Improvements in Serodiagnosis of Citrus Psorosis Virus

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ABSTRACT. A rabbit polyclonal antiserum to citrus psorosis virus (CPsV), already described as giving practical but modest results in DAS ELISA, has been used in a modified protocol to provide sensitive detection of all isolates so far tested. A purification method starting from locally and systemically infected *Gomphrena globosa* plants held at high temperature was used to prepare monoclonal antibodies (Mabs) (an IgM and an IgG) to the virus. The IgG Mab has been combined with the rabbit polyclonal in a TAS ELISA test that has also detected all isolates so far tested, and is again more sensitive than the improved DAS ELISA. Both types of ELISA should prove useful for field diagnosis of CPsV.

Evidence has been accumulating that the presence of citrus psorosis virus (CPsV), now placed in the new genus *Ophiovirus*, is strongly correlated with citrus psorosis disease and is probably the causal agent (2, 3, 5, 6, 8). The disease, widespread in the Mediterranean basin, South America and probably in Asia, has not been successfully indexed and controlled except in very few areas such as California, Spain and South Africa, where careful biological indexing has been applied. This procedure is slow and costly, and requires dedicated and trained personnel; thus inexpensive, simpler and more rapid diagnostic methods are urgently required.

An early antiserum to CPsV (2) reacted with only a few isolates of the virus and was not suitable for ELISA, and the first available monoclonal antibody (Mab) (4) was not suitable as a diagnostic reagent (1). An antiserum produced in Turin, able to detect CPsV by DAS ELISA (6) has been used for indexing CPsV in the south of Italy (5) but the test was not very sensitive. Mabs to an Italian isolate of CPsV have been used to distinguish different isolates of CPsV (9), but quantitative results on the sensitivity of detection using these Mabs are not yet available.

We now report improvements to the DAS ELISA protocol described in García et al. (6) that markedly increase its sensitivity. We also give the results of our program for producing Mabs to the CPsV coat protein. One of these Mabs, in a TAS ELISA format, detects a wide range of isolates with great sensitivity.

The CRSV-4 isolate (7), kindly provided by K.S. Derrick, was used for antibody production. Some other isolates were received as citrus budwood, and samples were also shipped to us as young citrus leaves sliced into strips, dried over calcium chloride or silica gel, and sealed in a polyethylene bag.

The DAS ELISA protocol (6) was modified as follows: (a) phosphate-buffered saline (0.1 M, pH 7.2) containing 0.05% Tween 20, 2% polyvinylpyrrolidone (PVP) and 2.5% defatted milk powder was the buffer used in the extraction and conjugation steps; (b) plates were coated with IgG at 0.5 µg/ml; (c) the conjugate was diluted to 1/1,000; and (d) most importantly, the plates, after each step, were washed with squirts from a wash bottle and emptied energetically five times by hand.

To obtain Mabs, the virus was purified according to (6) with modifications, from locally and systemically infected leaves of *Gomphrena globosa* plants grown at 30°/25°C (day/night) with supplementary lighting to give an 18 h day. Leaves were homogenized in TACM buffer (3) plus active carbon. After clarifi-

cation with Freon and precipitation twice with PEG-NaCl plus Nonidet P40, the preparation was centrifuged into a 10-40% cesium sulfate density gradient, and a virus-rich zone, detected by ELISA and by electron microscopy, was withdrawn, pelleted, and resuspended.

G. globosa raised at high temperature proved to be a better source of virus than *C. quinoa*. Preparations purified from *G. globosa* were somewhat cleaner and richer in virus particles, but were still not highly pure. A method (10) successful with the nucleocapsids of tospoviruses and with the particles of ranunculus white mottle *Ophiovirus* (12) was tried unsuccessfully.

Mabs were produced by standard methods, and selected in a TAS ELISA system, using sap of infected and healthy *G. globosa* as described below. We obtained two useful Mabs, an IgG and an IgM.

The TAS ELISA followed the method of Thomas et al. (11). Wells were coated with crude antiserum diluted 1/9,000 in coating buffer, for 4 h at 37°C. Plates were washed five times by hand with PBS-Tween in all washing steps. Healthy or infected citrus leaf was homogenized in 10 volumes (w/v) of the DAS ELISA extraction buffer, added to the plates, and incubated on a shaker at 4°C overnight. Mab-containing ascites fluid, diluted 1/32,000 in extraction buffer, was added to the plates and incubated for 90 min at 37°C. Alkaline phosphatase conjugates appropriate to either IgG or IgM, according to the Mab in question, diluted 1/20,000 in extraction buffer, were added and incubated for 90 min at 37°C. Substrate was added and the optical density read after 2-4 h.

For comparing ELISAs done at different times, we prepared "standards" of infected or healthy citrus or *G. globosa* leaf homogenized in 2 volumes (w/v) of extraction buffer. This was filtered through nylon stocking, centrifuged at low speed, mixed with an equal volume of glycerol, stored at -20°C, and used at final dilutions of 1/40 (citrus) or 1/200 (*G. globosa*), with very little loss of antigenicity over a period of several months.

The Bioreba method of homogenizing citrus leaves with buffer in a polyethylene bag, using ball bearings, was better than using a pestle and mortar or a Pollähne roller press, as it could homogenize mature as well as young citrus leaves without trouble.

The new DAS ELISA protocol gave infected/healthy (I/H) values generally five to ten times greater than the earlier protocol (6).

With the TAS ELISA, the crude antiserum could be coated directly at a dilution of 1/9,000, without absorption and purification. Mabs (ascites fluid) were best used at a dilution of 1/32,000. These relatively high dilutions make the test convenient and economic. Using homologous greenhouse material, H values, read at 2-4 h, were close to zero (generally from 0.003 to -0.002) after subtraction of readings from the buffer-only blanks, with I values up to 2.5, so that I/H ratios were commonly between 50 and 2,000. In one typical antigen dilution test (infected samples diluted in healthy extract) using the IgG Mab, a tissue dilution of 1/31,250 gave a mean I value (6 wells) of 0.036 ± 0.0003 and a mean H value of 0.006 ± 0.0005, with an I/H ratio of 6, considered positive.

TAS ELISA tests made with unpromising material (old leaves without symptoms, field samples taken in winter) have so far given very encouraging results (personal communications, P. Moreno in Spain, E. Dal Bo in Argentina).

The IgG Mab detected all the psorosis isolates we tested (psorosis being defined by indexing). The IgM Mab did not detect three isolates (Italian IAM-320X, Spanish RS-105 and Spanish P-121). Otherwise, both Mabs detected all of 10 North American, seven Spanish, six Argen-

tine, six Italian, and one Lebanese isolates.

With both the DAS and TAS ELISA formats, the two factors leading to improved performance were (1) addition of defatted milk to all buffers up to and including the conjugate buffer; (2) carefully washing and emptying the plates five times by hand. Three washes using a commercial plate washer were insufficient and led to high *H* values; five careful washes by hand were important in reducing this background without significantly reducing *I* values, especially when milk was used.

Although allowing infected leaf material to air-dry at room temperature led to loss of antigenicity, we had good ELISA readings from leaf material dried over silica gel or calcium chloride. Thus, a convenient method of shipping test samples is available, especially as this material is no longer infectious.

In conclusion, we have improved the DAS ELISA protocol, and present a TAS ELISA system that is highly sensitive and able to detect a wide range of CPsV isolates. So far, we have not encountered any isolate, indexing psorosis-positive and proved by DAS ELISA to be present in the leaf sample, that was not detected by the IgG-based TAS ELISA. Therefore, the target epitope may be common to many isolates from different parts of the world and, hopefully, may be universally present in all isolates.

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