Detection of Turnip Mosaic Virus Isolates Using an Antiserum to Coat Protein Breakdown Products

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ABSTRACT

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The identification of four major pathotypes of turnip mosaic virus, TuMV-C1,-C2,-C3, and -C4, was based on the specific resistance found in Chinese cabbage (*Brassica campestris* L. subsp. *pekinensis*) cultivars from China and Japan. Initial tests with an antiserum to TuMV Florida isolate (TuMV-FL) from another source did not detect TuMV-C2 in sodium dodecyl sulfate (SDS)–immunodiffusion and indirect enzyme-linked immunosorbent assay (ELISA). In order to develop an antiserum with a broader spectrum of reaction, purified virus of TuMV-C1 was stored for 1 week, to ensure cleavage of the coat protein, before being used for immunization. Polyclonal antiserum produced to TuMV-C1 reacted with the four TuMV pathotypes and 15 other isolates in SDS-immunodiffusion and double antibody sandwich and indirect ELISA. In Western blot, this antiserum reacted mainly to breakdown products of 29K and 27K of the coat protein; in contrast, antiserum to TuMV-FL reacted primarily to the native coat protein (38K). Broader reactivity of the TuMV-C1 antiserum should make it useful for screening breeding lines and for general diagnosis of TuMV.

Turnip mosaic potyvirus (TuMV) is transmitted in a nonpersistent manner by several aphid species but not through seeds (25). It is distributed worldwide and can infect species of more than 20 dicotyledon families, including crucifers (7,10,12,17–19,22,23,25,26,29), legumes (3,6,16), ornamentals (18,25,28), and weeds (24).

During the evaluation of 46 Chinese cabbage cultivars (*Brassica campestris* L. subsp. *pekinensis* (Lour.) Olsson) from China and Japan for resistance to TuMV, Provvidenti (17) identified four major pathotypes (TuMV-C1, -C2, -C3 and -C4) among a large number of isolates of this virus. The four pathotypes had been collected in New York State, and recovered from cabbage, Chinese cabbage, turnip, or other hosts. Immunity to TuMV-C1 and resistance to TuMV-C2 were found in Japanese as well as in Chinese cultivars of Chinese cabbage. Plant introductions from mainland China were diverse in reaction to TuMV isolates, but provided sources of immunity or resistance to TuMV-C3 and resistance to TuMV-C4. Immunity or resistance to TuMV isolates C1 to C4 were present in PI 419857, and appeared to be inherited independently of each other. Thus, TuMV-pathotype-specificity must be considered in breeding programs to develop resistant cultivars, since cultivar performance will depend upon the pathotypes used to evaluate material in the program. The four pathotypes have been found in several areas of the world and researchers have also identified additional pathotypes and sources of resistance (7,10,23,26).

In breeding programs for TuMV, selection of resistant plants based on symptoms or recovery tests is laborious and time consuming. When properly used, serological tests, especially enzyme-linked immunosorbent assays (ELISA), can facilitate the accurate identification of resistant germ plasm. In our initial tests, an antiserum obtained from Florida failed to react with TuMV-C2 in indirect ELISA and sodium dodecyl sulfate (SDS)–immunodiffusion tests. Thus, we were particularly interested in developing an antiserum that would react with a range of TuMV isolates.

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MATERIAL AND METHODS

Virus isolates. The four original pathotypes TuMV-C1, -C2, -C3, and -C4 were still available from a previous study (17) while 15 other isolates were secured from different sources (Table 1). All were maintained in the turnip cultivar Presto grown in the greenhouse and used as source for virus purification and other tests. An antiserum prepared by D. E. Purcifull (*unpublished*) against a Florida isolate of TuMV (20) was used. The same Florida isolate was designated as TuMV-T by McDonald and Hiebert in their study (13).

Virus purification and antiserum production. Ten to 20 days after inoculation, turnip leaves infected with TuMV-C1 were harvested and stored at –20°C for 1 week. Virus was obtained by the method of Hiebert and McDonald (8) with some minor modifications. Partially purified virus preparation was stored overnight at 4°C and further purified by CsCl density gradient centrifugation. Purified virus preparations were kept for 1 week at 4°C and were then dialyzed overnight with 0.02 M phosphate buffer, pH 7.5, and used for immunization. The purified virus was initially injected in 6 sites along the back of a white New Zealand rabbit using 1 ml of virus (1 mg/ml) emulsified with 1 ml of Freund's complete adjuvant. This injection procedure was repeated weekly two more times except that Freund's incomplete adjuvant was substituted for the complete

Table 1. Comparative serological reactions of turnip mosaic virus isolates

Isolate	Sodium dodecyl sulfate double diffusion		DAS-ELISA[b]		Indirect ELISA[c]		Western blot	
	FL[a]	C1[a]	FL	C1	FL	C1	FL	C1
TuMV-C1	+	+	0.81(+)	1.61(+)	0.97(+)	0.47(+)	+	+
TuMV-C2	–	+	0.19(+)	1.43(+)	0.02(–)	1.25(+)	+	+
TuMV-C3	+	+	0.47(+)	0.94(+)	0.68(+)	0.74(+)	+	+
TuMV-C4	+	+	0.15(+)	0.35(+)	0.64(+)	0.77(+)	+	–
ESC8-NJ	+	+	0.28(+)	1.72(+)	0.97(+)	0.69(+)	–	+
14 TuMV isolates	NT[d]	+	NT	1.51(+)	NT	0.38(+)	+	+
Healthy	–	–	0.01(–)	0.01(–)	0.01(–)	0.01(–)	–	–
Potyviruses[e]	–	–	NT	0.01(–)	NT	0.01(–)	NT	NT

[a] FL and C1 mean anti-TuMV-FL and anti-TuMV-C1 respectively.
[b] Doube antibody sandwich–enzyme-linked immunosorbent assay. An average OD_{405nm} reading taken only 10 min after addition of substrate. (+): OD_{405nm} reading over 0.100 and at least twice higher than that of healthy control was considered to be positive.
[c] An average OD_{405nm} reading taken 45 min after addition of substrate.
[d] NT = not tested.
[e] Potyviruses tested were papaya ringspot virus, zucchini yellow mosaic virus, bean yellow mosaic virus, and watermelon mosaic virus 2.

adjuvant in the last two injections. After the third injection, the rabbit was bled every week for a period of 5 months.

Electron microscopy. Purified virus particles were negatively stained with 2% phosphotungstic acid (PTA) or 1% uranyl acetate (UA) and observed using a JEOL JEM 100B electron microscope. For immunodecoration, antiserum was used to trap (1:10 dilution) and to decorate (1:100 dilution) virus particles as described by Milne and Luisoni (15).

SDS-immunodiffusion. SDS-immunodiffusion tests were conducted as described by Purcifull and Batchelor (21) in 0.8% Ionagar, 1.0% sodium azide, and 0.5% SDS. Crude antigens were prepared from freshly harvested leaves of infected turnip. One gram of tissue was ground in 1 ml of distilled water followed by the addition of 1 ml of 3% SDS to dissociate virus particles. The samples were expressed through cheesecloth and used immediately. For purified virus preparations, SDS was added to a final concentration of 1.5%.

ELISA. For double antibody sandwich (DAS) ELISA, optimum concentrations were standardized by coating plates with 4, 2 and 0.5 µg/ml of gamma-globulin that was purified by DEAE sephacel (Pharmacia, Uppsala, Sweden) (5). Extracts from infected and control tissues were diluted at 1/10, 1/100 and 1/1,000 with extraction buffer (phosphate-buffered saline [PBS]–Tween 20 + 2% polyvinylpyrrolidone [PVP]). The alkaline phosphatase (Sigma, St. Luis, Mo.) conjugate was prepared according to Clark and Adams (5) and modified by Bar-Joseph et al. (1). Optimum concentration of enzyme conjugate was standardized by twofold dilution beginning at 1/1000. The optical density was recorded by a MicroELISA auto reader (MR 580, Dynatech Laboratories, Inc., Chantilly, Va.) at 15-min intervals until 1 h after addition of substrate. Indirect ELISA was conducted by first coating plates with antigen prepared in coating buffer (0.05 M sodium carbonate, pH 9.6, 0.02% sodium azide). After reaction with 1 µg/ml of gamma-globulin, goat anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate (Sigma) was incubated and OD_{405nm} was measured after addition of substrate. Other procedures were similar to those described above for DAS-ELISA.

SDS-PAGE. Coat protein molecular weights were estimated by polyacrylamide gel electrophoresis (PAGE) on an SDS-polyacrylamide gel consisting of 5% polyacrylamide in stacking gel and 12% polyacrylamide in separating gel (11).

Western blot. Western blot analysis was conducted following the method described by Towbin et al. (27). Crude tissue extract was prepared according to the method of Berger et al. (2). Each 100 mg of fresh leaf tissue samples was ground with 200 µl of sample extraction buffer (750 mM Tris-HCl, pH8.8, 4% SDS, 4% 2-mercaptoethanol, and 40% sucrose). After boiling for 10 min, the sample was clarified by centrifugation for 4 min at 10,000 rpm, and a 20-µl sample was analyzed by SDS-PAGE. One gel was stained with Coomassie blue and the other duplicate gel was

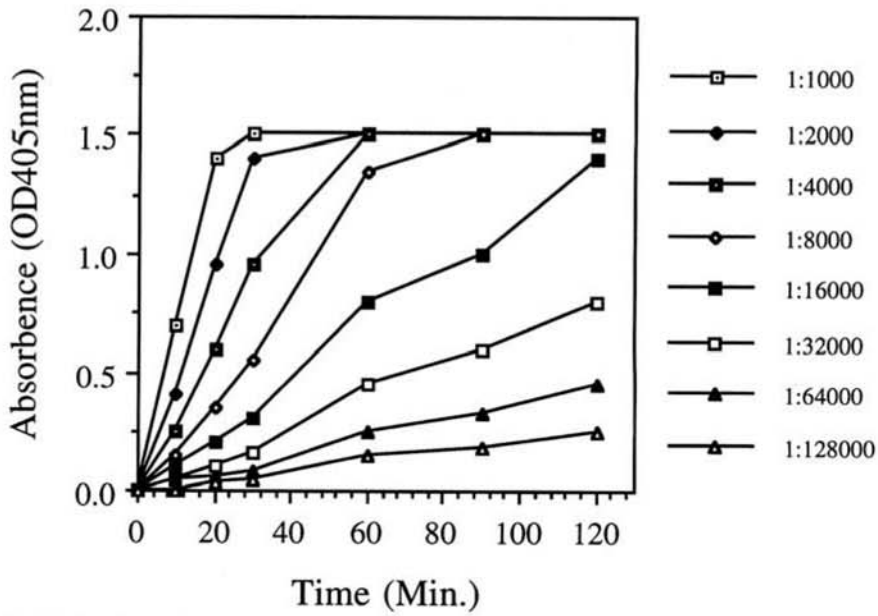

Fig. 2. Standardization of a freshly prepared alkaline phosphatase conjugate for double antibody sandwich–enzyme-linked immunosorbent assay (DAS-ELISA). Concentrations of enzyme conjugate were tested under conditions of 0.5 µg/ml of gamma-globulin for coating and 1/100 dilution of infected leaf tissue in ELISA.

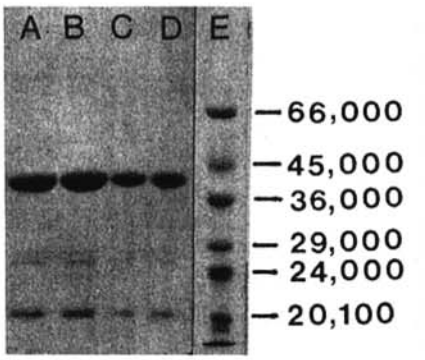

Fig. 1. Coomassie blue–stained coat protein profile of freshly purified turnip mosaic virus (TuMV) preparations analysized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The native 38K coat protein along with its breakdown products were detected. Four batches of virus preparation were loaded on lanes A, B, C and D respectively. Protein molecular weight marker contains 66K (bovine albumin), 45K (egg albumin), 36K (Glyceradehyde-3-phosphate dehydrogenase), 29K (Carbonic anhydrase), 24K (Trypsinogen) and 20.1K (Trypsin inhibitor) (Sigma, St. Louis, Mo.).

Table 2. Reciprocal tests of turnip mosaic virus pathotypes with doube antibody sandwich–enzyme-linked immunosorbent assay

Coating (2 µg/µl)	Antigen (1:20)	Conjugate	OD_{405nm}[a]
Anti-TuMV-FL	TuMV-C1	Anti-TuMV-FL	0.809
	TuMV-C2	(1:500)	0.194
	TuMV-C3		0.474
	TuMV-C4		0.154
Anti-TuMV-FL	TuMV-C1	Anti-TuMV-C1[b]	0.034
	TuMV-C2	(1:1000)	0.037
	TuMV-C3		0.022
	TuMV-C4		0.022
Anti-TuMV-C1	TuMV-C1	Anti-TuMV-C1[b]	0.877
	TuMV-C2	(1:1000)	0.871
	TuMV-C3		0.826
	TuMV-C4		0.867
Anti-TuMV-C1	TuMV-C1	Anti-TuMV-FL	1.033
	TuMV-C2	(1:500)	1.015
	TuMV-C3		1.001
	TuMV-C4		0.990

[a] OD_{405nm} readings were taken 30 min after addition of substract. At this stage, the healthy turnip control gave an OD_{405nm} reading of 0.032. A reading that was over 0.100 and at least twice of that of healthy control was regarded as positive reaction.

[b] Anti-TuMV-C1 alkaline phosphotase conjugate used in these tests had been kept for 6 years at 4°C after initial conjugation.

electro-transferred onto an Immobulin membrane (Millipore, Bedford, Mass.). The blotted membrane was blocked with nonfat milk and then incubated in 3 µg/ml of gamma globulin to TuMV antiserum for 4 h at 30°C. Antibody binding was detected using a protein A gold conjugate solution according to the manufacturer's instruction (Bio-Rad, Richmond, Calif.).

RESULTS

Biological properties. Infected Presto turnip displayed rather distinct symptoms with the four typical pathotypes of the virus. TuMV-C1 incited prominent leaf curling and mosaic, while TuMV-C2 caused a diffuse foliar mosaic and moderate stunting. Plants infected with TuMV-C3 displayed severe mosaic and necrosis, whereas those infected with TuMV-C4 reacted with prominent local lesions and foliar mosaic. The other 15 isolates of TuMV, which were recovered from various cruciferous hosts, caused a range of symptoms in Presto turnip that were similar to those incited by the four pathotypes.

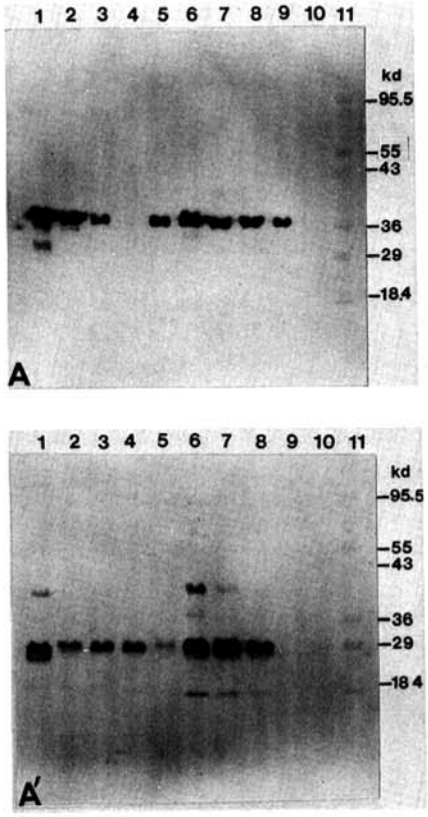

Fig. 3. Western blot analysis of different turnip mosaic virus (TuMV) isolates with antisera to TuMV-C1 (panel A′) and to TuMV-FL (panel A). Panels A and A′ were duplicate of the same samples. Lanes 1 to 9 were different isolates of TuMV, TuMV-Asgrow7 (lane 1); TuMV-Asgrow8 (lane 2); TuMV-H12 (lane 3); TuMV-ESC8NJ (lane 4); TuMV-Maine (lane 5); TuMV-C1 (lane 6); TuMV-C2 (lane 7); TuMV-C3 (lane 8); TuMV-C4 (lane 9). Lane 10 was healthy turnip control. Lane 11 was prestained standard protein molecular weight marker. Note that TuMV-FL antiserum reacts primarily to the 38K protein (Panel A) while the TuMV-C1 antiserum reacts to the 29K and 27K proteins (panel A′). TuMV-FL antiserum did not react with TuMV-ESC8NJ (A4) and TuMV-C1 antiserum did not react with TuMV-C4 (A′9).

Virus purification. Purified virus preparations collected after two cycles of CsCl density gradients appeared free of host contaminants under an electron microscope (data not shown). However, heterogeneous products were already observed in several batches of freshly purified preparations that were analyzed by SDS-PAGE (Fig. 1). These preparations were kept at 4°C for 1 week before being used for immunization.

Serology. In SDS-immunodiffusion, the dilution endpoint of the antiserum prepared to TuMV-C1 ranged from 1/256 to 1/2,048 depending upon the bleedings from which the antiserum was obtained. Diluted antiserum (1:32) formed more distinct precipitin lines than undiluted serum (data not shown). The antiserum gave prominent reactions with all TuMV isolates tested, and no cross-reaction was observed with antigens derived from plants infected with other potyviruses, including papaya ringspot, zucchini yellow mosaic, bean yellow mosaic, and watermelon mosaic virus 2.

In DAS-ELISA, an extremely high dilution of the antibody conjugate was necessary to obtain an optimum reàction. With 0.5 µg/ml gamma globulin for coating and 1:100 dilution of crude infected tissue for antigen, a strong reaction was observed even after the conjugate was diluted to 1/16,000 (Fig. 2). Even after 6 years of storage at 4°C, the enzyme conjugate was still effective but the optimum dilution was at 1:1,000 (Table 2).

The reactivity of the antiserum to TuMV-C1 and an antiserum to a TuMV isolate from Florida (20) to the four TuMV pathotypes (C1, C2, C3, and C4) and the ESC8NJ isolate were compared in SDS-immunodiffusion, ELISA (indirect and DAS), and Western blot tests (Table 1, Fig. 3). Additionally, the TuMV-C1 antiserum was also tested against 14 other isolates. This latter antiserum gave positive reactions in all tests with all of the isolates, except for TuMV-C4, to which it gave a negative reaction in Western blot (Fig. 3A′, lane 9). In comparison, antiserum to TuMV-FL did not react with TuMV-C2 in SDS-immunodiffusion and indirect ELISA (Table 1), and with ESC8NJ in Western blot (Fig. 3A, lane 4). The antisera also reacted differently in Western blots (Fig. 3). Antiserum to TuMV-FL reacted almost exclusively with the native 38K coat protein (Fig. 3A), while antiserum to TuMV-C1 reacted with the lower molecular weight breakdown products of the coat protein (Fig. 3A′).

Combination of antibodies from the different antisera were tested in DAS-ELISA using the four pathotypes as antigens. Coating with antibodies to TuMV-C1 in combination with conjugated antibodies produced against TuMV-C1 or -FL gave the best reactions (Table 2). However, coating with antibodies to TuMV-FL followed by a conjugate against antibodies to TuMV-C1 did not give positive reactions.

DISCUSSION

We have produced an antiserum to TuMV-C1 that reacted, in several types of commonly used serological tests, with 19 TuMV isolates including the four pathotypes that are used to differentiate sources of resistance to TuMV (17). In comparison, an antiserum prepared to an isolate of TuMV from Florida (20) did not react with the TuMV-C2 pathotype in SDS-immunodiffusion and indirect ELISA tests, but gave positive reactions in DAS-ELISA and Western blot. The reactivity of the TuMV-C1 antiserum to many different isolates and its strong reactions to the pathotypes in a number of serological tests should make it useful for screening breeding lines for TuMV resistance and for general diagnosis of TuMV. Moreover, this antiserum can be used at very high dilutions in ELISA and SDS-immunodiffusion tests and does not react to several common potyviruses.

Western blot analyses clearly showed that TuMV-C1 antiserum reacted almost exclusively to the presumably breakdown products of TuMV coat protein (4,9,14). This observation is not entirely surprising considering that coat protein heterogeneity of TuMV is increased by storage of viral preparations and that the antiserum we produced was obtained from a rabbit that was initially immunized with a virus preparation that had been stored for 1 week. The last immunization took place 4 weeks after the virus was purified. It is possible that the TuMV-FL antiserum was produced largely to intact virus particles since it reacted primarily to the native coat protein as observed by Western blot.

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