J. Phytopathology **150**, 292–296 (2002)

ISSN 0931-1785

Short Communication

International Center for Tropical Agriculture, Cali, Colombia

Partial Characterization of a Tymovirus Infecting Passion Fruit in Colombia, South America

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With 5 figures

Received October 31, 2001; accepted December 19, 2001

Keywords: *Passiflora edulis*, Colombia, Passion fruit yellow mosaic virus, tymovirus

Abstract

A tymovirus was isolated from passion fruit plants with conspicuous yellow mosaic leaf symptoms in Colombia. Partial sequencing of its genome showed that this virus is distinct from other tymoviruses for which nucleotide sequences are available; it had only 60–76% homology with the closest related tymovirus species, *Kennedya yellow mosaic virus*. However, the pathogenic and antigenic properties of the Colombian tymovirus are similar to those of *Passion fruit yellow mosaic virus* (PFYMV), a tymovirus that infects *Passiflora* spp that has previously been found in Brazil. Therefore, this name will be retained for the tymovirus occurring in Colombia until a Brazilian isolate of PFYMV is characterized at the molecular level.

Introduction

Passion fruit (*Passiflora edulis* Sims.) is one of the most important tropical fruit species consumed world-wide as fresh fruit and juice or as an additive to ice cream and preserves. Although Brazil is reportedly the centre of origin of passion fruit, it is now cultivated in all continents of the world. The main producers of the crop are Brazil, Colombia, Ecuador, Peru, Venezuela, Kenya, South Africa, Sri Lanka, Australia, New Zealand and Hawaii (Menzel et al., 1996). Of the various phytosanitary problems that affect passion fruit production world-wide, viral diseases are undoubtedly the most frequently mentioned. The following five viruses are reported to infect this crop in Brazil: *Passion fruit woodiness virus* (Chagas et al., 1981), *Cucumber mosaic virus* (Colariccio et al., 1984), *Purple passion fruit mosaic virus* (Chagas et al., 1984), *Passion fruit vein clearing virus* (Kitajima and Crestani, 1985), and *Passion fruit yellow mosaic virus* (Crestani et al., 1986). Colombia is the second largest producer of passion fruit in South America and shares geographical borders with Brazil. In an extensive survey of the main passion fruit-producing regions of Colombia (Varón et al., 1992; Chávez et al., 1999), two viruses were detected: a potyvirus, later identified as a strain of *Soybean mosaic virus* (Benscher et al., 1996), and an isometric virus tentatively identified as a tymovirus from its serological relationship to *Desmodium yellow mottle virus*, cytopathology (Francki et al., 1985), and particle morphology (Varón et al., 1992). Moreover, the latter virus induced symptoms (leaf and vein yellowing) similar to those of *Passion fruit yellow mosaic virus* (PFYMV), a partially characterized tymovirus occurring in Brazil (Crestani et al., 1986). The virus was detected in four (Antioquia, Caldas, Santander and Valle) of the 11 passion fruit-producing departments of Colombia (Chávez et al., 1999). Because of its current distribution and potential socio-economic impact, the present study was carried out to attempt to identity the causal virus.

Materials and Methods

The virus was originally isolated from a mosaic-affected (Fig. 1) passion fruit plant found in the municipality of Lebrija, department of Santander, in north-eastern Colombia. The virus was first transmitted to a virus-free passion fruit plant by grafting, and then maintained by mechanical inoculation using inoculum prepared with sterile distilled water (1 g infected tissue : 6 ml water).

Electron microscopy

Leaf extracts and purified preparations from infected passion fruit plants were negatively stained in 2% uranyl acetate, pH 3.7, and examined with a JEOL JEM-1010 electron microscope (JEOL, Peabody, MA, USA). Leaf tissue of symptomatic passion fruit plants was prepared for cytopathological examination as described earlier (Morales et al., 1990).

Fig. 1 Passion fruit leaf with early yellow mosaic symptoms

Virus purification

The virus was purified using a method developed at the Virology Research Unit of CIAT. Basically, 100 g of infected plant tissue were homogenized in 300 ml 0.1 M KPO_4 buffer, pH 7.0, containing 0.5% Na_2SO_3. The homogenate was centrifuged at 4100 ***g*** for 5 min, the pellet was discarded and the supernatant was centrifuged at 104 000 ***g*** for 90 min on a 20% sucrose cushion. The precipitate was resuspended in 30 ml 0.01 M KPO_4 buffer, pH 7.0, and the cycle of differential centrifugation was repeated. The resulting pellet was resuspended in 10 ml 0.01 M KPO_4 buffer, pH 7.0, in the presence of Freon 113 (1/3 vol) and 1% Triton X-100, prior to clarification at 3000 ***g*** for 5 min. The supernatant was then treated with 1/3 volume of chloroform, reclarified at 3000 ***g*** for 5 min and then centrifuged on a 10–40% sucrose density gradient at 104 000 ***g*** for 120 min in a SW28 rotor. The visible band was collected from the tubes, concentrated by ultracentrifugation at 103 000 ***g*** for 150 min, the final pellet being re-suspended in 0.4 ml of 0.01 M KPO_4 buffer, pH 7.5.

Serology

A polyclonal antiserum to the virus was produced at CIAT as described earlier (Morales et al., 1991). An antiserun [PVAS 155] to *Desmodium yellow mottle virus* (DYMoV) was purchased from the American Type Culture Collection (Rockville, MD, USA). The antiserum to *Passion fruit yellow mosaic virus* (PFYMV) was kindly provided by Dr Elliot W. Kitajima (Universidade de Brazilia, Brazilia, Brazil). Serologically specific electron microscopy (SSEM) and double antibody sandwich (DAS)-enzyme-linked immunosorbent assay (ELISA) were performed as described by Derrick (1973) and Clark and Adams (1977), respectively.

Electrophoresis

The molecular mass of the capsid protein of the purified passion fruit virus was determined in 10% polyacrylamide gels containing sodium dodecyl sulphate (SDS) as described by Weber and Osborn (1969). Bovine serum albumin (M_r 66 000), ovalbumin (M_r 45 000), and carbonic anhydrase (M_r 31 000), were used as size markers. For the analysis of dsRNA, healthy and diseased passion fruit plants were used as control and source of viral RNA, respectively, according to the procedure of Dodds and Bar-Joseph (1993). Approximate ds-RNA weights were estimated using a 1 kb ladder (Bethesda Research Laboratory, Bethesda, MD, USA).

Transmission tests

The following 27 plant species were tested for susceptibility by manual inoculation: *Cajanus cajan*, *Cannavalia ensiformis*, *Chenopodium quinoa*, *Cucumis sativus*, *C. melo*, *Datura stramonium*, *Glycine max* (four cultivars), *Gomphrena globosa*, *Lablab purpureus*, *Macroptilium atropurpureum*, *M. lathyroides*, *Nicotiana benthamiana*, *N. glutinosa*, *N. tabacum*, *Passiflora adenopoda*, *P. edulis*, *P. foetida*, *P. ligularis*, *P. pinnatistipula*, *P. quadrangularis*, *P. rubra*, *Phaseolus vulgaris* (11 cultivars), *Physalis angulata*, *P. floridana*, *Pisum sativum*, *Vigna radiata*, and *V. unguiculata*. The *Cerotoma*, *Colaspis* and *Diabrotica* species were used for insect transmission tests, using on average 170 individuals of each genus. The chrysomelid beetles were allowed 3 day-acquisition access periods on infected *Passiflora edulis* plants, and similar transmission access periods on a total of 10–24 virus-free passion fruit plants for each chrysomelid genus tested.

Partial molecular characterization of the virus

Viral RNA was extracted from purified virus suspensions using the Rneasy Plant Mini Kit, according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). Approximately 3 μg of viral RNA were transcribed into cDNA according to the method of Gubler and Hoffman (1983); the transcription reaction mixture of 50 μl contained 50 mM Tris-HCl, pH 8.3; 50 mM KCl; 10 mM $MgCl_2$; 10 mM dithiothreitol (DTT); 1 mM of each dNTP; 60 U Rnasin (Promega, Madison, WI, USA); 50 μCi of α^{32} dATP (3000 Ci/mmol, Amersham, Pharmacia Biotec, Buckinghamshire, UK); 1 μg random primers (Promega); and 600 U SuperScript II RT (Gibco/BRL, New York, USA), by incubation at 37°C for 1.5 h. The volume was increased to 100 μl, to reach concentrations of 40 mM Tris-HCl, pH 7.2; 90 mM KCl; 3 mM $MgCl_2$; 3 mM DTT; 50 μg/ml BSA; 30 U *Escherichia coli* DNA polymerase (Gibco); 2 U RNase (Promega), and incubating at 14°C for 4 h. The reaction was stopped by heating at 70°C for 10 min, followed by cooling in ice. The mixture was treated with 10 U of T4 DNA polymerase (Promega) and 3 mM dNTPs, prior to incubation at 37°C for 10 min The enzyme was inactivated with 5 μl 0.5M ethylenediaminetetraacetic acid.

The cDNA was fractionated in Sephacryl S-400 (Pharmacia AB Biotechnology, Uppsala, Sweden) and ligated into the *Sma*I site of pBlueScript II (+) plasmids (Stratagene, La Jolla, CA, USA), and then used to

transform (heat shock) DH5α *E. coli* cells (Stratagene). The transformed cells were selected in agar-ampicillin, X-Gal and IPTG (Stratagene). The transformed plasmids were identified by resuspending samples of white colonies in 50 μl of polymerase chain reaction (PCR) mix (1 μl 10 mM dNTPs, 5 μl 25 mM $MgCl_2$, 0.5 μl each of primers T3 and T7 (Stratagene); 1μU *Taq* DNA polymerase (Promega), 5 μl PCR 10× buffer (Promega) and H_2O. Samples were amplified in a model PTC 100, PCR machine (MJ Research, Watertown, MA, USA) following a 30 cycle programme (94°C/30 s, 55°C/30 s, 72°C/2 min, and a final extension at 72°C/5 min). The resulting products were analysed by electrophoresis in 1.3% agar gels (Gibco) and staining in ethidium bromide.

The transformed plasmids were sequenced using a Perkin Elmer ABI PRISM automated sequencer (Norwalk, CT, USA) The sequences obtained were analysed with the BLAST program supplied by the National Center for Biotechnology Information (Bethesda, MD, USA).

Results

Electron microscopy

Isometric virus-like particles approximately 30 nm in diameter were consistently present in negatively stained leaf extracts from mosaic-affected passion fruit plants. These particles were more readily distinguished as a mixture of solid and empty virions by SSEM, using homologous (PFYMV/Colombia) or heterologous (PFYMV/Brazil) antiserum (Fig. 2). Cytopathological examination of the diseased passion fruit tissue revealed the presence of peripheral vesicles in the chloroplasts (Fig. 3), and crystalline aggregates of 'empty' virus particles in the nuclei (Fig. 4).

Purification

The virus was successfully purified, with an average yield of approximatey 20.5 mg of virus per kg of infected passion fruit tissue.

Serology

SSEM with antiserum to PFYMV/Brazil resulted in a 440-fold increase in the number of particles of the Colombian passion fruit virus with respect to the untreated control. Unexpectedly, only a 170-fold increase in the number of particles was observed with the homologous antiserum (PFYMV/Colombia). DAS-ELISA was successful with the homologous polyclonal antiserum, using 1 : 1800 and 1 : 2000 gamma-globulin and conjugate concentrations, respectively. Average A_{405nm} values of 1.98 and 0.08 were obtained for positive samples and negative controls, respectively, 20 min after the addition of substrate.

Electrophoresis

Electrophoretic analyses of purified virus suspensions in the presence of SDS yielded a single capsid protein subunit of approximately M_r 22 600; the ds-RNA

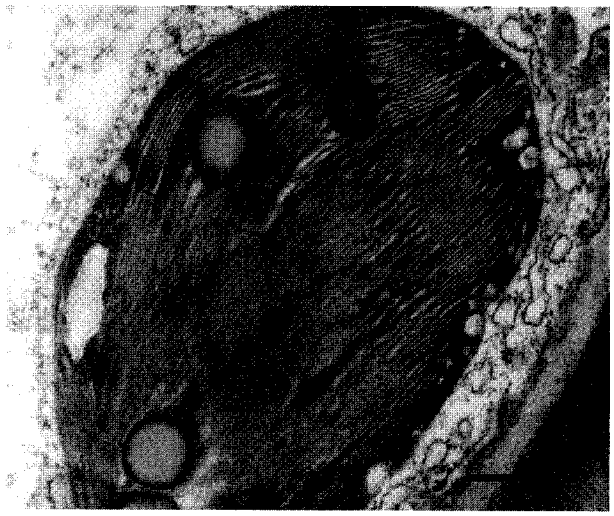

Fig. 3 Chloroplast with peripheral vesicles observed in mosaic-affected passion fruit leaf tissue

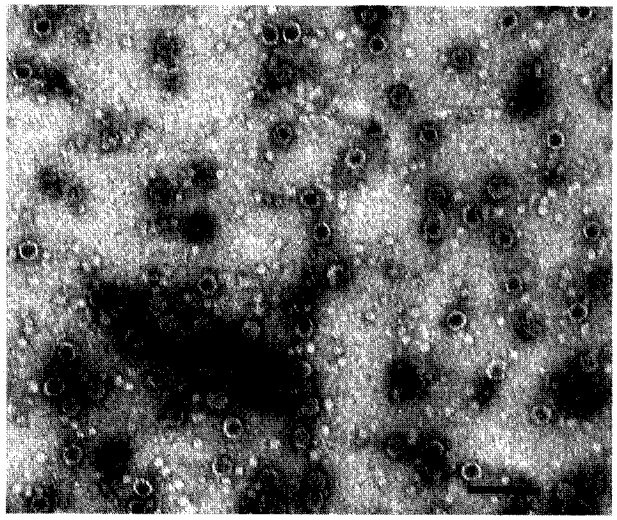

Fig. 2 Virus particles observed in serologically specific electron microscopy assays

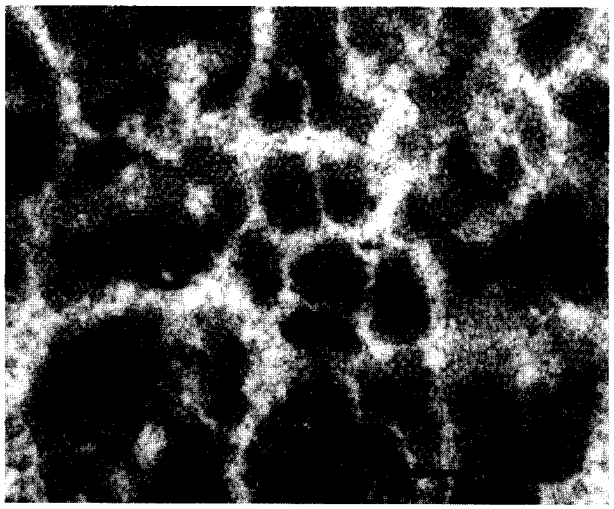

Fig. 4 Empty virus particle aggregates in the nucleus of an infected passion fruit leaf mesophyll cell

extracts from infected passion fruit plants produced a distinct band of approximately 6130 bp.

Host range

Of the 27 manually inoculated plant species, only *Passiflora adenopoda*, *P. edulis*, *P. pinnatistipula*, *P. quadrangularis*, *Physalis angulata*, *P. floridana*, and *P. peruviana*, developed systemic symptoms, and reacted positively in DAS-ELISA with the homologous antiserum prepared in Colombia. None of the test passion fruit plants that were inoculated using chrysomelids showed symptoms or reacted with antiserum to the Colombian virus isolate.

Molecular characterization

Two clones, of 473 (residues 81 through 555) and 324 (residues 3626–3949) bases, belonging to the putative ORF1 of the Colombian passion fruit virus, showed 62 and 66% nucleotide (nt) sequence identities, respectively, with the tymovirus *Kennedya yellow mosaic virus* (KYMV-Jervis Bay) from south-eastern Australia (Ding et al., 1990). These clones had amino acid (aa) sequence identities of 66 and 76%, respectively, with the corresponding sequences of KYMV. Two additional clones, of 442 and 493 bases, including the entire putative ORF3 (capsid protein) of the Colombian passion fruit virus, had consensus nt and aa sequence identities of 59.6 and 60% with the corresponding ORF3 of the KYMV-Bawley Point strain (Skotnicki et al., 1996). Clones 3 and 4 matched a region (residues 5462–6292) in ORF3 corresponding to the capsid protein (CP) gene of KYMV (residues 5711–6277), and had nt and aa sequence identities of 59.6 and 60%, respectively. The capsid protein of the Colombian passion fruit virus had a total length of 188 aa. The two putative ORF1 clones of the Colombian virus had lower (< 60%) aa sequences with corresponding genomic regions of *Turnip yellow mosaic virus*, *Ononis yellow mosaic virus*, *Physalis mottle virus* and *Eggplant mosaic virus*. However, sequences of ORF1 of only six tymoviruses are available. In the case of the putative CP gene (ORF3) of the Colombian virus, sequence data was available for 15 tymoviruses. A sequence comparison of the CP gene placed the Colombian passion fruit virus (PFYMV/Colombia) in a cluster with *Calopogonium yellow vein*, *Desmodium yellow mottle*, *Kennedya yellow mosaic*, *Okra mosaic*, *Clitoria yellow vein* and *Cacao yellow mosaic viruses* (Fig. 5).

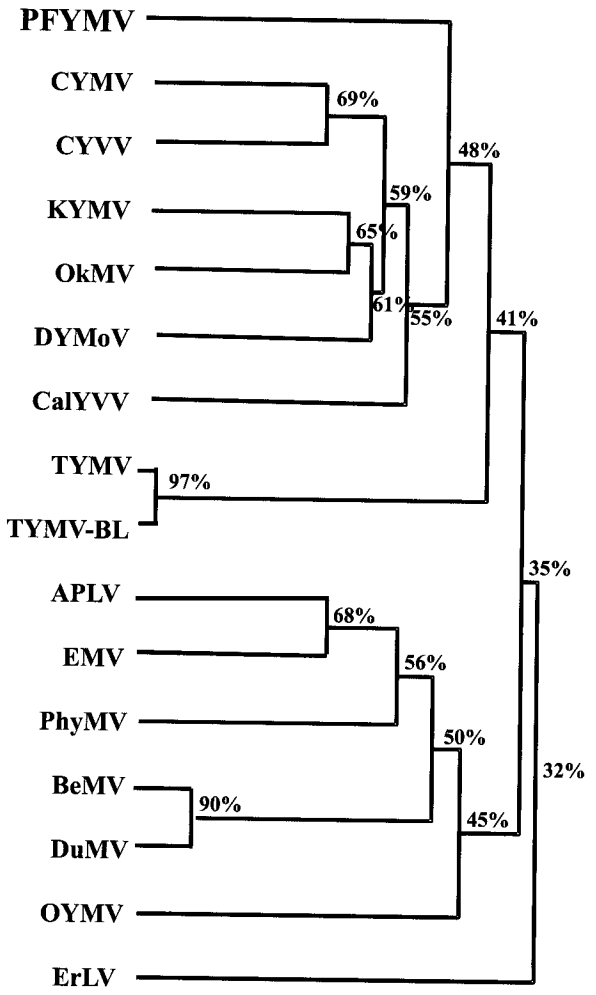

Fig. 5 Phylogenetic tree generated for the amino acid sequences in the capsid protein region of 15 different tymovirus species. PFYMV, Colombian tymovirus; CYMV, *Cacao yellow mosaic*; CYVV, *Calopogonium yellow vein*; KYMV, *Kennedya yellow mosaic*; OkMV, *Okra mosaic*; DYMoV, *Desmodium yellow mottle*; CalYVV, *Calopogonium yellow vein*; TYMV, *Turnip yellow mosaic* (BL = Blue Lake isolate); APLV, *Andean potato latent*; EMV, *Eggplant mosaic*; PhyMV, *Physalis mottle*; BeMV, *Belladona mottle*; DuMV, *Dulcamara mottle*; OYMV, *Ononis yellow mosaic*; ErLV, *Erysimum latent viruses*

Discussion

The morphology, cytopathology, antigenic properties, and partial molecular characterization of the isometric virus isolated from mosaic-affected passion fruit plants in Colombia clearly identifies it as a tymovirus (Koenig and Lesemann, 1979, 1981; Dreher et al., 2000). Its relatively low nucleotide and amino acid sequence identities with other tymoviruses sequenced to date shows that the Colombian tymovirus is a distinct species. However, the existence of a tymovirus (PFYMV) that induces similar symptoms on passion fruit in Brazil (Crestani et al., 1986), and is closely related serologically to the Colombian tymovirus, suggest that the two viruses are either closely related or the same. Consequently, it is proposed that the name *Passion fruit yellow mosaic virus* is retained for the tymoviruses found in both countries until a Brazilian PFYMV isolate is further characterized. Although the Colombian PFYMV was not transmitted by the chrysomelid species in the present tests, it is possible that they are the natural vectors of this tymovirus, as they are of the Brazilian isolate of PFYMV (Crestani et al., 1986). The Colombian PFYM tymovirus has a relatively restricted geographical distribution (present in four of 11 passion fruit-producing departments surveyed), unlike a *Soybean mosaic virus* strain that affects passion fruit wherever this crop is present in Colombia (Chávez et al., 1999). The management of chrysomelid populations in passion fruit-producing regions, and the present restricted

host range of the passion fruit tymovirus, should slow down the dissemination of this pathogen into other passion fruit production regions.

Acknowledgements

This investigation was supported by Biotechnology Division of the Colombian Institute for the Development of Science and Technology (COLCIENCIAS). The authors acknowledge the participation of Bibiana Chávez (CORPOICA) and Guillermo Guzmán (CIAT) in the collection of passion fruit samples and preparation of the graphic material used in this investigation. The Senior author is also employed by the International Plant Genetic Resources Institute (IPGRI).

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