Etiology

Purification, in situ Localization, and Comparative Serological Properties of Passionfruit Woodiness Virus-Encoded Amorphous Inclusion Protein and Two Other Virus Proteins

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ABSTRACT

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Passionfruit woodiness potyvirus (PWV) is one of the major limiting factors for growing passionfruit in Taiwan. To characterize this virus at the molecular level, a viral protein with M_r 51,000 (51K) was purified by a procedure developed in this study. Extracts from leaf tissues of PWV-infected passionfruit (*Passiflora edulis* f. *flavicarpa*) or tobacco (*Nicotiana benthamiana*) were treated with 5% Triton X-100 and centrifuged at 220 *g* for 10 min. The resulting pellets were further processed with two cycles of 5% Triton X-100 treatment and low-speed centrifugation (900 *g* for 10 min). The 51K protein was then purified from the pellets by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and antisera were produced in New Zealand white rabbits. Light microscopic immunostaining with the IgG to the 51K protein and protein A-gold complex demonstrated that the cytoplasmic amorphous inclusions were the major location of the 51K protein. Immunogold labeling with the IgG to the 51K protein in electron microscopy also revealed that gold particles were mainly distributed on the cytoplasmic amorphous inclusions and not on organelles such as nuclei and chloroplasts. The localization of the 51K antigen was distinct from that of the cylindrical inclusion protein (CIP, 66K) and the coat protein (CP, 36K), which were mainly concentrated on cytoplasmic cylindrical inclusions and virions, respectively. These results verified that the 51K protein is the amorphous inclusion protein (AIP) of PWV. Serological analyses by immunodiffusion, indirect enzyme-linked immunosorbent assay (ELISA), and immunoblotting of different viral antigens produced in vivo indicated that the purified AIP is serologically distinct from CP and CIP of PWV. Among the 14 potyviruses tested by SDS-immunodiffusion, antiserum to PWV CP reacted with extracts from plant tissues infected with New York and Florida isolates of watermelon mosaic virus 2 (WMV-2), and black eye cowpea mosaic virus (Florida), showing spur with the homologous antigen. The antiserum to PWV CIP, on the other hand, only spur-reacted with sap from plant tissue infected with Florida isolate of MWV-2 and did not react with any of the other heterologous antigens tested.

Additional keywords: light microscopy, serological relationships.

The potyviruses represent the largest and one of the economically most important virus groups affecting many cultivated plants. Their particles are flexuous rods of 680–900 × 11–15 nm that have a monopartite, single-stranded, positive-sense RNA genome of about 10 kb with a genome-linked protein (VPg) at the 5′-end and a poly (A) stretch at the 3′-end (10,13,20). In infected cells, potyviruses induce the synthesis of viral nonstructural proteins to form distinct structures known as inclusion bodies, which include the cylindrical inclusion (CI), the amorphous inclusion (AI), and the nuclear inclusion (NI) (12). The CIs are found in the cytoplasm of potyvirus-infected plant cells and consist of a protein monomer with a relative molecular mass (M_r) of 67,000–73,000 (67–73K) (11,16,17). The NIs are associated with infections by several members of the potyvirus group and some of these have been demonstrated to contain two proteins, the large NI protein (54K) and the small NI protein (49K) (4,11,21). Certain potyviruses induce AIs, which are irregular-shaped inclusions occurring in cells infected with papaya ringspot virus strains W and P (PRSV-W and PRSV-P), pepper mottle virus (PepMoV), pepper venial mottle (PVMV), potato virus-Y (PVY), turnip mosaic virus R-strain (TuMV-R) (6,13), watermelon mosaic virus 2

(WMV-2) (29), and zucchini yellow mosaic virus (ZYMV) (30,31). The AIs induced by PepMoV, PRSV-W (8), PRSV-P (35), WMV-2 (29), and ZYMV (30,31) have been purified and characterized. The AI consists of a virus-specific protein of 46–51K. Antisera to AI bodies produced by PepMoV and PRSV reacted with the in vitro translation products of the corresponding viral RNA, and the immunoprecipitation products were indistinguishable from those immunoprecipitated by an antiserum to the helper component (HC) protein of tobacco vein mottling virus (8,9,18). The direct evidence to link the AI protein with the HC protein was shown by Baunoch et al (1) by immunoblotting and immunogold-labeling techniques.

Passionfruit woodiness virus (PWV), first described by McKnight (27), is a member of the potyvirus group, with flexuous particles about 750 nm in length (20,32). The disease caused by PWV is characterized by foliar symptoms of mosaic, distortion and rugosity, and production of woody and severely malformed fruits (33). This virus was found in Taiwan in 1981 (5) and has since become widespread in the passionfruit-growing areas across the island. PWV greatly shortens the economic life of plants and is a major limiting factor for the production of the passionfruit cv. Tainung No. 1 (TN-1, a hybrid of *Passiflora edulis* Sims × *P. edulis* f. *flavicarpa* Degener) (3,5). The coat protein (CP, 36K) and the CI protein (CIP, 66K) of PWV have been purified and characterized (3,26). Antisera against the virions or CP of PWV have been widely used for detection of PWV (3,25). Evidence of cytoplasmic virus-specific vesicles as the possible site for virion assembly was found and CIs of PWV were shown at sites on endoplasmic reticula (26).

To further characterize PWV at the molecular level, the methods for the purification of the AI protein (AIP) of PRSV (8,35) and for the purification of nuclear inclusion proteins (NIPs) of TEV (21) and BYMV (4) were initially attempted in order to purify the AIP of PWV. However, the inadequacy of previously published methods prompted us to develop a new purification procedure for PWV AIP. A viral protein with M_r of 51,000 (51K) from the PWV-infected plants was purified and an antiserum against it was produced. Antisera to the 51K protein and the other two PWV-encoded proteins CIP and CP were used to localize the three antigens in PWV-infected cells by light microscopic immunostaining and immunoelectron microscopy. Also, serological properties of the three antigens in PWV-infected plant tissues were compared with those of 14 other potyviruses.

MATERIALS AND METHODS

Virus and hosts. The Taiwan virus isolate PWV-TW, originally obtained from diseased plants of TN-1 passionfruit grown at Fengshan Tropical Horticultural Experiment Station in Kaoshiung and identified as passionfruit woodiness virus (3,33), was used in this study. The virus was maintained in golden passionfruit (*Passiflora edulis* f. *flavicarpa* Degener) and *Nicotiana benthamiana* Domin.

Light microscopy of cytoplasmic inclusions. The cytoplasmic inclusions in epidermal strips of the leaves of PWV-infected golden passionfruit or *N. benthamiana* were examined under a light microscope, after clearing the cells by immersing the tissue in 5% Triton X-100 for 3–5 min and staining with the Calcomine orange–Luxol brilliant green BL (O/G) according to the method of Christie and Edwardson (6). Comparable noninfected tissues from the two host species were used as controls.

Partial purification of AIP. Leaves of PWV-infected *N. benthamiana* or golden passionfruit were harvested 10–14 days after mechanical inoculation and homogenized with the extraction buffer (0.1 M potassium phosphate, pH 7.5, containing 0.25% sodium sulfite; 3 ml per gram of tissue) in a Waring blender for 3 min. The homogenate was filtered through four layers of cheesecloth and the resulting filtrate was stirred with 5% Triton X-100 (final concentration) in a cold room for 1.5 h, then centrifuged at 220 *g* for 10 min in a Beckman JA-14 rotor. The pellets were then resuspended in the resuspension buffer (0.02 M potassium phosphate buffer, pH 8.2, containing 0.5% sodium sulfite; 1.3 ml per gram of leaf tissue) and centrifuged at 900 *g* for 10 min. The resulting pellets were resuspended in the resuspension buffer and further treated with two cycles of 5% Triton X-100 and low-speed centrifugation (900 *g* for 10 min). Finally, the pellets were resuspended in a small volume of 20 mM Tris-HCl, pH 8.2. This preparation was referred as partially purified PWV AIP.

To monitor the distribution of AIP during purification, samples saved from each step of the partial purification were electrophoresed in polyacrylamide minislab gels. The dissociated proteins were loaded on 12% sodium dodecyl sulfate (SDS)–polyacrylamide gels and electrophoresed at 180 V for 50 min (24). Protein bands were visualized by staining with Coomassie blue R-250.

Further purification of the AIP by gel electrophoresis. The partially purified AIP was further purified by preparative slab-gel electrophoresis as described by Yeh and Gonsalves (34) with some modifications. Partially purified proteins were dissociated by adding one-third volume of 4× dissociation buffer (0.4 M Tris-HCl, pH 6.8, 10% SDS, 20% 2-mercaptoethanol, 20% sucrose, and 0.005% bromophenol blue), heated in boiling water for 3 min, centrifuged at 2,500 *g* for 5 min to eliminate insoluble materials, and then loaded onto the stacking gel. Electrophoresis was conducted at 60 V for 16 h. A 51K protein was visualized by soaking the gel in cold 0.25 M KCl and was eluted from the gel using an ISCO electrophoretic concentrator. Yields of the purified protein were estimated by absorbance at 280 nm, and then stored at −20 C.

Purification of CIP and virus coat protein. CIs were purified from PWV-infected leaf tissue of *N. benthamiana* as described by Yeh and Gonsalves (34). The virus was also purified from leaves of PWV-infected *N. benthamiana* by chloroform-carbon tetrachloride clarification, polyethyleneglycol precipitation, and two cycles of Cs_2SO_4 density gradient centrifugation (14). Purified virus was resuspended in PEN buffer (0.01 M sodium phosphate, pH 7.0, 0.001 M EDTA, and 0.01 M N_aN_3), and stored at 4 C. The CIP and CP of PWV were further purified by preparative gel electrophoresis and electroelution as described above.

Production of antisera. Antisera to the CP, CIP, and 51K protein of PWV-TW were produced in New Zealand white rabbits as described by Yeh and Gonsalves (34) with modifications. One milligram of each purified protein in 1.0 ml of 0.004 M Tris-acetate, pH 8.4, containing 0.002 M EDTA, was emulsified with 1.0 ml of Freund's complete adjuvant, and injected intramuscularly into the hind legs of individual rabbits. The antigens, emulsified with Freund's incomplete adjuvant, were then administered three times at weekly intervals. Rabbits were weekly bled after the fourth injection for a period of 4–6 mo. The titters of the antisera were determined by SDS-immunodiffusion tests.

Light microscopic immunostaining. A light microscopic immunostaining method (23) was used to verify that the 51K protein was the AIP of PWV. Fresh epidermal strips from PWV-infected leaves of golden passionfruit were treated with 5% Triton X-100 in distilled water for 5 min and then floated on a solution of 10% pectinase and 4% cellulase in 20 mM phosphate-buffered saline (PBS, pH 7.2) for 30–60 min. They were incubated first with antisera against the CIP or the 51K protein (1:400 dilution) and second with protein A-gold complex (Sigma, P-1039; diluted at 1:6), each at 37 C for 30 min. These tissues were extensively rinsed with PBS after each treatment. Immunostained tissues were mounted on slides in 10% glycerine and viewed under a light microscope with a blue filter. Nonspecific binding of antibodies was checked by staining the infected tissues with normal serum or exposing healthy tissues to the antiserum against CIP or 51K protein.

Immunoelectron microscopy. An immunoelectron microscopy method was used to further confirm that the 51K protein was the AIP of PWV. PWV-infected leaf tissues of golden passionfruit were fixed with 5% glutaraldehyde, dehydrated in a ethanol series, and embedded in LR White according to the method of Ko (22). After embedding and polymerization, ultrathin sections were cut with a glass knife and collected on copper grids (75 mesh) coated with carbon-backed Formvar films. Sections on grids were placed in 1% bovine serum albumin (in 20 mM PBS, pH 7.2) to prevent

nonspecific binding of antibodies, and then incubated with individual antisera to CP, CIP, or the 51K protein at 1:400 dilution for 30 min. The grids were washed with PBS and then incubated with a 1:20 dilution of protein A-gold complex for 30 min. The grids were further washed in PBS, rinsed in distilled water, and poststained with 2% uranyl acetate for 15 min followed by 0.5% lead citrate for 1.5 min. The samples were examined by a JEOL 200 LX electron microscope. Normal serum and healthy tissues were also used as controls.

SDS-immunodiffusion test. SDS-immunodiffusion tests were conducted as described by Purcifull and Batchelor (28). The agar plates contained 0.8% agar, 1.0% sodium azide, and 0.5% SDS. Purified virus and inclusion proteins were dissociated in 1.0% SDS. Crude antigens were prepared from freshly harvested leaves of PWV-infected golden passionfruit, 1 g of tissue was ground in 1 ml of distilled water followed by the addition of 1 ml of 3% SDS. The samples were strained through cheesecloth and used immediately or stored at −20 C. Antisera to PWV 51K protein (AIP), CIP, and CP, collected 21, 42, and 28 days after the fourth injection, respectively, were used. Undiluted antisera were added to appropriate wells and the plates were incubated in a moist chamber at room temperature for 18–36 h.

The serological relationships of PWV with other potyviruses were studied using SDS-dissociated antigens prepared from virus-infected plant tissues. Bean common mosaic virus (BCMV-NY15), bean yellow mosaic virus (BYMV, BV 2 strain), clover yellow vein virus (ClYVV, B1 type), soybean mosaic virus (SbMV 7516), peanut mottle virus (PeMoV-Necrotic), WMV-2 (NY isolate), and TuMV (C1 type) were kindly provided by R. Provvidenti, New York State Agricultural Experiment Station, Cornell University. Cowpea aphid-borne mosaic virus (CABMV) and peanut stripe virus (PStV) were kindly provided by S. K. Green, Asian Vegetable Research and Development Center, Shanhua, Tainan, Taiwan. BlCMV (Florida) and CABMV (Morocco) were kindly given by D. Gonsalves, New York State Agricultural Experiment Station, Cornell University. Papaya ringspot virus type W (PRSV-W, Florida) was kindly given by C.-H. Huang, Taiwan Agricultural Research Institute, Wufeng, Taichung. The SDS-dissociated antigens in extracts of three virus-infected tissues, BCMV (type strain), BYMV (B-3 strain), blackeye cowpea mosaic virus (BlCMV, Florida), were kindly provided by C.-A. Chang, Taiwan Agricultural Research Institute, Wufeng, Taichung. Papaya ringspot virus type P (PRSV-P), tobacco etch virus (TEV), ZYMV, and potato virus-Y (PVY) were from our laboratory.

Indirect ELISA. Indirect enzyme-linked immunosorbent assay (ELISA) was used to examine the serological relationships and the purity of the PWV CP, CIP, and AIP preparations, and to detect the antigens in PWV-infected passionfruit tissues. This procedure was performed as described by Yeh and Gonsalves (34). Purified antigens were resuspended in 0.125 M Tris-HCl, pH 6.8, containing 0.5% SDS at 1 mg per milliliter and stored as a stock. Wells of polystyrene microtitration plates (Linbro, EIA microtitration plate) were coated with purified antigen or crude sap, diluted by a fivefold series in coating buffer (0.05 M sodium carbonate, pH 9.6, containing 0.01% sodium azide), and incubated at 30 C for 4–6 h. Plates were then rinsed three times with PBS containing 0.05% Tween 20. IgGs to CP, CIP, or 51K (AIP) were prepared from the corresponding antiserum by ammonium sulfate precipitation and DEAE column chromatography (7). Two hundred microliters of the purified IgG was added to the wells at 1 μg per milliliter diluted in PBS containing 2% PVP-40 and 0.2% ovalbumin and incubated at 30 C for 4 h. Wells were rinsed three times with PBS containing 0.05% Tween 20, then 200 μl of goat antirabbit IgG conjugated with alkaline phosphatase (Sigma, A-8025) was used at 1:1,000 dilution in the same buffer as the IgG and incubated at 30 C for 4 h. After further rinsing, the bound conjugate was detected by adding 200 μl of the substrate nitrophenyl phosphate at 1 mg per milliliter in 0.1 M diethanolamine buffer, pH 9.6. The plates were incubated at room temperature and reactions were recorded by an autoreader (Dynatech MR 700) 5–60 min after the addition of the substrate. The reactions were stopped by the addition of 50 μl of 3.0 M NaOH.

Immunoblotting. Immunoblotting was conducted according to the method described by Gooderham (15). Purified PWV CP, CIP, and AIP, each at 5 μg, or 30 μl of leaf extracts of PWV-infected golden passionfruit or *N. benthamiana* in dissociation buffer (3 ml per gram of tissue) were loaded onto a 12% polyacrylamide gel, electrophoresed, and transferred to a nitrocellulose membrane (0.45-μm pore size, Bio-Rad) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) at low temperature (cooled by ice) for 2 h at constant 100 V using a Bio-Rad mini trans-blot cell. After transfer, the membrane was incubated with a blocking solution containing 3% gelatin in TBS buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) for 30–60 min. The membrane was then incubated for 1–2 h with antisera against CP, CIP, or 51K protein (AIP), diluted 1:2,000 in antibody buffer (TBS containing 0.05% Tween 20 and 1% gelatin). After two washes with TBST (TBS containing 0.05% Tween 20), the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) at 1:3,000 dilution in antibody buffer for 1 h. After two 5-min washes with TBST and one 5-min wash with TBS, the reaction pattern was visualized by incubating the membrane in substrate solution, consisting of 20 ml of the color development reagent 4-chloro-1-naphthol (3 mg per milliliter in ice-cold methanol) and 100 ml of 0.018% hydrogen peroxide in TBS, at room temperature for 10–30 min. Reactions were stopped by rinsing the membrane in distilled water.

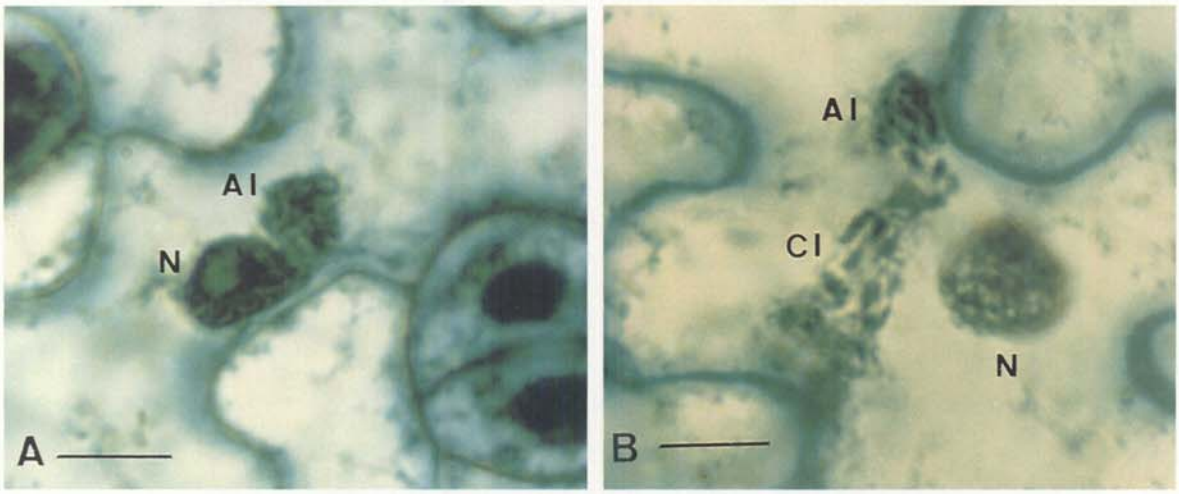

Fig. 1. Light micrographs of passionfruit woodiness potyvirus–infected tissues showing cytoplasmic inclusions in situ. The epidermal layers of virus-infected golden passionfruit (**A**) and *Nicotiana benthamiana* (**B**) were stained with the O/G stain. N = nucleus, CI = cylindrical inclusion, AI = amorphous inclusion. Bars represent 15 μm.

RESULTS

Light microscopy of cytoplasmic inclusions. Cytoplasmic CIs and AIs were observed in epidermal cells of PWV-infected golden passionfruit and *N. benthamiana* processed with O/G staining, but were not present in noninoculated plants. AIs visualized with the light microscope appeared to be imperfect spherical structures about the same size of the nuclei of the plant cells (Fig. 1A). Usually one or two AIs were found per cell, but not all of the cells contained AIs. CIs appeared to be short and platelike, and most of the infected cells contained them (Fig. 1B). Treatment with the O/G stain produced a green to olive brown coloration. No NIs were observed in PWV-infected tissues.

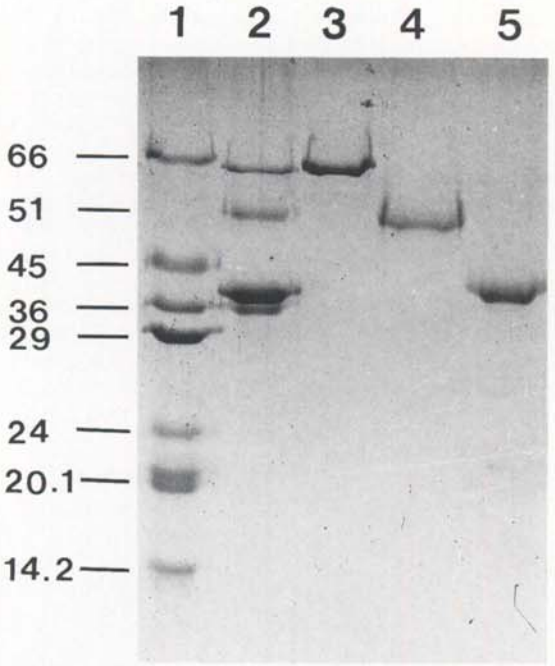

Fig. 2. Analysis of purified viral proteins induced by passionfruit woodiness potyvirus and determination of their relative molecular weights by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Lane 1, molecular weight markers: bovine albumin (66K), egg albumin (45K), glyceraldehyde-3-phosphate dehydrogenase (36K), carbonic anhydrase (29K), trypsinogen (24K), trypsin inhibitor (20.1K), α-lactalbumin (14.2K). Lane 2, a mixture of purified cylindrical inclusion protein, amorphous inclusion protein and coat protein of papaya ringspot virus type P (PRSV-P). Lane 3, purified PWV CIP. Lane 4, purified PWV 51K protein. Lane 5, purified PWV CP.

Purification of the AIP. A protein of 36K was obtained when purified virus was dissociated and electrophoresed through 12% SDS-polyacrylamide gels (Fig. 2, lane 5). A protein of 66K was obtained from purified CIs of PWV (Fig. 2, lane 3). A protein of 51K (AIP) was purified by the method developed in this study (Fig. 2, lane 4). All three viral proteins contained a single species of protein monomer.

In initial trials, the procedure used to purify PWV AIP was that described for the purification of PRSV AIP (8,35). However, since inclusions were not found and the AIP was not detected in the pellets following centrifugation at 4,000 *g*, the methods for the purification of NIPs of TEV (21) and BYMV (4) were followed. Likewise, inclusions were not found in the pellets after a sucrose step gradient centrifugation at 7,000 *g* for 12 min and the 51K protein was not detected in the preparation as analyzed by minislab gels. Also, the purification procedure developed by Chang et al (4) for the NIP of ClYVV was followed. Only trace amounts of a 51K protein were detected in the final step, but a large amount of a 51K protein was detected instead in discarded pellets of the first 164 *g* centrifugation step. In an effort to minimize the loss of the inclusions, a new, simplified procedure for purification of AIP induced by PWV was established as described under Materials and Methods. The yields of the 51K AIP were spectrophotometrically estimated (A_{280} of 1.0 unit was taken as 1 mg per milliliter, not corrected by light scattering) to be 2–4 mg per 100 g of infected tissue.

Titers of antisera. Antisera to CP, CIP, and 51K protein of PWV were obtained from immunized rabbits. The titers of antisera to individual proteins, as determined in SDS-immunodiffusion gels by using crude antigens from PWV-infected tissue of *N. benthamiana,* ranged from 1/4 to 1/16 for the bleedings taken 2–5 mo after the first injection. None of the antisera reacted with the sap from the healthy plant.

Light microscopic immunostaining. Light microscopic immunostaining was used to verify whether the 51K protein was the AIP of PWV. In the epidermal cells of PWV-infected passionfruit, CIs were immunostained by the CIP antiserum and protein A-gold complex (Fig. 3A). When the tissue was stained by the antiserum to the 51K protein, the cytoplasmic AIs of infected cells were immunostained (Fig. 3B). There were no specific structures stained in PWV-infected tissues treated with the protein A-gold complex alone or with normal serum only, nor in healthy tissues incubated with the antiserum to 66K CIP or 51K AIP. The results indicated that the major location of the 51K protein is at the cytoplasmic AIs in the PWV-infected cells.

Immunoelectron microscopy. Electron microscopy of ultrathin sections labeled with immunogold was used to further confirm

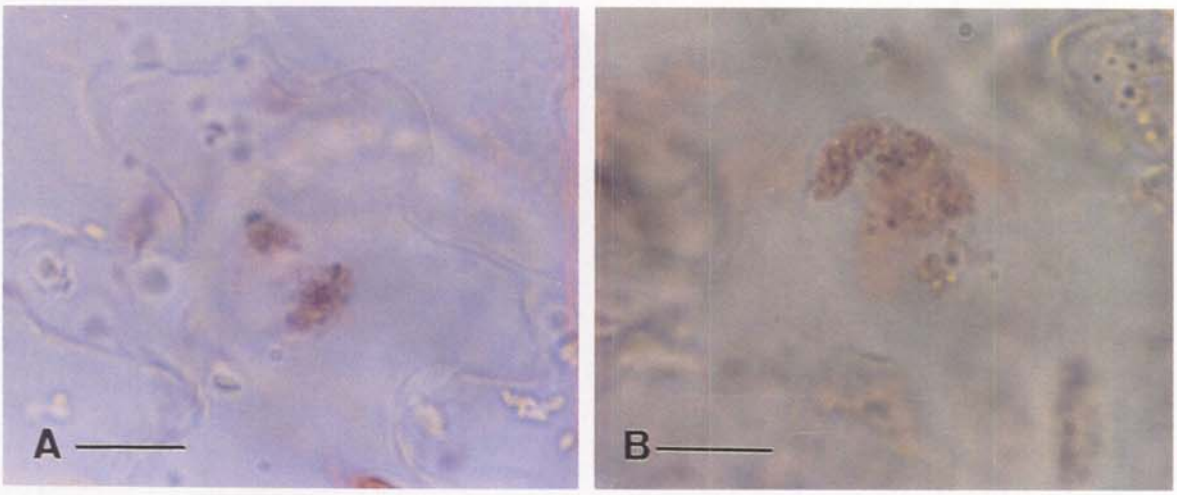

Fig. 3. Immunolight microscopy of cytoplasmic inclusions of passionfruit woodiness potyvirus (PWV)–infected passionfruit. The epidermal cells of PWV-infected passionfruit were treated with the antiserum to PWV cylindrical inclusion protein (**A**) or to a PWV protein with M_r 51K (**B**) and then stained with protein A-gold. Pink cytoplasmic inclusions were observed. Bars represent 15 μm.

that the 51K protein was the AIP of PWV. When sections of the leaf tissue of PWV-infected passionfruit were treated with the antiserum against PWV CP followed by protein A-gold complex, the gold particles specifically attached to PWV wherever the virions were present (Fig. 4A). When the antiserum against 66K CIP was used, the gold particles specifically labeled CIs, but not PWV virions, nor any other cell components (Fig. 4B). These CIs were seen as bundles in longitudinal section and as pinwheels in cross section. This confirms that the 66K protein is a component of PWV-specific cylindrical inclusion bodies (26). Gold particles were localized over cytoplasmic irregular-shape inclusions when the sections were incubated with the antiserum against the 51K protein. The size of the labeled inclusions ranged from 700–800 nm to 6–10 μm (Fig. 4C). Enlargement of these inclusions showed electron-dense ropelike structures in the homogenous matrix (Fig. 4D). Gold labels were not found on cell organelles, such as nuclei, mitochondria and chloroplasts; they were also absent from PWV-infected tissues treated with normal serum and healthy tissues treated with the three antisera. The results indicated that the 51K protein was the PWV-specific AIP.

Serological analyses of the AIP. SDS-immunodiffusion, indirect-ELISA, and immunoblotting methods were used to confirm the purity of the AIP used to immunize the rabbits and to ensure its freedom from PWV CP, CIP, and related proteins. In the SDS-immunodiffusion test, the antisera only reacted to their homologous antigens and there were no cross-reactions with the heterologous antigens (Fig. 5). These results clearly indicated that the purified AIP was serologically distinct from the CP and the CIP of PWV. None of the three antisera reacted with purified CP, CIP, or AIP of papaya ringspot virus (PRSV-P) (data not shown).

In indirect ELISA, when 1 μg per milliliter of IgG to PWV CP, CIP, or 51K AIP was used to react with different concentrations of purified CP, CIP, and 51K AIP, the homologous antigens were detected in concentrations as low as to 1.6 ng per milliliter, 200 ng per milliliter, and 40 ng per milliliter, respectively (Fig. 6). The results clearly showed that PWV CP, CIP, and AIP were serologically unrelated. Each of the three antisera detected the corresponding antigen in crude sap of PWV-infected passionfruit up to a dilution of 1:130,000 (data not shown).

Similar results were obtained with the immunoblotting analysis. The antisera against PWV CP, CIP, and 51K AIP only reacted with their homologous antigens and no cross-reactions were observed in the heterologous combinations (Fig. 7). Also, the antisera only reacted with the homologous antigens in crude sap of PWV-infected golden passionfruit and *N. benthamiana* (Fig. 7). These results further confirm that the AIP was a specific protein of PWV-infected plant tissue, and that it was serologically distinct from the CP and CIP of PWV.

Serological reactions with other potyviruses. In SDS-immunodiffusion tests, the antiserum against PWV CP reacted with sap from plant tissue infected with WMV-2 (New York), WMV-2 (Florida) and BlCMV (Florida), but the heterologous precipitation lines spurred with the homologous ones (Fig. 8A and B). No precipitin lines were observed when crude sap from plant tissues infected with BCMV, BYMV, ClYVV, SbMV, PeMoV, TuMV, CABMV, PStV, PRSV-W, PRSV-P, TEV, ZYMV, and PVY were tested against the PWV CP antiserum. Only the sap from plant tissue infected with WMV-2 (Florida),

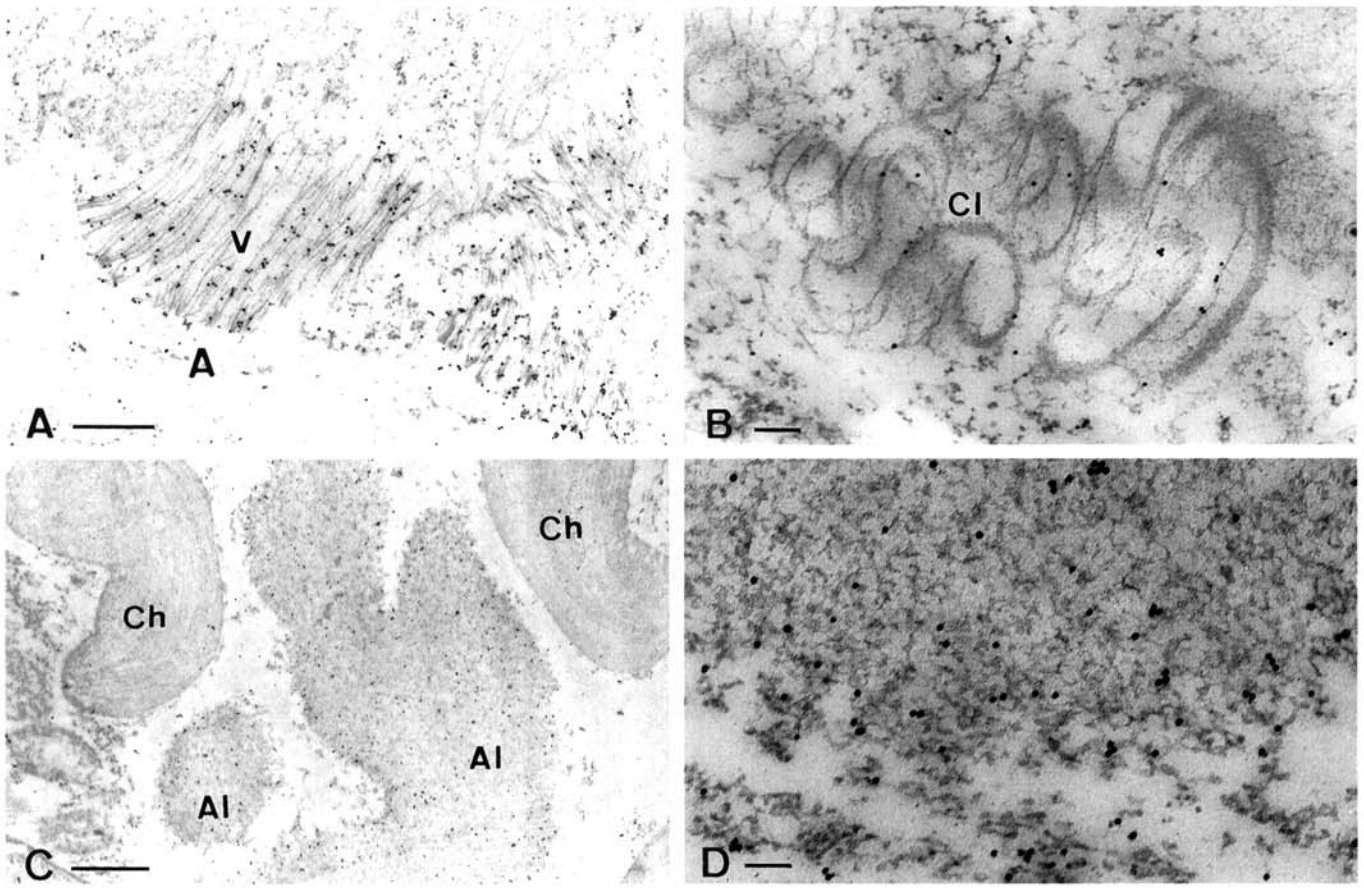

Fig. 4. Immunogold labeling using antisera to passionfruit woodiness potyvirus (PWV) coat protein (CP) (**A**), cylindrical inclusion protein (CIP) (**B**) or 51K amorphous inclusion protein (AIP) (**C, D**) to localize the corresponding antigens in PWV-infected cells of passionfruit. Ch = chloroplast, V = virus particles, CI = cylindrical inclusion, and AI = amorphous inclusion. **A,** Gold particles were specifically labeled on PWV particles in an ultrathin section of PWV-infected leaf tissue. Bar = 500 nm. **B,** Gold particles present at pinwheel (cylindrical) inclusions. Bar = 200 nm. **C,** PWV-infected passionfruit leaf cell showing amorphous inclusions (AI) to which gold particles were attached. Bar = 500 nm. **D,** Enlargement of an AI in **C**, electron-dense ropelike structures in the AI are shown. Bar = 100 nm.

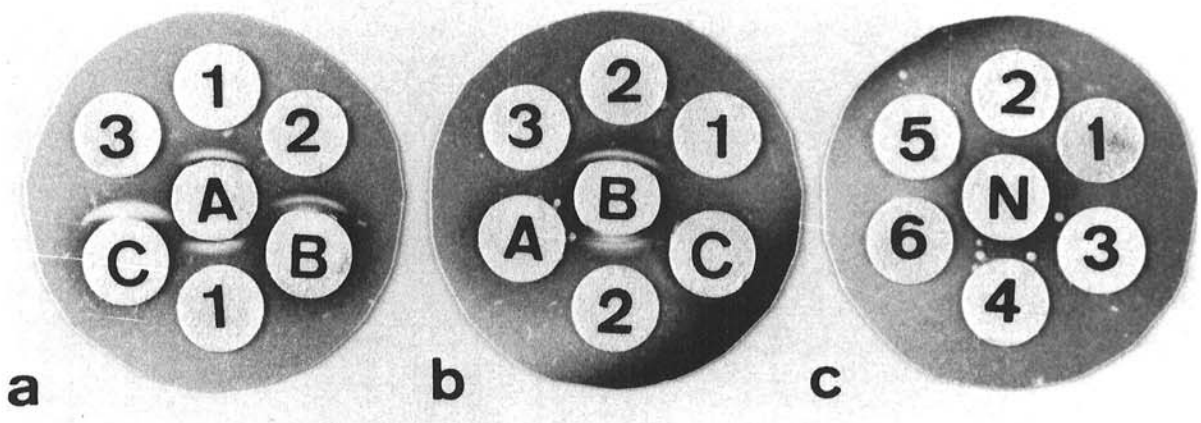

Fig. 5. Sodium dodecyl sulfate–immunodiffusion tests using antisera to passionfruit woodiness potyvirus (PWV) coat protein (CP), cylindrical inclusion protein (CIP), and amorphous inclusion protein (AIP). Twenty-five microliters of undiluted antisera or 25 μg of different antigens were added to the wells. Well contents: 1 = PWV CIP, 2 = PWV 51K AIP, 3 = PWV CP, 4 = PRV CIP, 5 = PRV AIP, A = antiserum to PWV CIP, B = antiserum to PWV 51K protein, C = antiserum to PWV CP, N = normal serum.

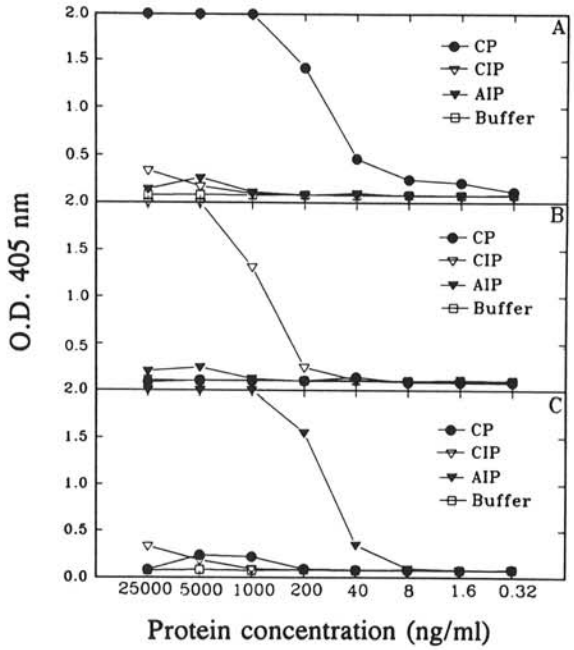

Fig. 6. Serological relationships among passionfruit woodiness potyvirus (PWV) coat protein (CP), cylindrical inclusion protein (CIP), and amorphous inclusion protein (AIP), detected by indirect enzyme-linked immunosorbent assay. Purified proteins were diluted by a fivefold series in coating buffer from the initial concentration of 25 μg per milliliter, IgG from antisera to PWV CP (A), CIP (B), and 51K AIP (C) was at 1 μg per milliliter, and goat anti-rabbit IgG (whole molecule) alkaline phosphatase at 1:1,000 dilution. Reading were recorded 30 min after the addition of substrate at 1 mg per milliliter.

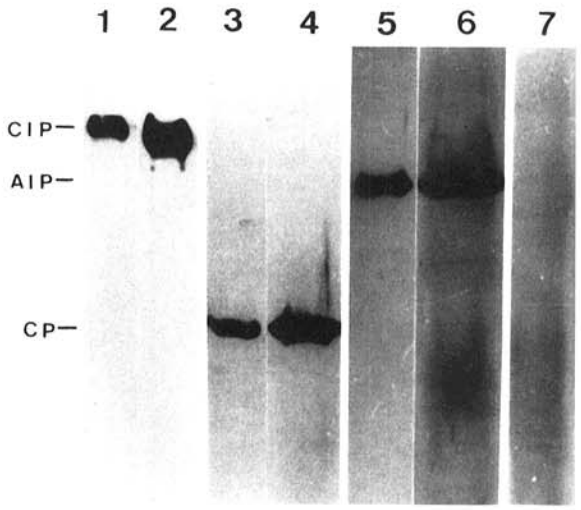

Fig. 7. Serological relationships among passionfruit woodiness potyvirus (PWV) coat protein (CP), cylindrical inclusion protein (CIP), and 51K amorphous inclusion protein (AIP), detected by immunoblotting. Antisera to PWV CIP (lane 1 and 2), CP (lane 3 and 4), or 51K AIP (lane 5, 6, and 7) were used at 1:2,000 dilution, goat anti-rabbit IgG (whole molecule) horseradish peroxidase conjugate at 1:3,000 dilution, and HRP color development solution (4-chloro-1-naphthol) at 0.5 μg milliliter. Lanes 1, 3, and 5 contain a mixture of purified CP, CIP, and 51K AIP of PWV. Lanes 2, 4, and 6 contain crude sap from PWV-infected *N. benthamiana*. Lane 7 contains crude sap from healthy *N. benthamiana*.

but not WMV-2 (New York) or BlCMV, showed slight spur reaction with PWV CIP antiserum (Fig. 8D). Antiserum against the AIP did not react with any of the other potyviruses tested (Fig. 8E).

DISCUSSION

An amorphous inclusion protein induced by PWV-TW was successfully purified from PWV-infected plants by a procedure developed during this study and a specific polyclonal antiserum was produced. Immunocytochemical methods using light or electron microscopy with protein A-gold labeling techniques were employed to ascertain the relationship of the purified AIP with the specific virus inclusions in situ. Immunolight microscopy using the antiserum to 51K protein showed that the cytoplasmic amorphous inclusions were the major location of the 51K protein. Immunogold labeling in electron microscopy also indicated that gold particles were mainly distributed on cytoplasmic AIs. These results provide direct evidence that this 51K protein was a major constituent of the AI observed in situ.

Because of differences in size and shape of the AIs induced by different viruses, the success of purification depends on a careful step-by-step evaluation of the treatments. We found that the AIs of PWV-TW were resistant to Triton X-100 treatment, a property that facilitated their purification. When the procedures for the purification of NIs of TEV (21) and BYMV (4), and for the AIP of PRSV (8,35) were initially tried, the AIs of PWV were pelleted during the first low speed of centrifugation (164–7,000 *g*). It appears that the AIs of PWV have a denser mass than those of the other potyviruses, thus the pellets after the first low-speed centrifugation for the purification of the AIs of PWV were

collected and this step was the key for the successful purification. The procedure developed in this investigation produced a high yield of PWV-TW AIP (2–4 mg per 100 g tissue), and may be applicable to the purification of AIPs of other potyviruses.

Although the SDS-treated AIP was used as immunogen, the resulting antisera were able to recognize the antigenic sites on the undenatured AI in situ. Apparently, the antigenic determinants of the AIP were preserved at least in part in the AI, despite the denaturing effect of the SDS-polyacrylamide gel electrophoresis. Similar results have been observed with antisera prepared to potyviral CIs, the NIs of TEV (17), the NIs of ClYVV and BYMV (4), and the AIs of PepMoV and PRSV-W (8).

The association of a distinct 51K protein with AI preparations indicated that the AI of PWV is made of a single kind of protein subunit. The preparation of the specific antiserum against the major component of the AI induced by PWV provides a useful probe for studying the time course of appearance of AI during the virus life cycle in infected tissues and protoplasts. The antiserum is also useful for studying the proteolytic processing and functions of PWV AIP.

In SDS-immunodiffusion tests, indirect ELISA, and immunoblotting, antisera prepared against CP, CI, and AI proteins of PWV-TW only reacted with their homologous purified antigens, and no cross-reaction was observed in the heterologous combinations. These results indicated that the three proteins are serologically unrelated.

Antiserum against the CP of PWV-TW reacted with antigens from WMV-2- or BlCMV-infected plant tissues, and the antiserum to PWV CIP reacted with slight spur with the sap from BlCMV-infected tissue. These results were different from those reported by Chang (3), since the antiserum to PWV virion reacted with BlCMV, WMV-2, BCMV, PStV, ZYMV, and pea seedborne mosaic virus (PSbMV), while the antiserum to PWV CIP failed to react with WMV-2 (3). This may be explained by the differences in antigenicity between the virus particles and the dissociated CP. Although the use of different strains of viruses may also affect the results, we believe that the PWV isolate used in this investigation and the one previously used by Chang (3) are of the same strain, since they were derived from the same location and were serologically indistinguishable (data not shown). Also, the discrepancy may be due to different antigen titers in the tested tissue. Other factors, such as the preparation of the antigen, immunogen dose, and animal response to antigens, as well as timing of immunization and bleedings, may also affect the results. More stringent studies with purified antigens and parallel comparisons with reciprocal antigen-antibody reactions should clarify these differences.

The antiserum to PWV-TW AIP did not react with crude sap from tissue infected with any of 14 potyviruses including WMV-2, BlCMV, and ZYMV. It seems that the AIP of PWV has more limited serological relationships with other potyviruses than those of the CP and the CIP. Although the concentrations of the viruses in the test tissues were high, as tested by local lesion assay (data not shown) and the CP antigens in tissues infected with WMV-2 and BlCMV reacted with PWV CP antiserum, negative results due to the insufficient amount of AIPs in the test sample cannot be ruled out. Heterologous AIP antisera are needed to ascertain the serological relationships of PWV AIP with other potyviruses.

The AIP of a potyvirus is considered the aphid transmission factor HC because of its molecular size and close serological relationship (9,18). The antiserum to the HC protein of PVY localized the antigen at the AIs in the cytoplasm (1). In addition to aphid transmissibility, the HC protein of potyvirus also functions as a cysteine-type proteinase that autocatalytically cleaves

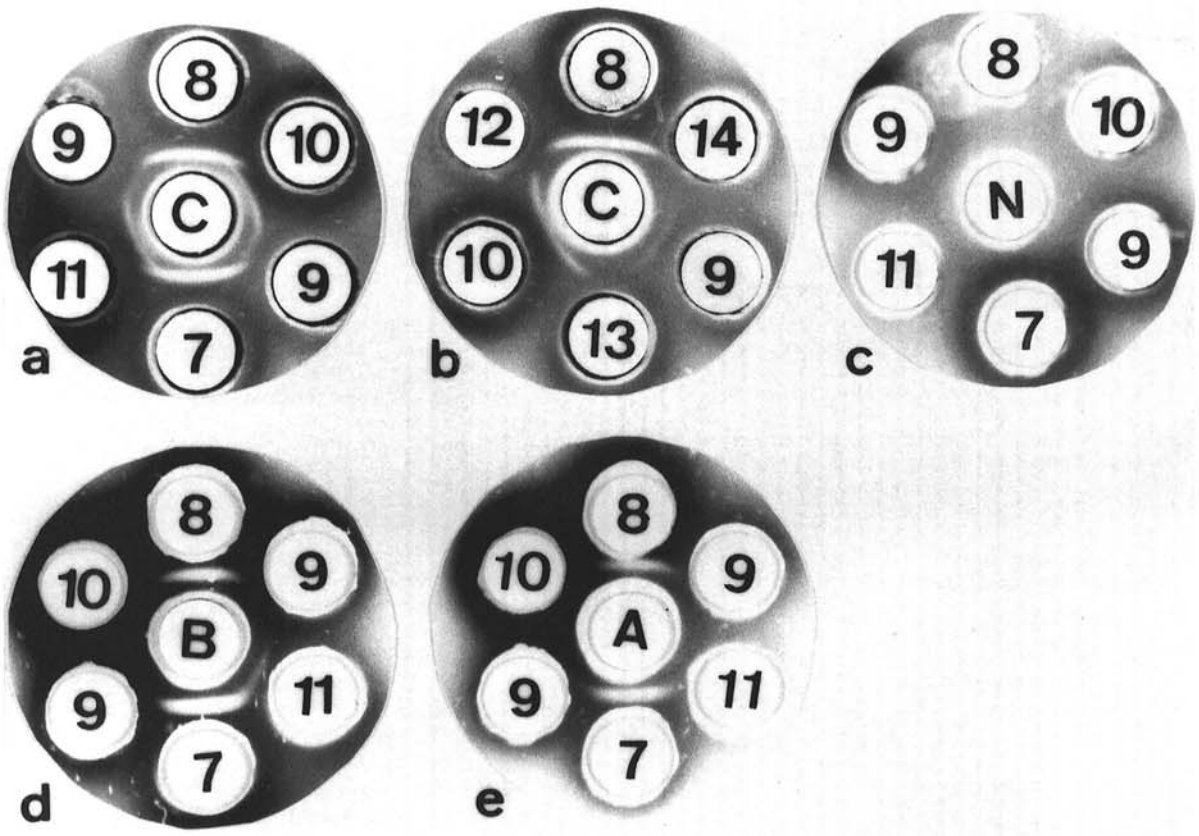

Fig. 8. Serological relationships among passionfruit woodiness virus (PWV), blackeye cowpea mosaic virus (BlCMV) and watermelon mosaic virus 2 (WMV-2). All antigens were from crude saps of virus-infected leaf tissues. C = antiserum to PWV coat protein, N = normal serum, B = antiserum to PWV cylindrical inclusion protein, A = antiserum to PWV 51K amorphous inclusion protein, 7 = crude sap from PWV-infected *N. benthamiana,* 8 = crude sap of PWV-infected passionfruit, 9 = crude sap of BlCMV (Florida)-infected blackeye cowpea, 10 = crude sap of WMV-2 (New York)-infected *N. benthamiana,* 11=WMV-2 (Florida)-infected *N. benthamiana,* 12 = healthy passionfruit, 13 = healthy *N. benthamiana,* 14 = healthy blackeye cowpea.

its C-terminus to liberate itself from the polyprotein (2). The molecular size of the AIP of PWV-TW is similar to that of PRSV (35,36), but in this study they were serologically unrelated. Immunogold labeling in electron microscopy showed electron-dense ropelike structures in AIs of PWV. These tubular structures are similar to the X bodies induced by TMV, which are considered the replication sites of the virus (19). The function and the role of the AI protein of PWV in relation to viral replication or aphid transmission require further studies.

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