A Strain of Soybean Mosaic Virus Infecting *Passiflora* spp. in Colombia

D. Benscher, S. S. Pappu, and **C. L. Niblett,** Plant Pathology Department, University of Florida, Gainesville 32611-0680; **F. Varón de Agudelo,** Instituto Colombiano Agropecuario, CNI, AA233, Palmira, Colombia; **F. Morales,** Virology Unit, Centro Internacional Agricultura Tropical, AA6713, Cali, Colombia; **E. Hodson** and **E. Alvarez,** Unidad Biologia Vegetal, Department Biologia, Pontificia Universidad Javeriana, Bogota, Colombia; **O. Acosta,** Department of Biochemistry, National University of Colombia, Bogota, Colombia; and **R. F. Lee,** Citrus Research and Education Center, 700 Experiment Station Road, Lake Alfred, FL 33850

ABSTRACT

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Viruses causing severe mosaic, epinasty, defoliation, and premature death of *Passiflora* spp. were isolated from west central Colombia. Western blot analyses indicated the presence of potyviruses. Two Colombian isolates (COL-22 and -GR) were compared to known potyviruses as to host range, symptomatology, serological activity, amino acid and nucleotide sequence similarity in the coat protein and 3′ noncoding region (3′NCR), and reactivity in dot-blot hybridization. Host range and symptomatology of COL-22 and -GR indicated they are similar to soybean mosaic virus (SMV). Also, both isolates reacted strongly with SMV antisera in immunodiffusion plates. Aphid transmission by *Aphis gossypii* and *Toxoptera citricida* was confirmed, but seed transmission was not demonstrated. In addition to COL-22 and -GR, five other Colombian isolates were cloned and sequenced. The deduced coat protein amino acid sequences for all of the Colombian isolates were virtually identical and shared a 98% similarity with SMV. Dot-blot hybridization experiments, using DNA complementary to the 3′NCR of the virus as a probe, further confirmed that the potyvirus infecting *Passiflora* spp. in Colombia is a strain of SMV. To our knowledge, infection of *Passiflora* spp. by a strain of SMV has not been previously reported.

The family *Passifloraceae* is comprised of about 550 species of woody and herbaceous plants. *Passiflora*, with over 400 species, is the largest of 12 genera in the family. Of the 60 edible species, only about 10 are well known for production worldwide. Popular species include *Passiflora edulis* (maracuyá púrpura), *P. edulis* f. *flavicarpa* (maracuyá amarillo), *P. cuadrangularis* (badea), and *P. ligularis* (granadilla) (33).

Due to the increased popularity and demand for the passionfruit flavor in juices and other food products, there has been an increase in *Passiflora* spp. production, especially in Latin America. About 4,000 ha of maracuyá and 2,500 ha of granadilla were in production in Colombia in 1991. Concurrent with the increased cultivation, there has been a dramatic rise in virus incidence, particularly from the potyvirus group (2,3,5,7,14,24,32).

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Corresponding author: D. Benscher
E-mail: dben@ifas.gnv.ufl.edu

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Most members of the potyvirus group have long, flexuous particles 680 to 900 nm long and 11 to 15 nm wide. The nucleic acid is single-stranded, positive-sense RNA of about 10,000 nucleotides, and it encodes a polyprotein of about 350 kD. The RNA is packaged by a single structural coat protein (CP) made up of about 2,000 repeating units (9). The CPs range in size from 30 to 45 kD. The 3′ noncoding region (3′NCR) and polyadenylated tail are immediately downstream to the CP gene (1,16). The CP amino acid and 3′NCR nucleotide sequences have been studied extensively for comparison and identification of potyviruses (13,23).

Soybean mosaic virus is a member of the potyvirus group and has been reported to infect 28 genera, 23 of which are in *Fabaceae* (4,6,10). The virus is easily transmitted mechanically, and transmission has been documented for at least 33 species of aphids (10). Soybean mosaic virus also can be seed transmitted (rates as high as 30%), which helps explain its worldwide distribution (4,6).

We report here the characterization and identification of a virus that is widespread and destructive in maracuyá and granadilla in west central Colombia. Its host range, coat protein amino acid sequence, and nucleotide sequence, as well as molecular hybridization data, indicate that the virus infecting *Passiflora* spp. is a strain of soybean mosaic virus.

MATERIALS AND METHODS

Viruses. Samples were collected from commercial fields of *Passiflora* spp. between Cali and Pereira, Colombia, a distance of about 150 km. Plants used for sampling were symptomless or exhibited severe decline, defoliation, mosaic, or ring spot symptoms. Viruses in the samples were transmitted mechanically from the different *Passiflora* spp. to maracuyá amarillo, granadilla, badea, and *Phaseolus vulgaris* cv. Black Turtle 2 (BT-2). Infected tissue was ground in 0.05 M K_2HPO_4 buffer, pH 8.5, at a 1:10 dilution (wt/vol) and rubbed on 400-mesh Carborundum-dusted leaves (Union Carbide, Buffalo, NY). For comparison, passionfruit woodiness virus (PWV-K; 14) was maintained on maracuyá and BT-2, and two isolates of soybean mosaic virus, SMV-76/6 and SMV-N (11), were maintained on BT-2.

Virus detection. Western blot analysis was performed on samples to detect the presence of potyviruses (29,31). Approximately 1 cm^2 of leaf tissue was ground with a mortar and pestle with 0.5 ml of extraction buffer (0.125 M Tris-HCl at pH 6.8, 4% sodium dodecyl sulfate [SDS], 20% glycerol, and 10% ß-mercaptoethanol), boiled for 4 min, briefly vortexed, and centrifuged. Ten microliters of the supernatant were run on a 12% SDS-polyacrylamide gel electrophoresis (PAGE) gel. The protein was transferred to a nitrocellulose membrane using a Mini Trans Blot (BIO-RAD, Hercules, CA) and probed with the potyvirus-specific PTY-1 antibody (20; Agdia, Inc., Elkhart, IN).

Ouchterlony double diffusion assays were performed using SMV antisera and methods described previously (15).

Polymerase chain reaction (PCR) using potyvirus-specific primers also was used for virus detection (see "Reverse transcription and amplification" below).

Host range and transmission studies. A host range comparison was performed by inoculating three *Phaseolus vulgaris* cultivars (Black Turtle 1 [BT-1], BT-2, and California Light-Red Kidney [RKB]), two *Pisum sativum* cultivars (Ranger and Bonneville), *Glycine max* cv. Kirby, maracuyá amarillo, and granadilla with the two Colombia isolates (COL-GR and COL-22), SMV 76/6, and PWV-K. Only BT-2 and maracuyá were inoculated with SMV-N.

Fifty maracuyá seedlings and 20 granadilla seedlings were inoculated in a separate test with partially purified SMV 76/6. Twenty grams of BT-2 tissue infected with SMV 76/6 was homogenized in 0.1 M KH_2PO_4, pH 8.0, containing 3% ß-mercaptoethanol. The homogenate was extracted with 1/10 volume of chloroform and centrifuged for 10 min at 3,000 × *g*. The supernatant was adjusted to 4% polyethylene glycol and 0.2 M NaCl and kept at 4°C for 90 min. The virus was pelleted by centrifugation at 12,000 × *g* for 10 min and resuspended in 2 ml of inoculation buffer (17). Twenty microliters of this preparation were placed on maracuyá or granadilla stems and slash-inoculated using a scalpel. Approximately 20 shallow axial cuts were made on each plant. Forty maracuyá and 10 granadilla stems were slash-inoculated, and leaves of an additional 10 plants of each were mechanically inoculated with the partially purified virus. BT-2 and BT-1 were inoculated as positive controls to monitor infectivity. Plants were maintained in a growth chamber at 25°C with 12 h of diurnal fluorescent light.

Seed transmission was investigated in both maracuyá and granadilla infected with the Colombian virus isolates. Seeds were collected from the fruit of several infected plants of each species, dried, and planted in autoclaved soil in flats. Seedlings were reared in an insect-free greenhouse and monitored for 12 weeks for symptom development.

Aphid transmission was studied using the melon aphid, *Aphis gossypii* Glover, and the brown citrus aphid, *Toxoptera citricida* Kirkaldy, in maracuyá and granadilla. After 6 h of starvation, aphids were permitted a 1-min acquisition feed on infected *Passiflora* spp., followed by a 1-min inoculation feed on healthy *Passiflora* spp. and removal of the aphids. Twenty aphids were used for each inoculation. Plants were observed for symptoms over 12 weeks.

Reverse transcription and amplification. Total nucleic acids were extracted from about 1 cm^2 of leaf tissue, which was either fresh, stored in 50% glycerol at –20°C, or desiccated. Coupled reverse transcription and subsequent amplification by polymerase chain reaction (RT-PCR) of the CP gene region were performed as described using selected primers (23). The amino acid regions WCIEN and QMKAAA are highly conserved among potyviruses. Genomic-sense, degenerate oligonucleotide primers for the amino acid regions WCIEN and QMKAAA, in concert with genome antisense, oligo-dT primers with 3′ terminal degeneracy (22,23,30) were used to generate fragments of 700 or 350 bp, respectively.

The fragments were identified on an 0.8% agarose gel when run with λ-*Hin*dIII size markers. The 700-bp fragment (WCIEN) included the C-terminal half of the CP gene, the adjacent 3′NCR, and 20 residues of the polyadenylated tail. The 350-bp fragment (QMKAAA) included the region coding for 50 amino acids from the C-terminus of the CP, with the adjacent 3′NCR and 20 residues of the polyadenylated tail.

Cloning and sequencing. The 700-bp WCIEN fragment was purified by electrophoresis on a 0.8% low-melting-point agarose gel (GIBCO-BRL, Gaithersburg, MD), treated with the Klenow fragment of DNA polymerase I (Promega, Madison, WI), phosphorylated with T4-polynucleotide kinase (US Biochemical, Cleveland, OH), and ligated into *Sma*I cut pUC 118, as previously reported (27). The ligated plasmid was used to transform competent cells of *Escherichia coli* DH5α. Plasmid DNA was prepared by a standard miniprep boiling procedure (27). Double-stranded plasmid DNA was sequenced by the dideoxy chain termination method, using the version 2.0 Sequenase kit (US Biochemical) (28). Sequence analyses were performed using the Seqaid-II (version 3.60) (26), University of Wisconsin Genetics Computer Group (8), Molecular Evolutionary Genetics Analysis (MEGA, version 1.01), and CLUSTAL V (18) programs.

Dot-blot hybridization. Templates were total nucleic acid extracts of healthy and infected plants. One gram of tissue was ground in liquid nitrogen, then triturated with 1.8 ml of extraction buffer (1 ml of 3-*N*-[Morpholino]propanesulfonic [MOPS] buffer [0.2 M MOPS, 0.05 M sodium acetate, and 0.01 M EDTA, pH 7.0], 1.6 ml of 37% formaldehyde, and 5 ml of formamide) and 0.6 ml of distilled water. The extract was heated at 65°C for 5 min, mixed with 2.4 ml of 20× SSC (3 M NaCl and 0.3 M sodium citrate, pH 7.2), and centrifuged for 10 min at 16,000 × *g* (13). One hundred microliters of 1:5 and 1:20 dilutions (in 10× SSC) of the supernatant were applied to a Gene Screen Plus nylon membrane (DuPont, NEN, Boston, MA) using a vacuum manifold (Schleicher and Schuell, Inc., Keene, NH).

Complementary DNA probes specific to the potyviral 3′NCR were prepared by using gel-purified PCR products of the respective 3′NCR fragments as templates (21) and random primers and Klenow in the presence of α^{32}P-dCTP (DuPont, NEN) (12). The membranes were probed for 8 h at 42°C with 1×10^6 cpm of the probe and then treated as previously described (21).

RESULTS AND DISCUSSION

A potyvirus was found to be widespread and causing extensive damage and yield reductions in *Passiflora* spp. in Colombia in 1991 and 1992 surveys. Vines of maracuyá infected with the virus showed intense chlorosis, mosaic, and epinasty (Fig. 1A), followed by hardening of the leaves, severe defoliation (Fig. 1B), and premature death. Ring spots on maracuyá fruit were also observed (Fig. 1C). Granadilla exhibited mosaic and anthocyanin pigmentation of the leaves, and the fruit showed ring spots (Fig. 1D). In many cases, all plants in a field were diseased. The presence of a potyvirus in affected plants was confirmed by Western blot analysis using the potyvirus-specific monoclonal antibody PTY-1 (20; Agdia)

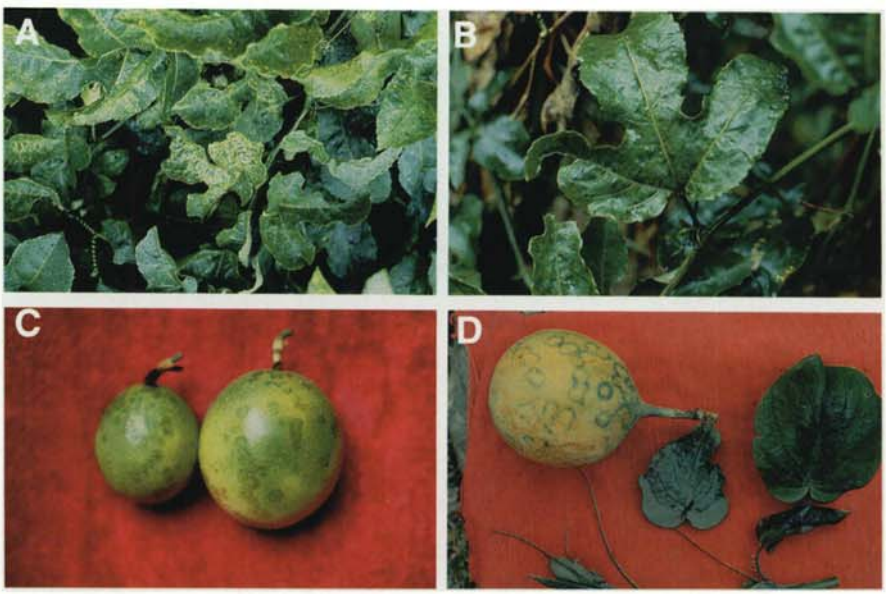

Fig. 1. Symptoms of Colombian potyvirus isolates COL-22 on maracuyá and COL-GR on granadilla *Passiflora* spp.: **(A)** Early infection of maracuyá showing chlorosis, epinasty, and mosaic symptoms on the leaves. **(B)** A later stage of infection of maracuyá exhibiting mosaic, leaf rugosity, and defoliation in the background. **(C)** Fruit of maracuyá showing characteristic ring spots. **(D)** Fruit and foliage of granadilla infected with the isolate COL-GR. The leaves show veinal pigmentation, while the fruit exhibit ring spots.

Table 1. Host plant reactions to Colombian passionfruit potyviruses inoculated on differential bean and pea cultivars, *Passiflora edulis* f. *flavicarpa* (maracuyá), and *P. ligularis* (granadilla) in comparison to known potyviruses

Virus	BT-1[a] bean	BT-2[a] bean	RKB[a] bean	Ranger pea	Bonneville pea	Maracuyá	Granadilla
COL-22[b]	AN[c]	S[c]	S	S	R[c]	S	S
COL-GR[b]	AN	S	S	S	R	S	S
SMV 76/6[b]	AN	S	S	R	R	0/50[d]	0/20[d]
PWV-K[b]	R	S	S	S	R	S	S

[a] BT-1 and BT-2 are Black Turtle-1 and -2 bean, respectively; RKB is California Light-Red Kidney bean.
[b] COL-22 and COL-GR are Colombian passionfruit potyviruses; SMV 76/6 is an isolate of soybean mosaic potyvirus; PWV-K is passionfruit woodiness potyvirus.
[c] Host reaction: AN = apical necrosis, followed by plant death; S = susceptible, systemic symptoms; R = resistant, free of any symptoms and recoverable viruses.
[d] No infection resulted from several inoculation methods.

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                                                                    50
COL-22    WCIDNGTSPDANGVWVMMDGKEQIEYPLKPIVENAKPTLRQIMHHFSDAA
COL-GR    --------------------------------------------------
COL-F     --------------------------------------------------
COL-I     --------------------------------------------------
COL-N     --------------------------------------------------
COL-S     --------------------------------------------------
COL-V     --------------------------------------------------
SMV-76/6  --------------------E-------------------------------
SMV-N     --------------------E-------------------------------
PWV-K     ----------V---------D--V------M---------------------

                                                                   100
COL-22    EAYIEMRNSESLYMPRYGLLRNLRDRELARYAFDFYEVTSKTPNRAREAI
COL-GR    --------------------------------------------------
COL-F     --------------------------------------------------
COL-I     --------------------------------------------------
COL-N     --------------------------------------------------
COL-S     --------------------------------------------------
COL-V     --------------------------------------------------
SMV-76/6  -----------P--------------------------------------
SMV-N     -----------P--------------------------------------
PWV-K     -------CASGP-------------KN----------NA--SD-----V

                                                                   151
COL-22    AQMKAAALSGVNNKLFGLDGNISTNSENTERHTARDVNQNMHTLLGMGPPQ
COL-GR    --------------------------------------------------
COL-F     --------------------------------------------------
COL-I     --------------------------------------------------
COL-N     ----------------------------------N---------------
COL-S     --------------------------------------------------
COL-V     --------------------------------------------------
SMV-76/6  ------------------------------------------------Q-
SMV-N     --------------------------------------------------
PWV-K     ---------N-T---------VA-I--D--------A-E---S------V-
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Fig. 2. Multiple alignment of deduced amino acid sequences of a portion of the coat protein (CP) gene of known potyviruses and isolates from Colombia using CLUSTAL V sequence analysis program. (–) denotes identical amino acid residues. Underlined regions show the location of the degenerate oligonucleotide genome-sense primers for the WCIEN and QMKAAA regions used for amplifying the CP gene and 3′ noncoding region (3′NCR), respectively. COL-X indicates the various isolates from Colombia, SMV-N and 76/6 indicate soybean mosaic virus isolates, and PWV-K indicates passionfruit woodiness virus.

(data not shown). This analysis resulted in the selection of seven virus isolates for further comparison. Extensive host range tests were performed only with isolates COL-22 and COL-GR, which were obtained from maracuyá and granadilla, respectively. Of the remaining isolates, four were collected from maracuyá (COL-F, -I, -N, and -V) and COL-S was collected from *P. cuadrangularis* (badea). In the field, COL-22 was associated with severe mosaic (Fig. 1A), rugosity of the leaves, subsequent defoliation, and premature death of maracuyà. In granadilla, COL-GR was associated with a mild mottle, veinal anthocyanin pigmentation in an oak-leaf pattern in leaves, and ring spots on the fruit (Fig. 1C). Foliar symptoms were reproduced in the greenhouse for both isolates on both *Passiflora* spp. Fruit symptoms on granadilla and maracuyà were not reproduced in the greenhouse because fruit did not set. Symptomatic fruit from the field were indexed by Western blot using PTY-1 antibody (20). Virus was detected in the fruit mesocarp, endocarp, and funiculi on the ovary wall, but it was not detected in the ring spots in the hard, thin pericarp (25). The potyvirus was detected in both the leaves and fruit of all the affected granadilla samples tested, but we did not demonstrate unequivocally that it caused the ring spots on the granadilla fruit.

Colombian isolates COL-22 and -GR exhibited identical host ranges. Both isolates caused apical necrosis resulting in plant death in BT-1 and systemic mosaic of BT-2, RKB, Ranger pea, soybean, maracuyá, and granadilla (Table 1). Subsequent inoculations were performed from each plant to a new set of host plants, and the same results were observed. The reactions in bean and soybean were very similar to those caused by SMV 76/6. However, SMV 76/6 was unable to infect Ranger pea, maracuyá, or granadilla. Similar differential reactions of SMV isolates on pea have been demonstrated previously (19).

Ouchterlony double diffusion tests were performed using an antibody to SMV to confirm infection and determine the relationship between the viruses (15). With extracts from symptomatic plants, precipitin lines formed and fused for COL-GR, COL-22, and SMV 76/6 without spur formation, which indicated that serologically the viruses are quite similar. Extracts from nonsymptomatic Bonneville pea plants did not form precipitin lines against the SMV antibody (data not shown). Maracuyá plants leaf- or slash-inoculated with partially purified SMV 76/6 failed to develop symptoms, and the virus was not detectable serologically. Representative maracuyá plants inoculated with SMV 76/6 or SMV-N were also indexed by RT-PCR and produced no potyvirus-related PCR products. Control plants of BT-1 and BT-2 inoculated with the same preparations readily developed characteristic symptoms in all of the plants inoculated. The host range and symptoms caused by PWV-K were distinct from both the Colombian and SMV isolates (Table 1). To detect the possible presence of watermelon mosaic virus II and cucumber mosaic virus, two cucurbits, *Cucurbita pepo* cv. Seneca hybrid and *Cucumis sativus* cv. Marketmore, were included in host range tests. Marketmore is susceptible to WMV-2 but resistant to CMV, whereas Seneca hybrid is highly

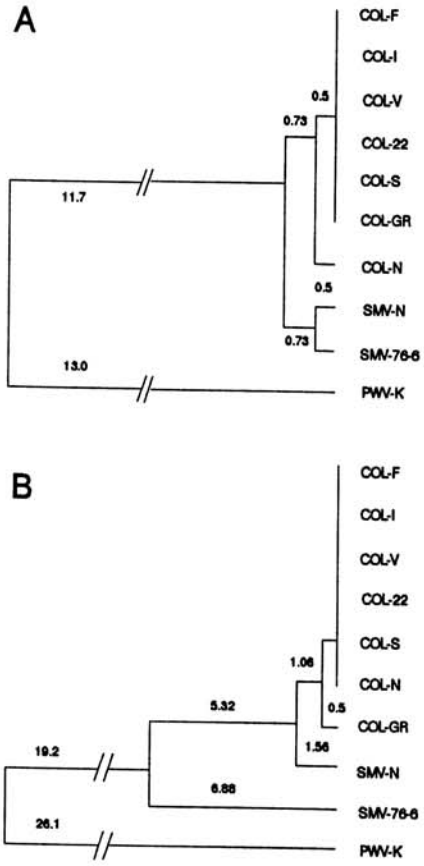

Fig. 3. Dendrograms of the sequence analysis of potyvirus coat protein (CP) and 3′ noncoding region (3′NCR) using an unweighted pair-group method with arithmetic means (UPGMA-MEGA). **(A)** Dendrogram comparing the deduced amino acid sequences in the CP region. The Colombian isolates share a 98 and 83% similarity to soybean mosaic virus (SMV) and passionfruit woodiness virus (PWV-K), respectively. **(B)** Dendrogram comparing the 3′NCR. The calculated distances in both figures are a measure of sequence divergence.

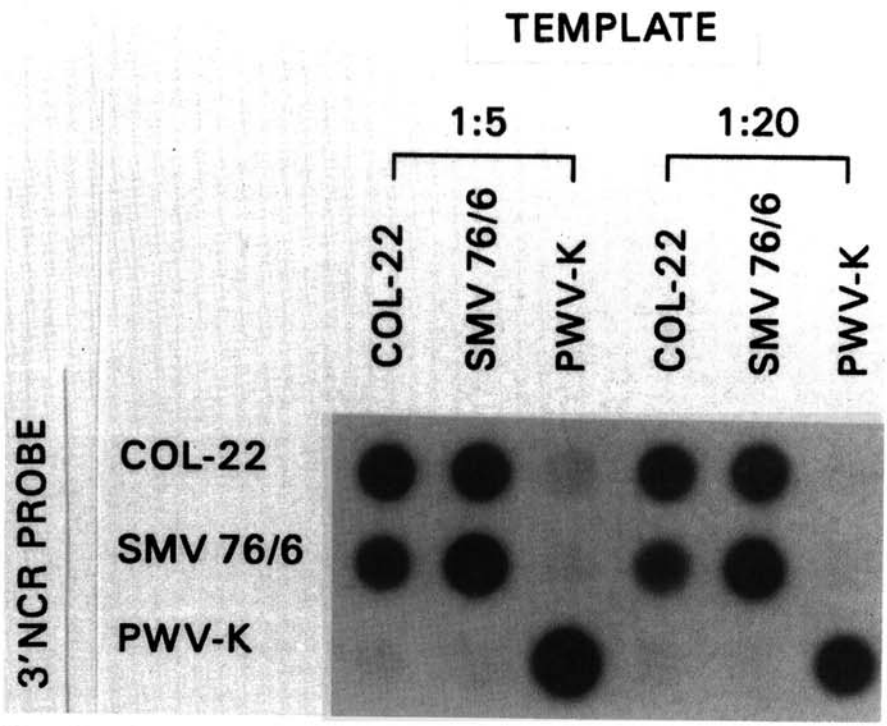

Fig. 4. Autoradiogram showing dot-blot hybridization of cDNA probes of the 3′ noncoding region (3′NCR) of the indicated viruses and extracts from infected tissue. Tissue extract dilutions of 1:5 and 1:20 were prepared as templates for COL-22 (Colombian potyvirus isolate), SMV 76/6 (soybean mosaic virus), and PWV-K (passionfruit woodiness virus). The blots were probed with the α^{32}P-dCTP-labeled 3′NCR of the same viruses. Film exposure was for 30 min.

susceptible to both viruses. Neither virus was detected (data not shown).

In the seed transmission tests, over 5,000 maracuyá seedlings and 1,000 granadilla seedlings were grown and monitored for symptom expression, but no symptoms were observed.

Aphid transmission of the Colombian virus by *A. gossypii* was demonstrated in 23 of 28 plants tested, confirming earlier work (32). *T. citricida* was also able to transmit the virus, although inefficiently, infecting only eight of 68 plants tested. Assays were performed on symptomatic and nonsymptomatic maracuyá and granadilla plants of various ages from the field, seed, and aphid transmission tests. Western blot, RT-PCR, and mechanical inoculation indicated that the potyvirus was present only in symptomatic plants (data not shown).

The two Colombian isolates, COL-22 and -GR, were identical in the CP amino acid sequences, as were four of the five other Colombian isolates from separate field collections (COL-F, -I, -S, and -V). Although silent third-base nucleotide substitutions did occur, the only difference found among the seven Colombian isolates in the CP was at amino acid 136. Here, COL-N had an asparagine residue, while the other Colombian isolates had an aspartic acid residue. The Colombian isolates differed in only two or three amino acids from the two SMV isolates, but there were many differences between the Colombian isolates and PWV-K (Figs. 2 and 3A). Comparison of the 3′NCR sequences resulted in a similar relationship between the viruses (Fig. 3B). COL-22 and -GR shared a 98% similarity in the CP region (Fig. 2) and a 90 and 98% similarity in the 3′NCR with SMV 76/6 and that reported for SMV-N (11), respectively. However, the Colombian isolates differed in 26 amino acids from the sequence reported for PWV-K (14), sharing only an 83% similarity in the CP region and a 55% similarity in the 3′NCR.

For comparative hybridization studies, tissue samples were prepared from infected BT-2, as this cultivar yields a high virus titer. Since the Colombian isolates and the SMV isolates were similar, one representative of each (COL-22 and SMV 76/6, respectively) was included in the blot. Passionfruit woodiness virus was included as an heterologous control. When probed with either the SMV or COL-22 3′NCR, strong hybridization occurred with the COL-22 and SMV samples, but not with the 3′NCR of PWV-K. The PWV-K probe hybridized only with its homologous sample (Fig. 4). Similar hybridization results were obtained using tissue extracts from infected maracuyá, but virus titer was lower and more erratic than when BT-2 was used. No hybridization occurred between any of the probes and healthy tissue (data not shown). These results correlate well with the sequence data. The 3′NCR is one of the more variable regions in the potyvirus genome; thus comparisons of sequence and hybridization of the 3′NCR have proven to be useful methods for characterizing and differentiating potyviruses and their strains (13,23).

On the basis of host range, symptomatology, serology, sequences of nucleic acids and deduced proteins, and hybridization data, we conclude that a single virus, soybean mosaic virus, is infecting at least three *Passiflora* spp. (maracuyá, granadilla, and badea) over a large geographic area in west central Colombia. However, we were unable to infect maracuyá or granadilla with two strains of soybean mosaic virus from the United States. In this research, we developed methods and probes to detect and identify potyviruses infecting *Passiflora* spp. These methods should be useful in detecting and characterizing potyviruses infecting other crop species.

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