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Immunological detection of passionfruit woodiness virus

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Abstract. Antiserum produced against capsid protein of passionfruit woodiness virus (PWV) efficiently detected PWV in infected leaf extracts by immunodot blotting (IDB), enzyme-linked immunosorbent assay (ELISA), Western blotting (WB) or leaf dip serology using the gold-labelled antibody decoration (GLAD) technique. The antiserum did not react with healthy leaf extracts in IDB, ELISA or WB tests and did not react with the sweet potato latent and papaya ringspot viruses in GLAD. PWV-capsid protein antiserum was superior in specificity to antiserum prepared against intact PWV for detection of PWV by IDB, ELISA or WB. The practical applications of these techniques were also demonstrated.

Key words: ELISA; Immunodot blotting; Immunoelectron microscopy; Passionfruit woodiness virus; Western blotting.

Introduction

Passionfruit woodiness virus (PWV), a member of the potyvirus group (Taylor and Greber, 1973), was first recorded in Taiwan in 1981 (Chang *et al.*, 1981) and has since become a major limiting factor for economic production of passionfruit (*Passiflora edulis* Sims.) on the island. The disease caused by PWV is characterized by mosaic and rugose leaves with woody and severely malformed fruits. It greatly shortens the economic life of plants. Since passionfruit is commercially propagated by grafting, the indexing of scions and root stocks for freedom from virus infection becomes important to the passionfruit industry.

Detection of PWV in infected plants by serological means has not been much studied (Moghal and Franki, 1976, 1981). Moghal and Francki (1976) prepared antiserum against intact viruses and showed distant serological relationships between PWV and bean common mosaic virus or potato virus Y by immunodiffusion tests. In this report, we produced antisera against intact PWV and dissociated viral capsid protein electro-eluted from polyacrylamide gels. The suitability of these two antisera for detection of PWV in infected leaves by methods of immunodot, enzyme-linked immunosorbent assays, Western blotting and gold-labelled antibody decoration was demonstrated.

Materials and Methods

Viruses and Hosts

Passionfruit woodiness virus (PWV) was

originally isolated from diseased plants of passionfruit. After three successive local lesion passages through *Chenopodium quinoa* Willd., the virus was maintained in *Nicotiana benthamiana* Domin. Papaya ringspot virus (PRV) and sweet potato latent virus (SPLV) used in the gold-labelled antibody decoration were obtained from S. D. Yeh, (Department of Plant Pathology, National Chung Hsing University, Taichung 40027, Taiwan) and M. L. Chung (Chiayi Agricultural Experiment Station, Chiayi, Taiwan), respectively. PRV was maintained in *Cucumis metuliferus* (Naud.) Mey, Acc. 2459, Line 35 and SPLV in *N. benthamiana.*

Virus Purification

Leaves of PWV-infected *N. benthamiana* were ground in 0.5 M phosphate buffer, pH 7.4, containing 5 mM EDTA and 1% 2-mercaptoethanol. Leaf extract was emulsified with 0.5 ml of a mixture of chloroform and carbon tetrachloride (1:1) per gram of leaf tissue. After low speed centrifugation, the aqueous phase was saved, treated with 8% PEG-6000 and centrifuged again. The resuspended virus in 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA (PE) was then treated with 2% Triton X-100 and centrifuged at 45,000 rpm for 1 h in a Hitachi RP 50 rotor over a 5 ml 20% sucrose cushion. Virus particles suspended in PE buffer were further purified by isopycnic centrifugation in $CsSO_4$ solution.

Antisera Preparation

Antisera were prepared in rabbits against PWV virions (Anti-PWV serum) and PWV capsid protein (Anti-PWV-CP serum) electroeluted from the SDS-polyacrylamide gel (Brakke *et al.*, 1987) by four intramuscular injections at weekly intervals, each with 1 mg protein. Antigen was emulsified in an equal volume of Freund's complete adjuvant for the first injection and of incomplete adjuvant for the latter injections. Bleedings were done 10 days after the forth injection. Antiserum titer determined by immunodiffusion test was 1:16 for anti-PWV and 1:8 for anti-PWV-CP.

Gold-labelled Antibody Decoration (GLAD)

Infected plant leaves were collected from the field and extracted in distilled water. After floating on the leaf extracts, grids were incubated in anti-PWV or anti-PWV-CP serum (a 1/200 dilution), followed by treatment with gold-labelled goat anti-rabbit IgG as described (Lin, 1984).

Immunodot Blotting (IDB)

Healthy or infected leaves of passionfruit and *N. benthamiana* were homogenized in 0.5 M phosphate buffer, pH 7.4, 1 ml per g of leaves. After passing through two layers of cheesecloth, the extract was centrifuged for 10 min at 3,000 rpm to remove the cell debris. The pellet was discarded and the supernatant fluid was saved for use.

The IDB procedure was performed in a Bio-Dot apparatus (Bio-Rad, Richmond, USA). The nitrocellulose membrane sheet was pretreated in 10 mM phosphate buffered saline, pH 7.4, (PBS) for 10 min and assembled in the Bio-Dot apparatus. After washing with PBS, membrane was dried by vacuuming. Ten microliters samples were then applied along the wall to wells on nitrocellulose membrane, allowed to stand for 10 min and dried. The membrane was then removed from the apparatus and placed in a tray that contained 10 ml of blocking solution (1% bovine serum albumin in PBS buffer). The blotted samples were then incubated with diluted anti-PWV serum or anti-PWV-CP serum, followed by reactions in steps with goat anti-rabbit IgG and horseradish peroxidase conjugates as described previously (Lin *et al.*, 1987).

Enzyme-linked Immunosorbent Assay (ELISA)

The indirect ELISA procedure was modified from that of Lommel *et al.* (1982). Leaf extracts were prepared as described for immunodot blotting, and serially diluted in 0.05 M carbonate buffer, pH 9.6. Two hundred microliters of sample was added to each well on the microtiter plate and the plate stored at 4°C overnight or at room temperature for 2 h. Plates were washed three times with PBS containing 0.05% Tween-20 (PBST) and then blocked with 2% BSA in PBS for 1.5 h. Diluted anti-PWV or anti-PWV-CP serum was added and the plate was incubated for 2 h at 37°C. After removal of antiserum and washing, the plate was incubated with alkaline phosphatase conjugated goat anti-rabbit IgG in conjugation buffer (PBST containing 2% polyvinyl pyrrolidone-40 and 0.2% ovalbumin) for 2 h at 37°C. The plate was again washed and *p*-nitrophenyl phosphate in diethanolamine substrate buffer (1 mg/ml) was then added. The absorbance values at 405 nm were read in a Bio-Tek EIA plate reader (Bio-Tek Instruments, Burlington, USA).

SDS-polyacrylamide Gel Electrophoresis

The leaf extracts of infected and healthy plants were prepared as described for immunodot blotting. The extracts were concentrated by methanol precipitation and the precipitates were disrupted in protein cracking buffer by heating at 100°C for 3 min (Lane, 1978). Samples were then electrophoresed in an SDS-polyacrylamide gel using discontinuous buffers (Laemmli, 1970). The protein bands were visualized by Coomassie Blue staining.

Western Blotting (WB)

The blotting of proteins was performed according to the method of Kyhse-Andersen (1984) using a Semidry Electroblotter (Sartorius, Göttingen, W. Germany). The protein blots were immunostained as described previously (Lin *et al.*, 1987).

Results

Gold-labelled Antibody Decoration (GLAD)

In leaf dip preparations of infected passionfruit leaves, virions were positively stained with anti-PWV or anti-PWV-CP serum and gold-IgG complexes (Fig. 1A). No virus particles in dips of PRV-infected (Fig. 1B) and SPLV-infected tissues (data not shown) were labelled

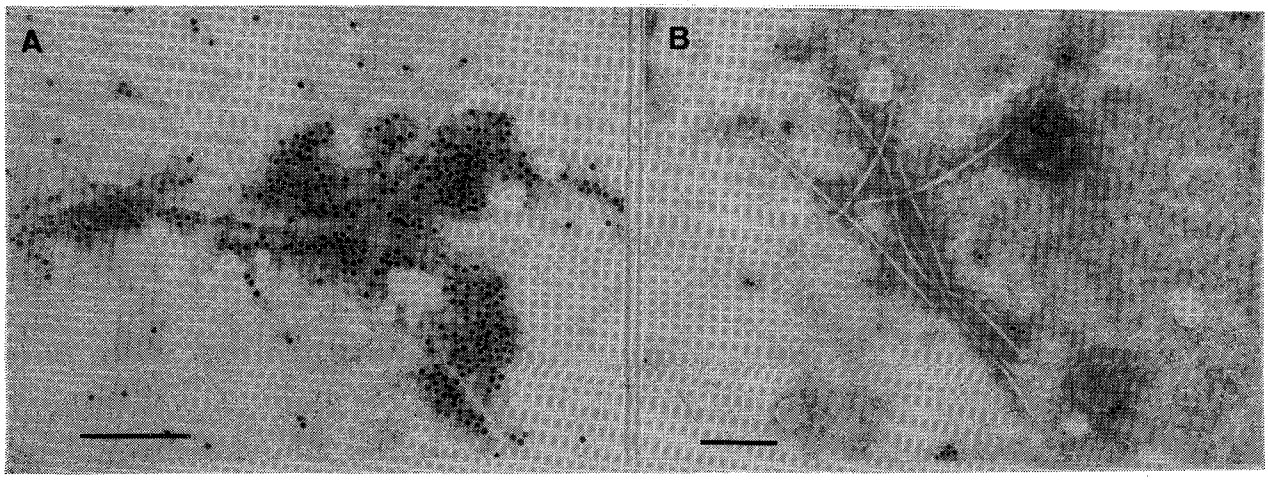

Fig. 1. Electron micrographs of (A) passionfruit woodiness virus and (B) papaya ringspot virus in infected leaf extracts of their respective host plants. The virus particles on grids were treated with anti-PWV serum and then gold-labelled goat anti-rabbit IgG complexes. Bars represent 200 nm.

with the antiserum. Leaf dips prepared from PWV-infected plants also gave no evidence of gold labelling when they were pretreated with normal rabbit serum, followed by a treatment with gold-labelled goat anti-rabbit IgG complexes (data not shown). The anti-PWV and anti-PWV-CP sera were equally effective for detection and identification of PWV in leaf dips by GLAD.

PWV could be easily detected in the leaf sap of infected passionfruits diluted up to 1:2048 by the decoration technique. At higher dilutions, detection of virus was less consistent. In different experiments, 17, 7, 4 of 20 fields on the grids were found to have virus particles all labelled when the dilution was made 1:4096, 1:8192, and 1:16384, respectively.

Immunodot Blotting (IDB) and Enzyme-linked Immunosorbent Assay (ELISA)

In the preliminary study, the IDB was unable to discriminate between healthy and PWV-infected *N. benthamiana* leaf extracts when either unabsorbed anti-PWV serum (Fig. 2A) or anti-PWV serum absorbed with healthy sap (data not shown) was used. Addition of 2% Triton X-100 to the blocking buffer removed the green color of leaf sap on the blots. However, it did not reduce the background nonspecific stain (Fig. 2B). In constrast, anti-PWV-CP serum detected PWV in leaf extracts from infected *N. benthamiana* diluted up to 1:1024 whether there was Triton X-100 in the blocking solution or not (Fig. 2C and 2D). It did not cross react with leaf extracts of uninfected plants. The light gray spots representing the healthy controls were due to the green color of leaf sap photographed in a black-and-white picture (Fig. 2C). These spots

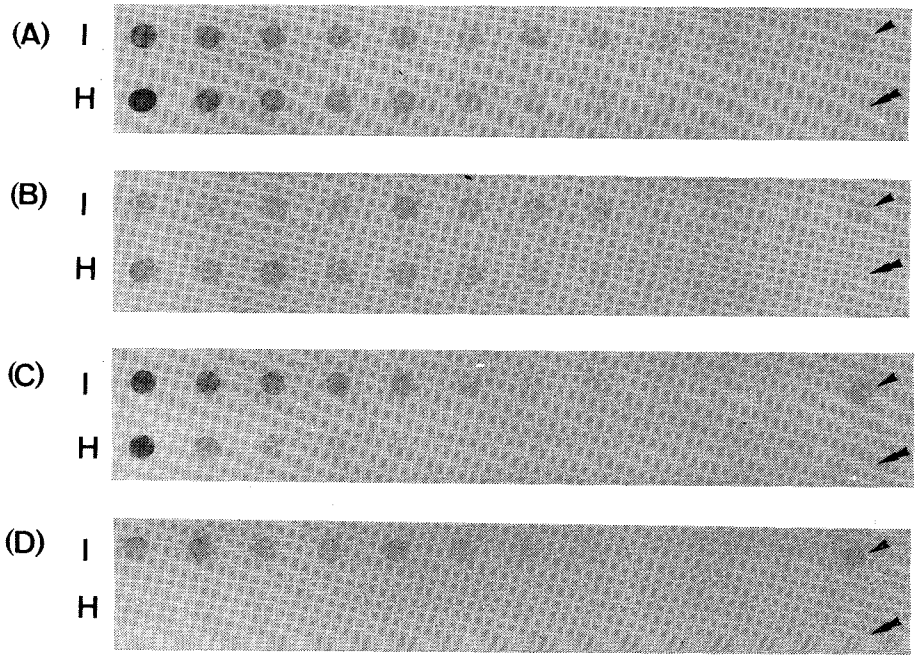

Fig. 2. Dot immunoassays of crude antigen in leaf extracts from PWV-infected (I) and healthy control (H) *N. benthamiana* using anti-PWV serum (A, B) or anti-PWV-CP serum (C, D). (A, C), 1% BSA in PBS as blocking solution. (B, D), blocking solution contained additional 2% Triton X-100. Samples (10 μl) in two-fold serial dilutions, starting from 1:16, were dotted on nitrocellulose paper in a Bio-Dot Apparatus. The assay procedure is described in the text. ←, purified virus. ←← PBS as mock antigens.

were easily distinguishable from the purplish spots of infected extracts in the original blots.

In further diagnostic IDB tests, passionfruit leaves were collected from the fields at different localities. Using either anti-PWV or anti-PWV-CP sera, PWV was successfully detected in saps of infected leaves diluted up to 1:1024 (data not shown). No positive reaction was observed in the healthy controls.

When indirect ELISA was performed, anti-PWV and anti-PWV-CP sera were equally well for detecting PWV from infected passionfruit (Fig. 3), but anti-PWV-CP serum was higher in specificity for detection of PWV from inoculated *N. benthamiana* (data not shown). ELISA was four-fold more sensitive than IDB. It could detect PWV in leaf sap diluted up to 1:4096.

Western Blotting (WB)

About 20 mg of leaf extracts of infected passionfruit or *N. benthamiana* was loaded onto a 10% polyacrylamide gel, electrophoresed, and Western blotted. Healthy material was included as controls in each electrophoretic run. Fig. 4A shows that the PWV capsid protein (36 kD)

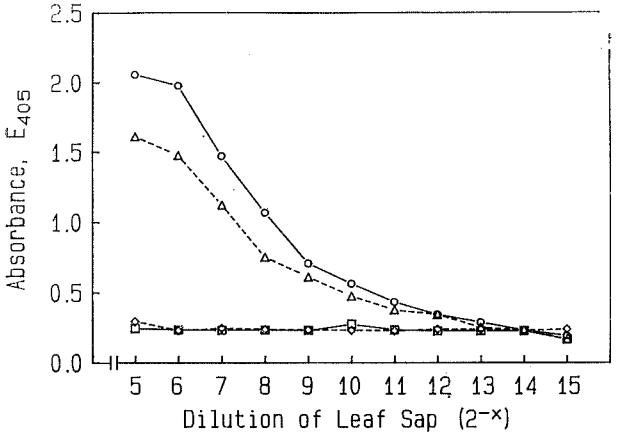

Fig. 3. The detection of PWV in leaf extracts of infected passionfruit by indirect ELISA. ○—○ Infected leaf sap treated with anti-PWV-CP serum. □—□ Healthy leaf sap treated with anti-PWV-CP serum. △···△ Infected leaf sap treated with anti-PWV serum. ◇···◇ Healthy leaf sap treated with anti-PWV serum.

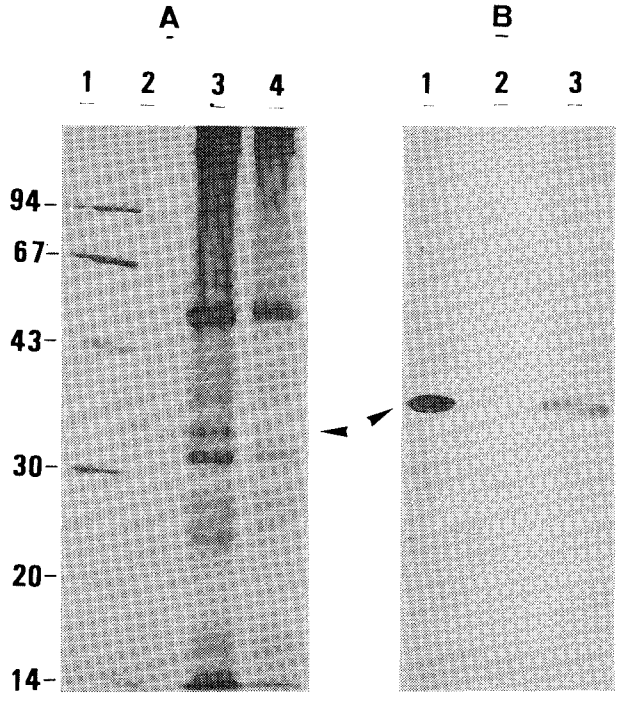

Fig. 4. SDS-polyacrylamide gel (10%) electrophoresis of total PWV-infected and healthy leaf extracts, blotted and probed with rabbit anti-PWV capsid protein serum. (A) Coomassie Blue stained gel. Lane 1, marker proteins ($MW \times 10^{-3}$) from top to bottom: myosin, β-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and α-lactabumin. Lane 2, purified PWV virions. Lane 3, proteins from infected leaf extracts. Lane 4, proteins from healthy control leaf extracts. (B) Immunoblot of the gel. Lane 1, purified PWV virons. Lane 2, healthy leaf extracts. Lane 3, PWV-infected leaf extracts. Arrow heads point to the PWV capsid protein band.

could be detected in extracts from infected leaves of *N. benthamiana* (Lane 3) but not in the healthy preparations (Lane 4). By WB analysis, a 1:150 dilution of anti-PWV-CP serum in PBS reacted only with the 36 kD capsid protein from both purified virus preparations and infected leaf extracts (Fig. 4B, Lane 1, 3). It did not react with any of the protein bands of healthy preparations (Fig. 4B, Lane 2). The anti-PWV serum used in this study failed to detect the PWV capsid protein specifically from

the *N. benthamiana* leaf extracts. It reacted with other protein bands originating from the healthy plants.

Discussion

The purified PWV preparations showed only one discrete band in the SDS-PAGE when a normal amount of protein (1-3 μg) was loaded, however, antiserum produced against these preparations (Anti-PWV serum) did not specifically detect the PWV in the infected *N. benthamiana* leaves by IDB, ELISA or WB. Probably the purified samples contained minute amounts of host components which could not be detected in Coomassie Blue-stained gels, but induced strong immunological response in the immunized animals. Thus, the anti-PWV serum reacted strongly with the host components of *N. benthamiana* and was unable to detect PWV in leaf extracts of that host efficiently. However, this antiserum successfully detected PWV in infected passionfruit plants. We then prepared antiserum against capsid protein electro-eluted from the excised gel band corresponding to the capsid protein (MW 36 kD) (Anti-PWV-CP serum). This serum did not react with any host proteins from either *N. benthamiana* or passionfruit, and was successfully used as a probe for PWV by immunoassays.

All four kinds of immunoassays employed in this study are simple and highly specific. Table 1 summaries the quantitative comparisons of three immunoassays for detection of PWV in the leaf extracts from infected passionfruit plants collected from the field. Although GLAD is the most sensitive method and takes only 10 min for each test (Lin, 1984), it may involve much labor for a large number of samples. ELISA is a suitable method for rapid analysis of large numbers of samples for the plant virus infection. However, IDB has been shown to have same potential at a lower cost than ELISA (Powell, 1987).

Table 1. *Quantitative comparisons of various immunoassays for the detection of PWV in infected passionfruit leaf extracts*

Dilutions of infected passionfruit leaf extracts	Immunoassays		
	GLAD	IDB	ELISA
1:2	+	+	+
1:4	+	+	+
1:8	+	+	+
1:16	+	+	+
1:32	+	+	+
1:64	+	+	+
1:128	+	+	+
1:256	+	+	+
1:512	+	+	+
1:1024	+	+	+
1:2048	+	−	+
1:4096	+	−	+
1:8192	+	−	−
1:16384	+	−	−

By WB analysis, extracts of PWV-infected plants, contained only the viral capsid protein with a molecular weight of 36 kD. Neither precursor forms nor degradation products were present in infected plants. There was no serological relationship observed between the PWV capsid protein and other PWV-specific proteins, such as cylindrical inclusion protein (MW 66,000) and amorphorus inclusion protein (MW 51,000) (Fig. 4) (Hsu, unpublished data). Therefore, WB provides a useful method for qualitative identification and molecular weight determination of capsid protein and for determination of serological relationship of plant viruses.

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百香果木質化病毒之免疫偵測

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利用百香果木質化病毒 (Passionfruit woodiness virus, PWV) 之鞘蛋白抗血清，以免疫墨點方式 (Immunodot blotting, IDB)，酵素聯結免疫測定法 (Enzyme-linked immunosorbent assay, ELISA)，西方墨點法 (Western blotting, WB) 或利用金標定抗血清 (Gold-labelled antibody decoration, GLAD) 之免疫粗汁液法，可以有效的偵測感病粗汁液中之病毒。以 IDB, ELISA 或 WB 方式偵測時，製備之抗血清不會與健康植物之汁液起反應，用 GLAD 法時，亦不會與蕃薯潛伏病毒 (sweet potato latent virus) 或木瓜輪點病毒 (papaya ringspot virus) 反應。病毒鞘蛋白之抗血清較病毒血清有較高之專一性，利用這些方式都可以直接應用到百香果田間之病毒偵測。