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Purification and Characterization of a Tospovirus Systemically Infecting Cucurbits in Taiwan

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ABSTRACT

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A tospovirus that infects cucurbits systemically was purified and compared with a New York TSWV isolate (NY) as well as an isolate of INSV(I). On SDS-PAGE, the cucurbit-infecting virus yielded three virion proteins of M_r 84(G1), 53(G2) and 31K(N) daltons, respectively. Both NY and I gave a protein profile that differs from above by having a 29K N protein. In electroimmunoblotting, antisera produced to the cucurbit isolates G and WMY reacted with all three virion proteins of the cucurbit-infecting isolates, though most strongly with the 31K one. They also reacted with the 84 and 53K proteins but not the 29K protein of NY. Likewise, antisera to NY and to the type strain of TSWV (formerly known as TSWV-L), did not react with N protein (31K) of the cucurbit-infecting isolates, though they reacted positively with the 84 and 53K proteins of these isolates. Polyclonal antisera produced to the N proteins of WMY and NY reacted with homologous antigens only. ELISA results indicate a distant serological relationship between the two virus groups. Antiserum to INSV was found without reactivity to cucurbit isolates in both ELISA and immunoblotting tests. In the present study, a simple procedure was developed using Percoll density gradients for purifying isolates of the cucurbit-infecting tospovirus and proved also suitable for TSWV.

Key words: Tospovirus, tomato spotted wilt virus, watermelon silver mottle, virus purification, serology

INTRODUCTION

Tomato spotted wilt virus (TSWV), the type member of the newly established *Tospovirus* genus (19), is unique among plant viruses by its complex particle morphology, unusual genetic organization and transmission by several species of thrips which may also act as its alternative host (3,18,47). It has an extremely wide host range, including 500 dicotyledonous and monocotyledonous plant species in 50 different families, and is worldwide in its distribution (9,10,20,39). TSWV virions are spherical or quasi-spherical, enveloped, about 80-100 nm in diameter (34,36) and contain four different structural proteins. Two of these, G1 and G2, are components of the projections on the viral envelope and are glycosylated (35,36,42). The N protein is closely associated with viral RNAs to form the nucleocapsids. A fourth which occurs in a few copies and may also complex with viral RNAs is possibly associated with polymerase activity of the virus (13,35,36,42).

The genome of TSWV consists of three single-stranded RNAs of different sizes, denoted large(L) RNA, medium(M) RNA and small(S) RNA (15,35,45). The L RNA (8897 nucleotides) is of negative polarity, believed to encode a putative RNA polymerase of 331.5 kDa (13). The ambisense M RNA (4821 nucleotides) encodes a nonstructural protein (NS_m) of 33.6 kDa and a protein of 127.4 kDa which acts as precursor for the G1 and G2 glycoproteins (27). The S RNA (2916 nucleotides) is also ambisense in coding strategy to encode a nonstructural protein (NS_s) of 52.4 kDa and the N protein of 29 kDa (14,28). These features are in common with arthropod-borne animal

viruses in the family *Bunyaviridae*, hence the basis for grouping tospovirus in this large virus family (19).

Because of its complex morphology, TSWV is instable under in vitro conditions and difficult to purify. Only in recent years, polyclonal antisera of adequate specificity were prepared against the complete particles of TSWV isolates and related viruses (12,21,30). Monoclonal antibodies that specifically react with intact virus or individual virion proteins have also become available (1,22,41). Using a panel of such antibodies, TSWV isolates from various sources are differentiated into serogroups and serotypes (12,39). Some TSWV-like virus isolates with distinct host responses and/or serological characteristics have also been reported (24,30,38,40). Evidently, plant viruses previously known as TSWV constitute a heterogeneous plant virus group that contains members differing widely in host specificity, serology, and other properties to deserve more thorough characterization. Indeed, the formerly known serotype TSWV-I(TSWV-impatiens) is sufficiently distinct in serology and symptomatology to justify its establishment as a separate virus, impatiens necrotic spot virus (INSV) in the *Tospovirus* genus (30,31,32).

In this study, we developed a purification procedure that is simple and applicable to different tospoviruses and characterized a tospovirus which infects cucurbits systemically. Comparisons were made of the latter with the type strain of TSWV (TSWV-L) and the distinct INSV (30,39) in serology, protein composition and host reaction.

As the research was in progress, a paper comparing virus isolates in the Tospovirus genus from various sources, including those from Taiwan able to infect watermelon, appeared (2), with results consistent with ours in that the watermelon-infecting tospovirus has N protein of a relatively larger molecular weight, not recognized by antisera to TSWV and INSV, although serological cross-reactions occur for the glycoproteins. Parts of our work have been published elsewhere (6,7).

MATERIALS AND METHODS

Virus and plants

The cucurbit-infecting TSWV-like virus isolates used in this study were G (from wax gourd), WMY, WM5 (from watermelon), and MMN2, MMN3 (from muskmelon). A New York isolate of the type strain of TSWV (formerly, TSWV-L), herein designated NY (48,49) and a US isolate of INSV (30), designated I, were included and compared for serological and other characteristics. The isolates WMY and I were received from Dr. S. D. Yeh and referred to as TSWV-W and TSWV-I elsewhere, respectively (30,48,49). All virus isolates except I, were propagated and maintained in *Datura stramonium* L. Less frequently *Nicotiana benthamiana* Domin. and *N. tabacum* L. cv. White Burley were used for these purposes. INSV was propagated in *N. benthamiana*.

Infectivity assay

Virus samples were assayed for infectivity by rubbing on *Chenopodium quinoa* Wild. leaves with the virus inoculum prepared in 0.05 M phosphate, pH 7.4, containing 0.01 M Na_2SO_3 and 0.01 M EDTA, unless stated otherwise. Counting of the local lesions was made at 6-8 days after inoculation. Prior to inoculation, the test plants were darkened for 24 h and leaves dusted with carborundum. Cowpea plants [*Vigna unquigulata* (L.) Walp. cv. Black Eye] were also used as assay plant for NY in some tests, with their first leaves inoculated following 24 hours of darkening.

Purification of virus

The various tospovirus isolates were purified by a method modified from the procedure developed for purification of rice transitory yellowing virus (8). The procedure relies on the use of Percoll density gradients to concentrate the virus and a passage through Sepharose 4B column for removal of contaminating Percoll. As starting material, leaves with early systemic symptoms were collected from infected plants of *D. stramonium* or White Burley tobacco. The leaves, about 50-80 g, were homogenized in extraction buffer, consisting of 0.05 M phosphate, pH 7.4, 10 mM Na_2SO_3 and 10 mM EDTA. The homogenate was strained through cheesecloth and centrifuged twice at 7000 *g* for 5 min each. To the second supernatant, Percoll was added to a final concentration of 28%. The mixture was then centrifuged at 22500 rpm (67500 *g*) for 30 min in a Hitachi RPS 28SA swing rotor. The virus was concentrated as whitish to pale green zone near the bottom of the centrifuge tubes and was withdrawn. The virus sample was then diluted with a buffer consisting of 10 mM phosphate, pH 7.4, 10 mM Na_2SO_3 and 1 mM EDTA and centrifuged for 30 min at 25000 rpm (80000 *g*) in the swing rotor. This step concentrated the virus as a thin layer lying on top of a clear gelatin-like pellet. After removal of the supernatant, virus was resuspended in a small quantity of 10 mM Na_2SO_3, previously adjusted to pH 7.4 and then fractionated on a Sepharose Cl-4B (Pharmacia) column, 54 × 1.2 cm, to remove Percoll. Fractions were collected and their light absorbance at 260 nm determined. Those fractions which adsorbed light strongly were pooled for electron microscopy or other use.

Polyacrylamide gel electrophoresis

Purified virus samples, mixed with an equal volume of a pH 6.5 buffer consisting of 125 mM Tris-

HCl, 20% glycerol (v/v), 10% 2-mercaptoethanol, 4% SDS and 0.002% bromophenol blue, were analyzed by polyacrylamide gel electrophoresis (PAGE). The mixture was first boiled for 3 min. Electrophoresis was performed in a 10 or 12% gel, using a discontinuous buffer system (29). The gels were stained either with silver nitrate (46) or with Coomassie blue.

Antiserum preparation

Antisera to the cucurbit virus isolates G, WMY, and to NY were produced in New Zealand white rabbits by four successive intramuscular injections of purified virus. The virus samples were emulsified with an equal volume of Freund's complete (for primary injections) or incomplete adjuvants (for subsequent injections) before injection. The antisera had a titer of 1:4 to 1:8 as determined by double diffusion tests in agar gel. Polyclonal antisera to the N proteins of isolates WMY and NY were similarly prepared, except that the polyacrylamide gel-separated proteins were used as immunogen. The titer for anti-N protein of isolate WMY was 1:4 and of isolate NY was 1:8 by double diffusion tests.

Polyclonal antisera to the type strain of TSWV [previously, the common serotype TSWV-L (lettuce)] and INSV [previously, the serotype TSWV-I (30)], were obtained from Dr. J. W. Moyer of North Carolina State University.

Western blots

The polyacrylamide gel-separated virus proteins were transferred to nitrocellulose(NC) papers by the method of Towbin et al. (44) in a Bio-Rad Trans-Blot Cell, using a current of 200 mA applied overnight. After transfer, the papers were immunostained essentially as described by O'Connor and Ashman (37). Rabbit antisera to tospoviruses or individual virion proteins were used at 1:800 and the goat anti-rabbit immunoglobulin enzyme conjugate at 1:1000 as the primary and secondary antibodies, respectively.

For detection of glycoproteins, the Con A-peroxidase method of Clegg (11), modified by Faye and Chrispeels (17), was followed. Proteins were electro-transferred from gel to the NC paper and sequentially incubated with Con A (concanavalin A) and peroxidase. The blots were then immersed in a solution of enzyme substrate 4-chloro-1-naphthol in the presence of H_2O_2 to render the Con A-binding glycoproteins visible as purple bands on NC papers.

Glycoproteins were sometimes detected in the polyacrylamide gels by the periodic acid-Schiff staining procedure after electrophoretic separation (16).

ELISA

The indirect ELISA was performed as described by Lommel et al. (33). Leaves of infected *N. benthamiana* were homogenized in the coating buffer (0.05 M sodium carbonate, containing 0.02% NaN_3, pH 9.6) in a 1:9 ratio (w/v) and the extracts clarified at 5000 rpm for 10 min. The polystyrene microtiter plates (Nunc, Denmark) were coated with a 1 to 4 serial dilution of antigens, treated with a 1 to 800 dilution of primary antiserum from rabbits and reacted with alkaline phosphatase labeled goat anti-rabbit immunoglobulin diluted to 1:5000 in buffer. All the polyclonal antisera used as primary antibody were pre-adsorbed with healthy leaf extracts before use (21). *p*-nitrophenyl phosphate substrate was added and color reactions were read with a Bio-Tek Microplate reader (Bio-Tek Instruments, Burlington, VT) 30 min after the addition of the substrate. The relative immunoreactivities of each isolate to different antisera were compared.

Electron microscopy

Purified virus samples collected from effluents of the Sepharose CL-4B column were mixed with an equal volume of 2% uranyl acetate, pH 4.3, then transferred to carbon-coated Formvar membrane on grids. The stained specimens were examined in a JEOL 100B electron microscope at 80 kV.

RESULTS

Purification of TSWV

TSWV has been shown to aggregate easily and sediment on initial low speed centrifugation during purification (4). Loss of infectivity did not exceed 30% from clarifying leaf tissue extracts under our conditions. Infectivity was better retained in buffers containing chelating agents such as EDTA and DIECA. For example, homogenates of TSWV-NY infected *N. benthamiana* prepared in 50 mM phosphate buffers, pH 7.4, containing 10 mM Na_2SO_3, lost the infectivity completely in 2 hours at 24 C as assayed on *Chenopodium quinoa* leaves. However, infectivity of such homogenates with added EDTA or DIECA to 10 mM was not substantially reduced during same standing period.

Purified virus preparations thus obtained contained both intact virion and the viral core from which envelope had been lost (Fig. 1). The enveloped particles were spherical or nearly spherical, about 105 nm in diameter, and the core also spherical, with a diameter ranging from 75 to 100 nm.

The purification procedure adopted in this study was suitable for NY and all the cucurbit-infecting virus isolates tested. Infectivity was detectable in samples from the Percoll gradients (24% of the original) and the Sepharose CL-4B column effluents (0.1% of the original). Host materials, i.e., broken chloroplasts and

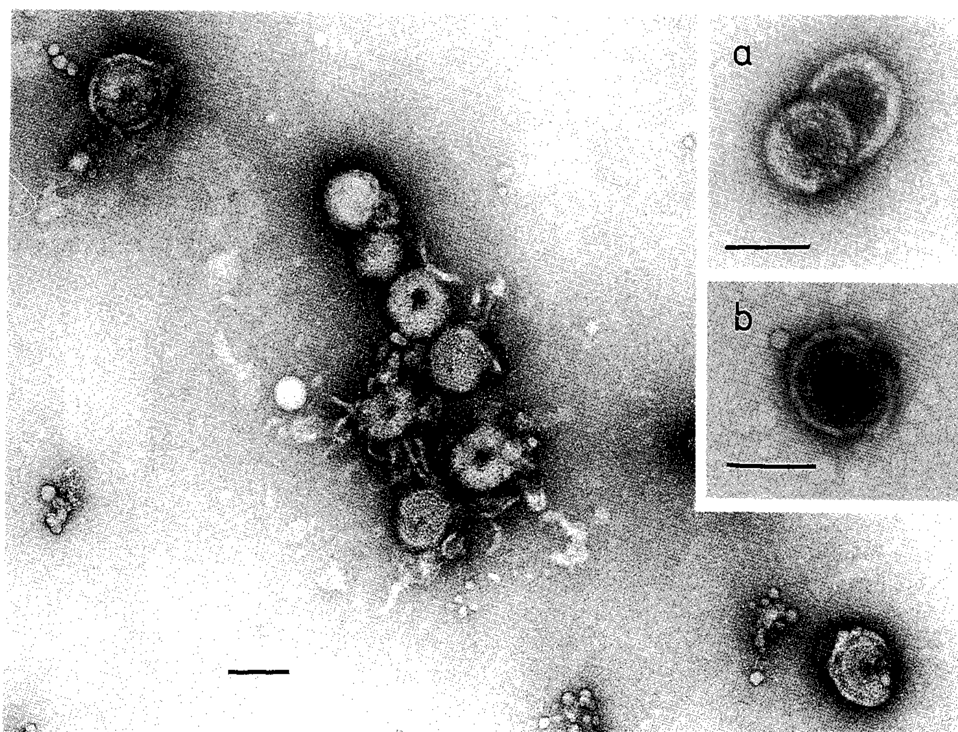

Fig. 1. Electron micrographs of purified WMY isolate stained with 2% uranyl acetate. Note that most particles had lost their envelope, leaving undamaged or damaged cores. The detached envelopes take a hairy look. Small particles 20–25 nm in diameter are contaminating Percoll particles. Insert (a) shows the detachment of envelope from a virus particle. In insert (b), an undamaged spherical particle is seen. All bars represent 100 nm.

other cell organells, were largely eliminated in these steps. A repetition of the Percoll gradient centrifugation step further minimized the contaminants of host origin. The virus-containing fractions of Sepharose column eluate were rather clean as evident from the protein profiles in polyacrylamide gels after electrophoresis (Fig. 2). These fractions were not completely freed from Percoll contamination. But the latter did not interfere with electrophoretic analysis.

Virion proteins of virus isolates

SDS-PAGE analysis showed that purified samples of the cucurbit-infecting virus isolates consistently yielded a protein profile that showed a major protein band at 31 K position and two less prominent protein bands at 84 K and 53 K positions. The protein profile of the NY isolate of TSWV differed from that of cucurbit isolates in that the major band occurred at 29 K position. The positions of the two minor bands were same as those of the cucurbit isolates. Healthy plant materials similarly processed gave a band at 53 K position only, but of far greater intensity than similarly positioned band detectable in virus samples (Fig. 2).

The three proteins were electro-transferred from gels to nitrocellulose papers and subjected to the Con A/peroxidase staining procedure for glycoproteins (11,17). Both the 84 K and 53 K (The estimate of molecular weight for glycoproteins by polyacrylamide gel electrophoresis may vary with the gel concentration. The molecular weight values for the two glycoproteins as obtained in this study have not been corrected by expotation.) proteins were stained as positive. The procedure failed to stain the 31 K protein of the cucurbit isolates and the 29 K protein of NY. Puzzlingly, the 53 K band in the healthy samples was also stained as positive (Fig. 3).

Serological comparison by ELISA

Results of a typical ELISA test are presented in Fig. 4. The cucurbit-infecting isolates G (from wax gourd) and WMY (from watermelon) reacted about equally strongly with antiserum prepared to either. There was a weak but positive reaction of the NY isolate with the antiserum to G (Fig. 4). In other tests, a weak reaction of NY with anti-WMY was also noted. Likewise, antiserum to NY reacted specifically with the homologous antigen. There was also a weak reactivity of anti-NY and, more conspicuously, anti-TSWV-L (received as a gift from J. W. Moyer) with the G and WMY antigens. In less extensive ELISA tests, isolates WM5, MMN2, MMN3 showed same serological properties as did G and WMY.

There was a lack of serological reaction of anti-INSV with the cucurbit isolates as well as with NY. A

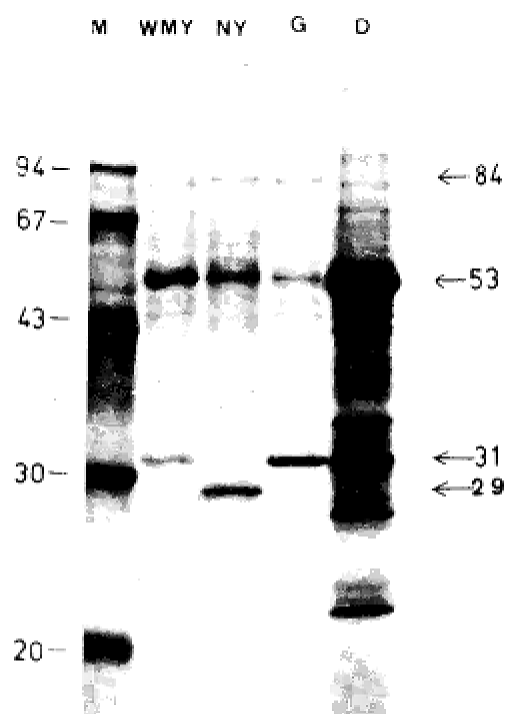

Fig. 2. Polyacrylamide gel electrophoretic analysis of virion proteins of TSWV-NY and the cucurbit-infecting virus isolates. Purified preparations of WMY, G and NY were electrophoresed, together with healthy control sample, in 10% polyacrylamide gel. The gel was stained with silver nitrate. Lanes WMY, NY and G represent virus samples of the respective isolates from infected *Datura stramonium*. Lane D represents healthy control sample from *D. stramonium* and Lane M the molecular weight markers.

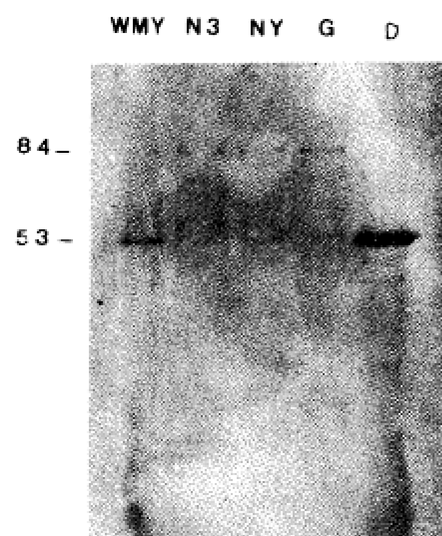

Fig. 3. Analysis of glycoproteins of TSWV-NY and cucurbit-infecting virus isolates. Polyacrylamide gel-separated proteins were electrotransferred to NC paper. Strips of the blotted paper were processed according to the Con A-peroxidase procedure for staining of glycoproteins. Lanes WMY, N3 and G represent cucurbit-infecting virus isolates originated from watermelon (WMY), muskmelon (MMN3) and wax gourd (G), respectively. Lane NY represents TSWV-NY and lane D healthy *Datura stramonium*. Note that a positive staining was obtained for the 84K protein of MMN3, NY and G and for the 53K proteins contained in WMY and healthy samples. A weak positive band at 53K position was barely detected for NY and G.

negative test was also obtained when leaf extracts from INSV-infected plants of *N. benthamiana* were tested as antigen to antisera specific for G, WMY and NY.

Western blotting and immunostaining

The serological relationships of the cucurbit-infecting virus isolates with NY and I were further studied by immunostaining of Western blots. When polyacrylamide gel-separated virion proteins were electrophoretically transferred to NC papers, protein N (31K) of the cucurbit isolates was positively stained either with anti-G or with anti-WMY used as primary antibody. The 31K protein was not stained when the primary antibody was anti-NY or anti-TSWV-L. Likewise, the N protein (29K) of NY was specifically stained by the polyclonal antisera to NY and TSWV-L as primary antibody. No positive staining was obtained when N protein of NY was allowed to react with anti-G and anti-WMY (Fig. 5).

Both G1(84K) and G2(53K) proteins of NY and the tested virus isolates systemically infecting cucurbits could be stained as positive with any of the polyclonal antisera produced to virion of G, WMY and NY (Fig. 5). However, the G1 band was consistently more conspicuous than the G2 band on the blots. We also found that the staining was more intense using anti-TSWV-L than using anti-G or anti-WMY as primary antibody in parallel tests. A relatively weak reactivity of the latter two antisera toward the glycoprotein antigens might account for such a result.

Using polyclonal antisera to gel-separated protein N as primary antibody, a similar result to that described above was obtained, i.e., anti-WMY-N specifically stained the N protein of all tested isolates which systemically infect cucurbits, and it failed to stain N protein of NY. Likewise, anti-NY-N positively stained N protein of NY and it did not stain the N protein of cucurbit-infecting isolates (Fig. 6). In these tests, neither antiserum reacted with the G1 and G2 proteins on the Western blots.

The cucurbit-infecting virus isolates were also compared with INSV in a limited immunoblotting test. When blotted, N protein of INSV was specifically stained by anti-INSV as primary antibody, but was not stained by anti-WMY, anti-G or anti-NY. Anti-INSV failed to stain the N protein of G, WMY and NY.

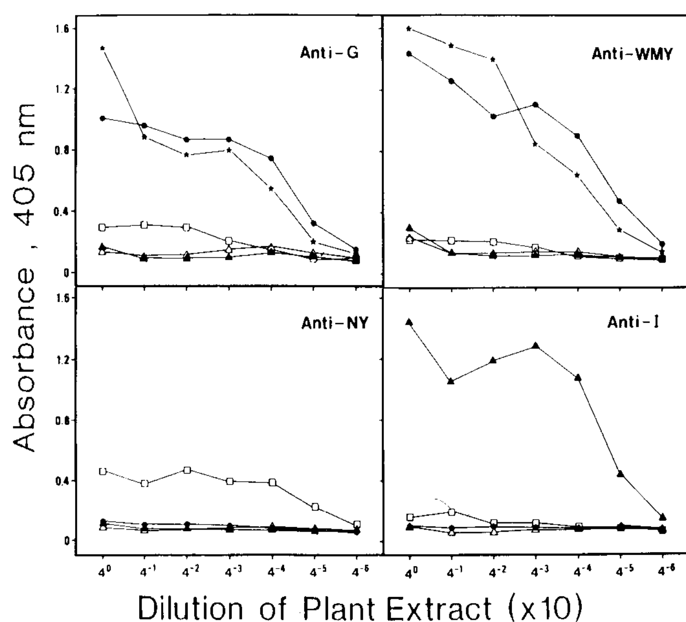

Fig. 4. ELISA determination of serological relationships of the virus isolates that systemically infect cucurbits with TSWV-NY and INSV using polyclonal antisera. The antigens were prepared from virus-infected *Nicotiana benthamiana* plants by homogenizing 1 g leaf tissue in 9 ml of coating buffer and the crude extracts clarified. The 4-fold dilution series were prepared from the clarified plant saps. All antisera were used at 1:800 after pre-adsorption with clarified sap prepared from healthy *N. benthamiana* plants. Alkaline phosphatase conjugated goat anti-rabbit IgG was used at 1:5000. Readings were taken 30 min after addition of the enzyme substrate. Antiserum used to react with the antigens is indicated for each panel. The reaction curves obtained for the different antigens are: G, ★——★; WMY, ●——●; NY, □——□; INSV, ▲——▲; Healthy control, △——△.

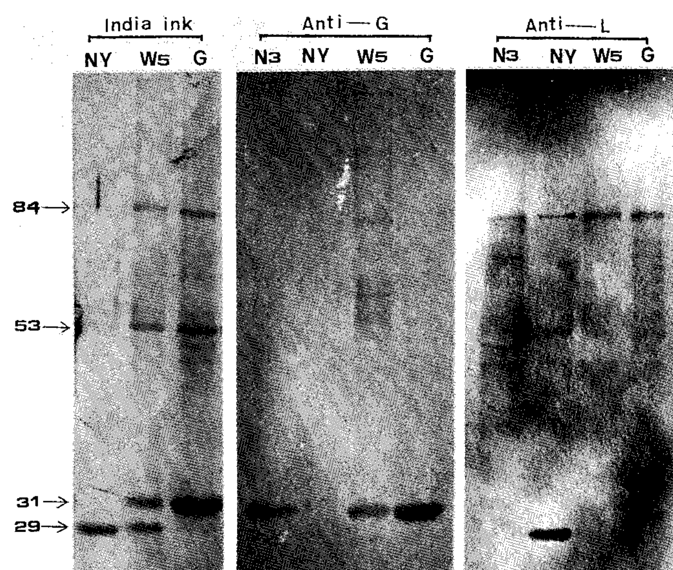

Fig. 5. Serological analysis of virion proteins of TSWV-NY and cucurbit-infecting virus isolates by Western blotting. Polyacrylamide gel-separated proteins of virus isolates were electro-transferred to NC paper for immunostaining. In the left panel, the blots were stained with India ink to mark the positions of G1(84K), G2(53K), and N(31K for cucurbit isolates and 29K for NY) proteins. In the middle panel, the blots were first probed with anti-G and then reacted with alkaline phosphatase conjugate of goat anti-rabbit IgG (1:2000) for a reaction time of 2 hours at room temperature. Color was developed by adding NTB (1 mg/4 ml) and BCIP (5 mg/ml) with a 30 min incubation at 37 C. In the right panel, the blots were probed with antiserum to TSWV-L, followed by treatments as in the middle panel.

Host plant reaction to virus isolates

The host reaction to virus isolates was determined by mechanical inoculation on young plants in Solanaceae, Cucurbitaceae, Leguminosae and Chenopodiaceae. Systemic mottling was induced in *N. benthamiana* by NY and all of the tested cucurbit-infecting virus isolates. Inoculated leaves showed no symptoms, except for NY isolate which induced chlorotic spots. In other *Nicotiana* plants, the symptoms mainly consisted of ring and necrotic spots on inoculated leaves and mottling, necrotic spotting and some distortion of the systemic leaves. Concentric rings were most commonly observed on *N. rustica* and White Burley tobacco plants. No significant symptom difference was found among the virus isolates. Usually, virus infection was fatal to inoculated plants of *N. benthamiana*, *N. glutinosa* L. and *N. edwardsonii* Christie and Hall. On *Datura stramonium*, isolate NY induced more typical mosaic symptoms than did the various cucurbit-infecting isolates, of which the main symptoms consisted of chlorotic and necrotic lesions and ringspots. Under the screenhouse conditions in summer months, old plants of *D. stramonium* infected with the cucurbit-infecting virus showed a silvery greyish green leaf color. The color intensity varied with virus isolates. Necrotic lesions were consistently produced by NY on *Chenopodium quinoa*, whereas inoculation results could be erratic for the cucurbit isolates.

Most significant differentiating symptoms between NY and the cucurbit-infecting isolates were found on watermelon [*Citrullus lanatus* (Thunb.) Matsum. & Nakai]. While the NY isolate of TSWV induced localized necrotic symptoms only, the cucurbit isolates caused a systemic disease manifesting mosaic, leaf rugosity, brown spotting, narrowing and upward curling of leaves, shortened internodes, and stunted plant growth.

The first leaves of *Vigna unguiculata* cv. Blackeye produced rather large chlorotic spots (3–4 mm) when inoculated with NY isolate. In contrast, inocultation

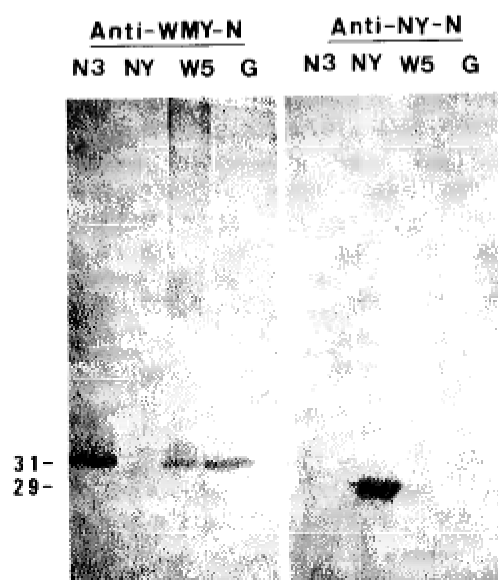

Fig. 6. Serological analysis of virion proteins of the cucurbit-infecting tospovirus isolates and TSWV-NY using polyclonal antisera produced to the N proteins of WMY and NY as primary antibody in Western blotting. Lanes N3, W5, and G indicate tospovirus isolates MMN3 (from muskmelon), WM5 (from watermelon), and G (from wax gourd), respectively. Lane NY represents TSWV-NY. The blots were first probed with anti-WMY-N in the left panel and with anti-NY-N in the right panel. Other procedural details are the same as given in Fig. 5.

with the cucurbit-infecting virus isolates (G, WMY, WM5, MMN3) led to the development of brilliant yellow small spots (1–2 mm). However, the latter reaction could be erratic.

Only a limited test was made with INSV for its host reactions. The virus caused localized chlorotic spots on *D. Stramonium* which rarely became systemic. Mottling and vein necrosis were observed on infected *N. benthamiana* plants. On *N. rustica, N. tabacum* cv. White Burley and *C. quinoa,* the inoculated leaves developed chlorotic spots which turned into necrotic later. On *N. edwardsonii,* these localized host reactions were often followed by systemic spread.

Table 1 summarizes the reactions of test plants to most of the virus isolates studied.

DISCUSSION

Numerous purification procedures have been described for TSWV (3,4,25,36,43), but few of these has gained wide acceptance. Most of the published procedures appear to be lengthy in operation and to involve the use of sucrose density gradients. Joubert et al. (25) pointed out that host derived contaminants could not be efficiently removed by sucrose density gradient centrifugation. Polyclonal antisera produced in rabbits immunized with such purified virus samples usually contain antibodies that react with host plant antigens (21,40,43).

The main advantage of the Percoll centrifugation procedure adopted in the present study is that it involves only a very few steps and could be completed in about 4–5 hours, an important factor to consider when an extremely instable virus like tospovirus is handled. Purified virus preparations obtained in this way retained some infectivity and proved suitable as immunogen for the production of antiserum. The method is mild and the results are reproducible.

Of the known four virion proteins of tospoviruses (L, G1, G2, and N), L protein was not detected in the purified virus preparations in our study. This could be caused by a minute quantity of the protein in virion or by its instability to lead to destruction during virus purification. In the healthy control samples, the presence of a 53K protein may be confusing. Whether the G2 protein (53K) might have been of host origin can not be answered for the moment. The possibility of contamination of G2 protein in purified virus samples with host-derived material of similar molecular weight, the larger chain of the ubiquitous ribulose bisphosphate carboxylase (53–55K) in particular, is not excluded. The latter has been shown not to be glycosylated, however (40a).

Like other large plant viruses (Rhabdoviruses for example), the amount of tospovirus occurring in host plants is very much affected by season. Infected plants produce milder symptoms and contain less virus in the winter months than in summer. In our work, leaves of infected *Datura stramonium* plants were used as starting material in virus purification trials. Greater amounts of virus were obtained from a given quantity of starting material collected in the summer months.

TSWV, the type member of the newly established *Tospovirus* genus, has once been considered as monotypic group of plant viruses based on its unique morphology, protein composition, genome organization and vector relationships (18,23). Serological and biological studies on virus isolates or strains of unlike geographical derivations indicate divergency within this group. The most clearly delineated serogroups by far have been the lettuce serogroup, TSWV-L (21,41) and the impatiens serogroup, TSWV-I known as INSV now (30). By symptoms and serology, the TSWV-like virus inciting silver mottle in watermelon in Japan (24) and that responsible for peanut bud necrosis in India (40) appear to differ from either of the above two.

Evidence obtained in this study indicates that the cucurbit-infecting virus from Taiwan is sufficiently distinct from TSWV and INSV. The N protein of the cucubit virus gave a molecular weight of 31K as compared to 29K for both TSWV and INSV. It did not react with polyclonal antisera produced against either of

TABLE 1. Comparative host reactions of tospovirus virus isolates systemically infecting cucurbits and other known tospoviruses

Plant species	Tospovirus isolates systemically infecting cucurbits					TSWV-NY	INSV
	G	WMY	MMN2	MMN3	WM5		
Solanaceae							
Nicotiana benthamiana	M	VN, M	M	M	VN, M	cs, M	VN, M
N. glutinosa	NS, M, TN	NS, M, TN	NS, M, TN	CS, NS, TN	NS, M	NS, M, TN	NS, M
N. edwardsonii	CS, NS, TN	CS, NS, TN	CS, NS, TN	CS, NS, TN	CS, NS, TN	CS, NS, TN	CS, NS
N. tabacum cv. White Burley	CS, NS, CNR	CS, NS, CNR	CS, NS, CNR	CS, NS, CNR	CS, NS, M, CNR	CS, NS, CNR	CS, NS
N. rustica	CS, NS, CNR	CS, NS, CNR	CS, NS, CNR	CS, NS, CNR	CS, NS, M, CNR	CS, NS, CNR	nt
Datura stramonium	CS, NS, M	CS, NS, M	CS, NS, MM	CS, NS, MM	CS, NS, M	CS, NS, M	CS, NS
Capsicum annuum	—	—	—	—	—	M	nt
Lycopersicon esculentum	NS, M, LC, St	NS, M, St	nt	nt	VN, M, LC, St	YS, VN, St	nt
Chenopodiaceae							
Chenopodium quinoa	cs, ns	ns	ns	ns	ns	cs, ns	ns
C. amaranticolor	—	—	—	ns	—	ns	nt
Cucubitaceae							
Citrulus lantus	M, LB, St	M, LB, St	VN, LB, St	VN, LB, St	VN, LB, St	ns	nt
Leguminosae							
Vigna unquiculata	cs(s)	cs(s)	—	cs(s)	CS(s)	cs(l)	nt

Explanations for abbreviations: CS, chlorotic spots; CNR, concentric necrotic rings; LB, leaf browning; LC, leaf curling; M, mosaic and/or systemic mottling; MM, mild mosaic; NS, necrotic spots; St, plant stunting; TN, top necrosis; VN, vein necrosis; YS, yellow spots; cs, chlorotic spots on inoculated leaves only; ns, necrotic spots on inoculated leaves only; nt, not tested; (l) large lesion size; (s) small lesion size. —, no symptoms or inoculation did not succeed.

the latter viruses by immunoblotting. In ELISA tests, a low level of cross-reaction was sometimes observed between NY (or TSWV-L) and the cucurbit virus isolates. This is believed to have resulted from reactions of the test antisera toward the virion glycoproteins which are conserved among tospoviruses (30). Biologically, the cucurbit virus can be easily separated from TSWV and INSV by vector relationships and by symptoms. While the cucurbit virus has been successfully transmitted by *Thrips palmi* Karny, the insect does not transmit TSWV (5,49). Both of *Thrips tabaci* Lindeman and *Frankliniella occidentalis* (Pergande) transmit TSWV as well as INSV but these insects are non-vector for the cucurbit virus (5,20). Ability to infect watermelon and other plants in Cucurbitaceae systemically is unique for the cucurbit virus and is not possessed by TSWV which causes only localized necrotic lesions. INSV has been shown not to infect cucurbits (30).

Recently the taxonomic position of the cucurbit-infecting virus has become the focus of interest at several laboratories. Working on a number of tospoviruses of different geographical origins, including two cucurbit isolates that were collected from Taiwan, namely, Tospo-WM (same as WHY used in this study) and Tospo-To (collected from tomato), Adam et al. (2) showed that the two Taiwan tospovirus isolates and the watermelon silver mottle virus (WSMV) reported from Japan (24) are serologically identical. The latter also had a 31K N protein (26) as did the Taiwan cucurbit virus (2,6,7,49). Comparing the nucleotide sequence of the N protein gene of the cucurbit virus, designated Tospo-W, with that of other tospoviruses, including TSWV and INSV, Yeh and Chang (48) reported that there is 54.4–55.9% identity, and the complete open reading frame (ORF) of the N protein gene of WSMV is 825 nucleotides long, 39 or 51 nucleotides longer than other tospovirus isolates. Thus, all evidence so far available indicates that the cucurbit virus occurring in Japan and Taiwan constitutes a distinct member in the *Tospovirus* genus.

Interestingly, the virus causing peanut bud necrosis from India also had N protein of 31K (40) and was serologically related to the cucurbit virus (2). Adam et al. (2) considered the peanut virus and the cucurbit virus being strains of a same tospovirus.

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LITERATURE CITED

1. Adam, G., Lesemann, D. E., and Vetten, H. J. 1991. Monoclonal antibodies against tomato spotted wilt virus: Characterization and application. Ann. Appl. Biol. 118:87-104.
2. Adam, G., Yeh, S. D., Ready, D. V. R., and Green, S. K. 1993. Serological comparison of tospovirus isolates from Taiwan and India with impatiens necrotic spot virus and different tomato spotted wilt virus isolates. Arch. Virol. 130:237-250.
3. Best, R. J. 1968. Tomato spotted wilt virus. Adv. Virus Res. 13:65-146.
4. Black, L. M., Brakke, M. K., and Vatter, A. E. 1963. Purification and electron microscopy of tomato spotted wilt virus. Virology 20:120-130.
5. Chen, C. C., Shy, J. F., and Yeh, S. D. 1990. Thrips transmission of tomato spotted wilt virus from watermelon. Plant Prot. Bull. (Taiwan) 32:331 (Abst.)
6. Chiu, R. J. 1992. Strains of tomato spotted wilt virus infecting cucurbits: Purification and partial characterization. Abstracts of Papers p.1. Cross-Taiwan Straits Symposium on Plant Pathology, August 20-25, 1992, Beijing. (in Chinese)
7. Chiu, R. J., Huang, C. R., Weng, M. S., Chen, Y. C., Chang, C. T., Lin, C. J., and Hsu, H. T. 1991. A watermelon strain of tomato spotted wilt virus: Purification and some properties. Plant Proct. Bull. (Taiwan) 33:427 (Abst.)
8. Chiu, R. J., Hsu, Y. H., Chen, M. J., Chen, C. C., Lee, R. C. R., Lin, M. C., Lin, S. M., and Kuo, T. T. 1990. Purification and partial characterization of rice transitory yellowing virus. Phytopathology 80:777-783.
9. Cho, J. J., Mau, R. F. L., Mitchell, W. C., Gonsalves, D., and Yudin, L. S. 1987. Host list of plants susceptible to tomato spotted wilt virus (TSWV). Univ. Hawaii Coll. Trop. Agri. Hum. Resour. Res. Ext. Series 078, 12 pp.
10. Cho, J. J., Mau, R. F. L., German, T. L., Hartmann, R. W., Yudin, L. S., Gonsalves, D., and Provvidenti, R. 1989. A multidisciplinary approach to management of tomato spotted wilt virus in Hawaii. Plant Dis. 73:375-383.
11. Clegg, J. C. S. 1982. Glycoprotein detection in nitrocelluose transfers of electrophoretically separated protein mixtures using concanavalin A and peroxidase: Application to arenavirus and flavivirus proteins. Anal. Biochem. 127:389-394.
12. de Avila, A. C., Huguent, C., Resende, R. de O., Kitajima, E. W., Goldbach, R. W., and Peters, D. 1990. Serological differentiation of twenty isolates of

tomato spotted wilt virus (TSWV). J. Gen. Virol. 71:2801-2807.

13. de Haan, P., Kormelink, R., Resende, R. de O., van Poelwijk, F., Peters, D., and Goldbach, R. 1991. Tomato spotted wilt virus L RNA encodes a putative RNA polymerase. J. Gen. Virol. 71:2207-2216.
14. de Haan, P., Wagemakers, L., Peters, D., and Goldbach, R. 1990. The S RNA segment of tomato spotted virus has an ambisense character. J. Gen. Virology 71:1001-1007.
15. de Haan, P., Kormelink, R., Peters, D., and Goldbach, R. 1990. Genetic organization and expression of the tomato spotted wilt virus genome. Pages 60-66 *in*: Virus-Thrips-Plant Interactions of Tomato Spotted Wilt Virus. Proceedings of a USDA Symposium. Hsu, H. T., and Lawson, R. H. ed. USDA ARS-87, Beltsville.
16. Fairbanks, G., Steck, T. L., and Wallach, D. F. H. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membranes. Biochemistry 10:2606-2617.
17. Faye, L., and Chrispeels, M. 1985. Characterization of N-linked oligosaccharides by affinoblotting with concanavalin A-peroxidase and treatment of the blots with glycosidases. Anal. Biochem. 149:218-224.
18. Francki, R. I. B., Milne, R. G., and Hatta, T. 1985. Tomato spotted wilt virus group. in: Atlas of Plant Viruses. Vol. 1. Pages 101-110. CRC Press, Boca Raton, Florida.
19. Francki, R. I. B., Fauquet, C. M., Knudson, D. L., and Brown, F. 1991. Classification and nomenclature of viruses. Pages 281-283 *in*: 5th Rep. Int. Comm. Taxon. Viruses. Arch. Virol., Suppl. 2.
20. German, T. L., Ullman, D. E., and Moyer, J. W. 1992. Tospoviruses: Diagnosis, molecular biology, phylogeny, and vector relationships. Annu. Rev. Phytopathol. 30:315-348.
21. Gonsalves, D., and Trujillo, E. E. 1986. Tomato spotted wilt virus in papaya and detection of the virus by ELISA. Plant Dis. 70:501-506.
22. Hsu, H. T., Wang, Y. C., Lawson, R. H., Wang, M., and Gonsalves, D. 1990. Splenocytes of mice with induced immunological tolerance to plant antigens for construction of hybridomas secreting tomato spotted wilt virus-specific antibodies. Phytopathology 80:158-162.
23. Ie, T. S. 1970. Tomato spotted wilt virus. C.M.I./A.A.B. Descriptions of Plant Viruses No. 39.
24. Iwaki, M., Honda, Y., Hanada, K., Tochihara, H., Yonaha, T., Hokama, K., and Yokoyama, T. 1984. Silver mottle disease of watermelon caused by tomato spotted wilt virus. Plant Dis. 68:1006-1008.
25. Joubert, J. J., Hahn, J. S., von Wechmer, M. B., and van Regenmortel, M. H. V. 1974. Purification and properties of tomato spotted wilt virus. Virology 57:11-19.
26. Kameya-Iwaki, M., Hanada, K., Honda, Y., and Tochihara, H. 1988. A watermelon strain of tomato spotted wilt virus and some properties of its nucleocapsid. Abstracts of Papers, 5th Intern. Cong. Plant Pathol. p. 65.
27. Kormelink, R., de Haan, P., Meurs, C., Peters, D., and Goldbach, R. 1992. The nucleotide sequence of the M RNA segment of tomato spotted wilt virus, a bunyavirus with two ambisesense RNA segments. J. Gen. Virol. 73:2795-2804.
28. Kormelink, R., Kitajima, E. W., de Haan, P., Zuidema, D., Peters, D., and Goldbach, R. 1991. The non-structural protein (NS_S) encoded by the ambisense S RNA segment of tomato spotted wilt virus is associated with fibrous structures in infected cells. Virology 181:459-468.
29. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of bacteriophage T4. Nature (London) 227:680-685.
30. Law, M. D., and Moyer, J. W. 1990. A tomato spotted wilt-like virus with a serological distinct N protein. J. Gen. Virol. 71:933-938.
31. Law, M. D., Speck, J., and Moyer, J. W. 1991. Nucleotide sequence of the 3′ non-coding region and N gene of the S RNA of a serologically distinct tospovirus. J. Gen. Virol. 72:2597-2601.
32. Law, M. D., Speck, J., and Moyer, J. W. 1992. The M RNA of impatiens necrotic spot tospovirus (Bunyaviridae) has an ambisense genomic organization. Virology 188:732-741.
33. Lommel, S. A., McCain, A. H., and Morris, T. J. 1982. Evaluation of indirect enzyme-linked immunosorbent assay for the detection of plant viruses. Phytopathology 72:1018-1022.
34. Milne, R. G. 1970. An electron microscope study of tomato spotted wilt virus in sections of infected cells and in negative stain preparations. J. Gen. Virol. 6:267-276.
35. Mohamed, N. A. 1981. Isolation and characterization of subviral structures from tomato spotted wilt virus. J. Gen. Virol. 53:197-206.
36. Mohamed, N. A., Randles, J. W., and Francki, R. I. B. 1973. Protein composition of tomato spotted wilt virus. Virology 56:12-21.
37. O'Connor, C. G., and Ashman, L. K. 1982. Application of the nitrocellulose transfer technique and alkaline phosphatase conjugated anti-immunoglobulin for determination of the specificity of monoclonal antibodies to protein mixtures. Jour. Immunol. Methods. 54:267-272.
38. Pang, S. Z., Slightom, J. L., and Gonsalves, D. 1993. The biological properties of a distinct tospovirus and sequence analysis of the S RNA. Phytopathology 83:728-733.
39. Peters, D., de Avila, A. C., Kitajima, E. W.,

Resende, R. de O., de Haan, P., and Goldbach, R. W. 1990. An overview of tomato spotted virus. Pages 1-14 *in*: Virus-Thrips-Plant Interactions of Tomato Spotted Wilt Virus. Proceedings of a USDA Symposium. Hsu, H. T., and Lawson, R. H. ed. USDA ARS-87, Beltsville.

40. Reddy, D. V. R., Ratna, A. S., Sudarshana, M. R., Poul, F., and Kiran Kumar, I. 1992. Serological relationships and purification of bud necrosis virus, a tospovirus occurring in peanut (*Arachis hypogaea* L.) in India. Ann. Appl. Biol. 120:279-286.

40a. Siegel, M. I., Wishnick, M., and Lane, M. D. 1972. Rubulose-1, 5-diphosphate carboxylase. Pages 169-192 *in*: Boyer P. D. ed. The Enzymes. vol. 6. Academic Press, New York.

41. Sherwood, J. L., Sanborn, M. R., Keyser, G. C., and Myers, L. D. 1989. Use of monoclonal antibodies in detection of tomato spotted wilt virus. Phytopathology 79:61-64.

42. Tas, P. W. L., Boerjan, M. L., and Peters, D. 1977a. The structural proteins of tomato spotted wilt virus. J. Gen. Virol. 36:267-279.

43. Tas, P. W. L., Boerjan, M. L., and Peters, D. 1977b. Purification and serological analysis of tomato spotted wilt virus. Neth. J. Path. 83:61-72.

44. Towbin, H., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA 76:4350-4354.

45. Van den Hurk, J., Tas, P. W. L., and Peters, D. 1977. The nucleic acid of tomato spotted wilt virus. J. Gen. Virol. 36:81-91.

46. Wedrychowski, A., Olinski, R., and Hnilica, L. S. 1986. Modified method of silver staining of proteins in polyacrylamide gels. Anal. Biochem. 159:323-328.

47. Wijkamp, I., Van Lent, J., Kormelink, R., Golbach, R., and Peters, D. 1993. Multiplication of tomato spotted wilt virus in its insect vector, *Frankliniella occidentalis*. J. Gen. Virol. 74:341-349.

48. Yeh, S. D., and Chang, T. F. 1995. Nucleotide sequence of the N gene of watermelon silver mottle virus, a proposed new member of the genus *Tospovirus*. Phytopathology (in press).

49. Yeh, S. D., Lin, Y. C., Cheng, Y. H., Jih, C. L., Chen, M. J., and Chen, C. C. 1992. Identification of tomato spotted wilt-like virus on watermelon in Taiwan. Plant Dis. 76:835-840.

摘　要

邱人璋[1]、陳慶忠[2]、徐惠迪[3]. 1994. 一種感染瓜類植物之 tospovirus 之純化與特性測定. 植病會刊 3:198-208. (1. 台中市　國立中興大學遺傳工程中心, 2. 彰化縣　台灣省台中區農業改良場, 3. 美國農部農業研究服務中心花卉與種苗試驗室)

一種引起瓜類植物系統性感染之 tospovirus，已被純化成功。該病毒與蕃茄斑點萎凋病毒 (Tomato spotted wilt virus, TSWV) 之 NY 分離株及鳳仙花壞疽斑病毒 (Impatiens necrotic spot virus, INSV) 之美國分離株比較結果，前者之純化物經凝膠電泳後，可檢出三種蛋白質，依其分子量大小順序，分別爲 84， 53 與 31 kDa。相當於文獻記載之 TSWV 所含 G1， G2，與 N 蛋白。其中 31K 蛋白含量較高， 84K 與 53K 者檢出量均甚低， NY 與 I 兩分離株在凝膠中所呈現之蛋白質帶與台灣瓜類病毒分離株不同，主要之蛋白質帶，出現在 29K 之位置，而非 31K 之位置，其他二種蛋白在凝膠中之位置，則與瓜類分離株同。利用免疫漬染法，瓜類各分離株病毒之 84， 53 及 31K 三種蛋白與 WMV 及 WG 兩分離株之抗血清均有反應。而以 31K 蛋白反應產生之染色帶，最爲明顯。NY 所含之 84K 及 53K 兩種蛋白，與上述抗血清亦起反應，但 29K 蛋白則否。同一試驗中， NY 以及典型之 TSWV 病毒 (TSWV-L) 抗血清，未能與瓜類分離株之 31K 蛋白反應，但與 84K 及 53K 兩種蛋白則有反應。利用 WHY-N 與 NY-N 蛋白之抗血清，進行電泳免疫漬染法試驗，供試血清分別僅與同源之 N 蛋白起反應。ELISA 測試之結果，前述兩類分離株間，顯示微弱之血清學關係，此係由於二者所含 84K 及 53K 蛋白之共通血清學特性使然。在 ELISA 與免疫漬染試驗時，抗 INSV 血清對各瓜類分離株，未呈現反應性。本研究採用 Percoll 梯度密度離心法，將病毒濃縮於靠近管底之區域，抽出後，使之通過 Sepharose Cl-4B 形成之管柱，藉以移除所污染之 Percoll；方法簡便，不僅可用於純化瓜類病毒分離株，同時亦適用於 TSWV 之純化。

關鍵詞：Tospovirus，蕃茄斑點性萎凋病毒，西瓜銀色斑駁病，病毒純化，病毒血清學。