Purification of Tomato Spotted Wilt Virus Isolated from Tobacco Plants in Morioka, Japan*

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Abstract

A virus isolated from tobacco plants in Morioka was confirmed as tomato spotted wilt virus (TSWV) and designated as TSWV-M isolate. The virus shows severe symptoms on several plants including tobacco, tomato and *Nicotiana benthamiana*. TSWV-M was used for the purification study. Out of seven kinds of buffers for extraction of TSWV, 0.1 M phosphate buffer containing 0.01 M Na₂SO₃ and 0.01 M EDTA·2Na was suitable with high infectivity in crude sap checked by half-leaf method using *N. tabacum* cv. Xanthi nc. In a purification method with isopycnic linear sucrose density gradient with concentration from 15.0% to 60.0% (w/w), the peak fraction of TSWV infectivity was indicated at 42.0% (w/w) sucrose fraction. By this method, however, the host components could not be eliminated from the peak fraction. In isopycnic step-wise sucrose density gradient method with trap of 40.0% (w/w), host components could be excluded from 42.0% (w/w) sucrose fraction with high infectivity. Intact TSWV particles were observed in the fraction by the electron microscopy. With the passage of the TSWV purification process, specific infectivities in the final step were calculated as 44 times at OD₂₈₀ and 46 times higher at OD₂₈₀ in the ratio to the first step preparation.

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Key words: tomato spotted wilt virus, purification, isopycnic linear sucrose density gradient, isopycnic step-wise sucrose density gradient, specific infectivity.

INTRODUCTION

Tomato spotted wilt virus (TSWV), which has very wide host range²⁾, causes severe chlorotic, necrotic and malformed symptoms on host plants in tropical, sub-tropical and temperate zones^{9,13)} and is transmitted by thrips^{1,18)}. In Japan, TSWV causes damages to tomato^{12,25)}, tobacco^{22,23)}, green pepper¹⁹⁾, dahlia⁸⁾ and watermelon⁹⁾.

TSWV particle is constructed with ribonucleic acid^{26,27)}, protein^{17,20)}, phosphorus and lipid. Electron micrographs of ultrathin sections of infected leaves^{7,14,15)} and negatively stained infected leaf sap⁵⁾, show the virus particles with envelope. The virus particles are spherical with spike structure on the envelope and their diameters are about 85 nm. Recently, TSWV is classified to Bunyaviridae from the characteristics of the virus¹⁶⁾.

Since organic solvents and/or surface-active agents have been used for purification of ordinary plant viruses, both reagents can not be used for TSWV. Joubert *et al.*¹⁰⁾ purified TSWV

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fixed with glutaraldehyde by electrophoresis with column after sucrose density gradient centrifugation. On the other hand, Tas *et al.*²⁰ reported that TSWV was purified by excluding host components using antisera to host components. Other purification methods may not have succeeded in purification of particles coated with envelope^{2,3,11,17,18}. This report demonstrates a method of purification to obtain the intact TSWV with high infectivity using step-wise sucrose density gradient.

MATERIALS AND METHODS

Virus and plants. TSWV-M isolated from tobacco in Morioka, Japan in 1983²¹) was used in this study. This virus was mechanically inoculated to several differential host plants. Plants for inoculation were grown in a glass chamber at 25°C for 16 hr light and 20°C for 8 hr dark periods with 60–70% humidity. Inoculation buffer was 0.1 M phosphate buffer (PB) pH 7.0 with 0.01 M Na₂SO₃.

Electron microscopy. TSWV-M infected sap of *Nicotiana tabacum* cv. Xanthi nc leaves was treated with 5% (v/v) glutaraldehyde and mounted on grids coated with a collodion film, and stained with 2% (w/v) phosphotungstic acid pH 7.0 as previously described^{5,6,14}. Samples for ultrathin sections were obtained from systemically infected tobacco (*N. tabacum* cv. Burley 21) leaves, which showed systemic symptoms in 5–6 days after inoculation. Uranyl acetate and lead citrate were used for staining in ultrathin sections. Examinations were done on a JEM-1200 EX electron microscope.

Extraction buffer. Seven kinds of buffers (Table 1) were compared using the same plant source at the same time. Three grams of infected leaf pieces were homogenized in 27 ml of each extraction buffer with mortars and pestles. Their crude sap were filtered through two layers of aseptic gauze and centrifuged at 10,000 × *g* for 10 min. Supernatants and the precipitates were separately resuspended in 1 ml of 0.1 M PB with 0.01 M Na₂SO₃. These crude sap, supernatants and precipitates were inoculated to leaves of tobacco (*N. tabacum* cv. Xanthi nc) for infectivity assay with half-leaf method.

Purification of TSWV. Upper leaves of infected tobacco (*N. tabacum* cv. Burley 21) with typical TSWV symptoms in 4–5 days after inoculation were used as materials for virus purification. Isopycnic sucrose density gradient method in final step was used either linear or step-wise gradients for comparison. Purification of TSWV was performed according to the procedure shown in Fig. 1. All works were done in the cold room at 4°C.

Infectivity assay and purity check. Every fraction after each centrifugation step of isopycnic sucrose density gradient was measured by spectrophotometer at OD₂₆₀ and OD₂₈₀ and quantitatively assayed by inoculation to tobacco leaves (Xanthi nc) in half-leaf method using crude sap of systemically infected tobacco leaves as control, respectively. The fraction with the highest TSWV infectivity was observed with direct negative staining method by electron microscope. The recovery of TSWV from each step was measured by spectrophotometer at OD₂₆₀ and OD₂₈₀ and assayed by infectivity test.

RESULTS

Symptoms of differential host plants

Tomato (*Lycopersicon esculentum* cv. Fukuju-2) showed local necrotic lesions in 4–5 days after sap inoculation and systemic symptoms including malformation and curling of leaves, in 8–10 days, followed by severe mosaic, necrosis and stunting. *N. tabacum* cv. Burley 21 showed local lesions on the inoculated leaves and systemic symptoms that appeared yellowing along veins, followed by mosaic symptoms. Finally, the plants died with severe necrosis (Plate I-1). Inoculated leaf of *N. tabacum* cv. Xanthi nc indicated many necrotic lesions with chlorotic rings (Plate I-2), then systemic yellowing and mosaic appeared. Chlorotic local lesions on inoculated

Fig. 1. Procedure of TSWV purification. *: 0.02 M PB pH 7.0 contained 0.01 M EDTA·2Na and 0.1% (w/v) L-cysteine.

leaf of *N. benthamiana* appeared in 4–5 days after inoculation, followed by yellowing. *Petunia hybrida* (Pearlmix) showed only necrotic local lesions on inoculated leaves, in 3–4 days after inoculation. Inoculated leaf of *Vigna sesquipedalis* showed faint chlorotic ring spots in 5–6 days after inoculation, and followed by systemic yellowing and necrosis.

Electron microscopy

TSWV-M particles with envelope were observed in sap of infected tobacco cv. Xanthi nc (Plate I-3). Most of TSWV particles were coated with spike structural envelope around the core. The particles were about 85 nm in diameter and spherical cores with high electron density were about 60 nm in diameter. In mesophyll cells of systemically infected tobacco cv. Burley 21 leaves, many TSWV particles were observed inside the membrane-like structure in cytoplasm by ultrathin sectioning (Plate I-4). Most of the virus particles were recognized as intact.

Purification of virus

Effects of extraction buffers for extraction of TSWV-M from infected leaf tissues were shown in Table 1. Infectivities of crude sap homogenated with each buffer were shown in any buffers, respectively. Homogenate using 0.1 M PB containing 0.01 M EDTA·2Na kept the higher infectivity in the supernatant after centrifugation than other buffer solutions tested. The effect of buffer containing $MgCl_2$ on the infectivity was not observed. Consequently, 0.1 M PB pH 7.0 containing 0.01 M Na_2SO_3 and 0.01 M EDTA·2Na was used in the following purification.

Comparison with both linear and step-wise isopycnic sucrose density gradients after centrifugation were shown in Fig. 2. The infectivity in each purification process was shown to be the highest at 42.0% (w/w) sucrose concentration in linear gradient. This peak of infectivity, however, was included into broad peak from 33.6% to 38.6% (w/w) when measured at OD_{260} and OD_{280} . Therefore, as host components were contaminated to this fraction, no single narrow peak of TSWV was obtained. In electron microscopic observation, host components accompanied with TSWV particles were observed in this fraction. However, step-wise gradient formed

Table 1. Comparison of extraction buffers for TSWV purification measured by infectivity on tobacco leaves

Buffer	Infectivity ^{a)}		
	Sap	Ppt. ^{b)}	Sup. ^{b)}
1. 0.1 M Phosphate pH 7.0+0.01 M Na ₂ SO ₃	93	9	31
2. 0.1 M Phosphate pH 7.0+0.01 M Na ₂ SO ₃ +0.01 M MgCl ₂	133	13	19
3. 0.1 M Phosphate pH 7.0+0.01 M Na ₂ SO ₃ +0.01 M EDTA·2Na	221	9	76
4. 0.1 M Phosphate pH 8.0+0.01 M Na ₂ SO ₃	97	4	16
5. 0.01 M Phosphate pH 7.0+0.01 M Na ₂ SO ₃	88	7	37
6. 0.01 M Phosphate pH 8.0+0.01 M Na ₂ SO ₃	326	12	27
7. 0.3 M Phosphate pH 7.0+0.01 M Na ₂ SO ₃	115	6	30

a) Infectivity is expressed by relative value in percentage, the number of local lesions on the 3 inoculated half leaves of *N. tabacum* cv. Xanthi nc per a test divided by the number of local lesions on the other half leaves inoculated with control leaf sap in 0.1 M PB containing 0.01 M Na₂SO₃.

b) Precipitates (Ppt.) and supernatants (Sup.) after centrifugation of crude sap (Sap) at 10,000×g, 10 min.

Fig. 2. Fractionations of TSWV by ultracentrifugation in isopycnic linear sucrose density gradient from 15% to 60% (w/w) sucrose concentration (A) and isopycnic step-wise sucrose density gradient of 15%, 40% and 60% (w/w) sucrose concentration (B).

40.0% (w/w) sucrose step as trap could divide TSWV peak at 42.0% (w/w) from host components peak at 37.0% (w/w).

Electron microscopy of purified virus

The virus particles were observed by electron microscope using negative staining (Plate I-5). Most of TSWV particles were coated with envelope around the core.

Recovery of virus

Recovery of virus in each purification step was shown in Table 2. The infectivity was detected in all steps except in step 2. Absorbencies at OD₂₆₀ and OD₂₈₀ were increased, with the

Table 2. Recovery of TSWV from each purification step

	Step 1	Step 2 ^{b)}	Step 3	Step 4	Step 5
Volume (ml)	246	256	25	40	4
Infectivity ^{a)}	33	0	155	61	28
OD ₂₆₀	39.8	30.3	9.3	1.6	0.8
OD ₂₈₀	28.3	21.5	5.0	0.9	0.5
OD ₂₆₀ /OD ₂₈₀	1.41	1.41	1.86	1.92	1.46
Specific infectivity					
Infec./OD ₂₆₀	0.83	0.00	16.67	37.42	36.84
(ratio/Step 1)	1.00	0.00	20.08	45.08	44.39
Infec./OD ₂₈₀	1.17	0.00	31.00	71.76	53.85
(ratio/Step 1)	1.00	0.00	26.50	61.33	46.03
Total relative quantity of TSWV					
Infec. × vol.	8118	0	3798	2440	112
(%)	100.0	0.0	46.8	30.1	1.4
Infec. × OD ₂₆₀	9798	0	228	65	3
(%)	100.0	0.0	2.3	0.7	0.1
Infec. × OD ₂₈₀	6962	0	123	32	2
(%)	100.0	0.0	1.8	0.5	0.1

a) Infectivity is expressed by relative value as described in footnote of Table 1.

b) Discarded supernatant was measured.

passage of the purification process, and specific infectivities, measured by infectivity per unit UV optical density, in the final step were calculated as 44.4 times at OD₂₆₀ and 46.0 times higher at OD₂₈₀ in the ratio to the first step preparation. Total relative amounts of virus, which were calculated by infectivity multiplied by volume (ml), OD₂₆₀ or OD₂₈₀, respectively, were decreased gradually. Considerable amounts of TSWV included in plants were lost through the purification process.

DISCUSSION

It is clear that TSWV-M is different from another isolate obtained from tobacco plants in Okinawa (TSWV-R)^{22,23)} which shows yellow mottle without severe necrosis on tobacco plants. Host range of TSWV has been reported that covers 157 species of 29 families in dicotyledons and 6 species of 5 families in monocotyledons²⁾, and symptoms have been recorded in detail. The symptoms of plants inoculated with TSWV-M are similar to those reported previously^{2,6,8)} except TSWV-R²³⁾ and watermelon isolates⁹⁾ which also show similar symptoms to TSWV-R. *P. hybrida* and *N. tabacum* cv. Xanthi nc are useful for assay plants. *N. tabacum* and *N. benthamiana* are suitable as source plants for purification.

Particles of TSWV-M with envelope in membrane-like structure were observed by ultrathin sectioning as reported previously⁷⁾.

Purification of TSWV is known to be rather difficult^{4,23)}. There are several reasons and one reason is that TSWV is inactivated *in vitro* within 2–5 hr at room temperature⁴⁾. The other reason is that as TSWV was coated with envelope which consisted of lipid, phosphorus and protein^{17,20)}, and organic solvents and/or surface-active agents which are usually used for plant virus purification are not applicable to obtain intact TSWV and to remove host components.

Mohamed *et al.*¹⁷⁾ used rate-zonal linear sucrose density gradient method from 10.0% to 40.0% (w/v) sucrose concentration instead of organic solvent and/or surface-active agent. Joubert *et al.*¹⁰⁾ used zone electrophoresis of the column in fixing treatment with glutaraldehyde, linear sucrose density gradient of 10.0% to 40.0% (w/v). Since TSWV fixed with glutaraldehyde

lost the biological activities, infectivity of purified virus have not been checked. Tas *et al.*²⁰⁾ used antisera against host components for elimination of host components from partially purified virus suspension. This method showed poor separation between the virus and host components by our preliminary test. In the present study, sucrose density gradient was modified for excluding components of host plants. Sample contained TSWV was centrifuged on 15.0% (w/w) to 60.0% (w/w) isopycnic step-wise sucrose density gradient for 16 hr. This gradient tube was formed sucrose step on 40.0% (w/w) as a trap to separate TSWV from host components. After centrifugation, two peaks of absorbencies at OD₂₆₀ and OD₂₈₀ appeared on 42.0% (w/w) and 38.5% (w/w), respectively. The peak on 42.0% (w/w) had high infectivity on tobacco leaves and also contained many intact TSWV particles with envelope observed under the electron microscope. From these results, this method is useful for TSWV purification. This isopycnic step-wise sucrose density gradient, however, took long time (more than 15 hr) and unstable viruses like TSWV may lost some infectivity. Though the estimated amounts of virus decreased in each passage of this purification process, specific infectivity was shown to increase. Therefore, TSWV was concentrated step by step.

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和 文 摘 要

津田新哉・夏秋啓子・都丸敬一：タバコから分離されたトマト黄化えそウイルスの精製

1983 年、岩手県盛岡市のタバコから分離されたウイルスは、トマト、タバコ、*Nicotiana benthamiana*、ペチュニアおよびササゲなどの植物への汁液接種の結果および電子顕微鏡観察によってトマト黄化えそウイルス (TSWV) と確認された。ショ糖密度勾配遠心法を用いて本ウイルスの精製を試みた。はじめに、感染葉からのウイルスの抽出を目的として、7 種類の磨碎用緩衝液を検討した結果、0.1 M リン酸緩衝液 (pH 7.0, 0.01 M 亜硫酸ナトリウムと 0.01M EDTA を含む) は、タバコ葉を用いた半葉法による感染価の検定によって適当と考えられた。40.0% (w/w) ショ糖濃度を階段状とした段階ショ糖平衡密度勾配遠心法を行った結果、宿主植物由来の物質のピークと TSWV 感染性のピークとは分離され、TSWV 感染性の最大値を示す分画には、電子顕微鏡観察で TSWV 粒子が多数観察された。精製各過程における比感染価は、精製の進行に伴って増大し、第一段階の感染価に対して最終的には OD₂₆₀ 値で約 44 倍、OD₂₈₀ 値では約 46 倍となった。

Explanation of plate

Plate I

1. Systemic symptoms of *N. tabacum* cv. Burley 21 by TSWV-M sap inoculation. The arrow shows inoculated leaf.
2. Local lesions on inoculated leaf of *N. tabacum* cv. Xanthi nc.
3. Electron micrograph of TSWV particles with envelope in the dip preparation of systemically infected *N. tabacum* cv. Burley 21. Bar indicates 100 nm.
4. Ultrathin section of a mesophyll cell of systemically infected *N. tabacum* cv. Burley 21. Bar indicates 200 nm.
5. Electron micrograph of purified TSWV in the fraction with 42.0% (w/w) sucrose concentration following isopycnic step-wise sucrose density gradient centrifugation. Bar indicates 100 nm.

Plate I