spain, Argentina and Israel, confirming the widespread presence of viruses similar to the original Florida isolate, associated with these symptoms. It was confirmed that a capsid protein of 48-50 K was present and that the nucleic acid of the particle was

RNA. The virus particles appeared as rod-shaped structures with a sinuous profile, about 9 nm wide and 300-500 nm or 1500-2500 nm in length. The morphology indicated that the virus was of a novel type with no immediate affinities, since all known rod-shaped viruses had a width of at least 12-13 nm, and did not show a regularly sinuous profile. The name "Spirovirus" was proposed for this single member of a new virus group (Derrick *et al.*, 1993).

However, all this time, the virus was visualized in the EM by staining it with the contrasting agent uranyl acetate in the so-called positive-stain mode, in which the virus particle itself absorbs the stain. The next part of my discussion concerns the differences between positive staining and negative staining, so I will make a small digression to explain these.

Huxley and Zubay (1961) were the first to introduce uranyl acetate (UA) as a stain for electron microscopy. They noted that when used in the positive-stain mode, UA stains nucleic acid preferentially, while imparting very little contrast to protein. In the negative-stain mode, UA stains the background only, thus causing the particle to appear white (unstained). However, the whole shape of the particle becomes outlined in detail, including protein, nucleic acid or even lipid structures if present. Thus for example, with a particle such as the nucleocapsid of influenza virus, containing about 5 % nucleic acid and 95 % protein, Compans *et al.* (1972) noted that very clear, relatively large, and easily interpreted structures were seen in negative stain, but these appeared in positive stain as thin and ill-defined shadows - not surprising if such images represented only 5 % of the particle!

So what has psorosis got to do with influenza? The answer is that the particles of CPSaV, in our interpretation, are themselves nucleocapsids very similar in basic structure to those of influenza virus, except they are somewhat smaller. Thus negative staining reveals a great deal more fine detail in the particle than does positive staining; in fact it has permitted a new interpretation of the particle's structure and hence its probable taxonomic affinities. Our interpretation has been aided by the discovery in our laboratory of an apparently similar but "easier" virus in ranunculus (ranunculus white mosaic, RWMV; Vaira *et al.*, 1997) and the finding in Japan of another similar virus in tulip (tulip mild mottle mosaic, TMMMV; Morikawa *et al.*, 1995). RWMV and TMMMV have capsid proteins of 42 K and 46 K respectively, not far from the 48-50 K of CPSaV. More details of some of the present work are reported in Garcia *et al.* (1994, 1997) and Milne *et al.* (1996).

The main isolate studied was CtRSV-4 from Florida (Garnsey and Timmer, 1980). Other isolates from the USA, Spain and Italy had similar particle morphology.

For electron microscopy, dilutions in water were prepared from partially purified material, either "top" or "bottom" fractions from velocity density gradient preparations. Alternatively, leaf samples about 6 mm^2 were directly homogenized in 50 µl of 0.1 M phosphate buffer pH 7 containing 2 % PVP. Grids were loaded with a drop of the extract, rinsed after a few seconds with water, and negatively stained with 1 % aqueous uranyl acetate following a standard procedure (Milne, 1993).

In negative stain, the particles were observed to come in at least two size classes, small and large, corresponding to top and bottom components, as already noted above; however, further levels of complexity became apparent. The particles observed by Derrick and co-workers, and described as rod-shaped, turned out to be composed of two closely twined filaments, each about 3 nm in diameter. These filaments were in reality one continuous circular thread, looping back on itself at each end. It is interesting that if an elastic band or a closed loop of string is twisted and then allowed to take its own shape, it can assume the form of the particles seen in the EM, complete with a periodic sinuous wave along the length of the structure. Thus we think that the thread composing the virus particle is in some way internally coiled.

If these particles are in fact composed of a thinner circular thread, more open circular forms should sometimes be apparent. In fact the majority of particles in the virus preparations were seen in open circular form, though the circles were not smooth in outline but highly kinked or looped, again suggesting a degree of internal twisting. We observed that in fresh preparations, the majority of particles were in the open form, but that as time passed, they tended to collapse into the duplex or linear form.

Available photographs of TMMMV from Japan show top and bottom particles, both in the open form. Particles of RWMV (top and bottom) are seen in open circular form when resuspended in dilute Tris buffer pH 8 containing the reducing agent dithiothreitol (DTT) but can be converted to the duplex linear form by resuspension in citrate buffer pH 6 (Vaira *et al.*, 1997). These images have confirmed and supported our interpretation of the structure of CPSaV particles.

As our results are somewhat in contrast with those of Derrick and colleagues, some specific questions arise, as well as more general ones. For example, Derrick and associates did not observe the open circular particles, after positive staining, probably because these were simply not visible, having low contrast. In addition, they only observed particles after incubation of the extracts on antiserum-coated grids, but never directly in crude or partially purified extracts. Perhaps this was because, as noted by us, long incubation allowed a greater proportion of the open circular particles to collapse into the more easily detected duplex form.

What can the new interpretation of the particle structure of CPSaV suggest about the relationships of this virus to other plant and animal viruses? First, it is likely that CPSaV, RWMV and TMMMV will turn out to form a group or genus, and we have proposed the name Ophiovirus for such a genus (Garcia *et al.*, 1994). The name is derived from the Greek "ophis", a snake, in reference to the serpentine form of the particles. While the name "spirovirus" has clear priority, it is based on a misconception of the structure of the virus particle, and is also confusingly similar to *Spiroplasma citri*, the name of the agent of citrus stubborn disease, and to the name "Spiromicrovirus", already used for a genus of bacteriophages.

More broadly, it is already well documented that Tenuiviruses such as rice stripe virus have particles closely resembling those of the "ophioviruses", in being present in at least two size classes, and in

forming both open circular and apparently rod-shaped particles of similar dimensions and physical behaviour (Francki *et al.*, 1985; Ishakawa *et al.*, 1989; Ramirez and Haenni, 1994; Toriyama *et al.*, 1994a). Further, the Tenuiviruses are now recognized to be closely related to members of the Bunyaviridae, a family of mostly mammalian viruses that also includes the Tospoviruses such as tomato spotted wilt virus (Murphy *et al.*, 1995). The suggestion arising out of a study of particle morphology is therefore that the "ophioviruses" will in time be recognized as a genus within the Bunyaviridae.

Although molecular studies on "ophioviruses" are not far advanced, it is known in two cases (CPSaV, RWMV) that RNA extracted from the particles is found in both single-stranded and double-stranded forms. Exactly similar behaviour is found with the Bunyaviridae and Tenuiviruses, but not with other virus groups. The current interpretation is that *in vivo* the genomic RNA is single-stranded but that strands of both polarities become encapsidated; upon deproteinization *in vitro*, the strands of opposite polarity combine to form dsRNA while excess strands of a single polarity remain unpaired, as ssRNA (Ramirez and Haenni, 1994).

We have noted that the particles of "ophioviruses" are circular. Here it is interesting that the particles of Tenuiviruses and the nucleocapsids of the Bunyaviridae are also circular, because the basically linear genomic RNAs are circularized by a so-called panhandle structure of complementary bases at the 5' and 3' ends of each RNA (see Toriyama *et al.*, 1994b; Murphy *et al.*, 1995). Such terminal base-pairing could provide the reason why the particles of CPSaV and its relatives appear circular, but the relevant sequence data are not yet available to show whether or not this is so.

What implications are there for other properties of "ophioviruses"? Many of the Bunyaviridae and all Tenuiviruses have insect or at least arthropod vectors, in which they multiply. This suggests that "ophioviruses" may also be found to have propagative arthropod vectors, although in no case has such a vector yet been reported.

The work described above will help to explain how CPSaV fits into the known world of viruses, and will help to predict some further properties that the virus may possess. The more practical aspects of how to detect the virus and how to measure the amount of variation among different isolates are also beginning to go beyond the biological indexing stage. Garcia *et al.* (1997) have reported partial sequences for CtRSV-4 and the preparation of primers that allow the virus and some psorosis isolates to be detected by RT-PCR. These authors have also described for the first time an antiserum good enough to be used in DAS-ELISA; although this antiserum is, by other standards, relatively poor, it has permitted DAS-ELISA detection of psorosis isolates from the USA, Argentina, Spain and Italy, and has formed the basis for field and nursery surveys for psorosis in the Puglia region of Italy, as also reported at the MNCC meeting in Adana, 1996.

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