Serological Properties of Aphid-Transmissible and Aphid-Nontransmissible Pea Enation Mosaic Virus Isolates

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ABSTRACT

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An aphid-transmissible isolate of pea enation mosaic virus (T-PEMV) was compared biologically and serologically with an aphid-nontransmissible isolate (NT-PEMV) of the same virus. Although both isolates were easily transmitted by sap inoculations, NT-PEMV was not transmitted by the pea aphid, despite attempts using three different acquisition methods. Isolate T-PEMV was efficiently transmitted by the pea aphid by all three acquisition methods. Antisera produced in rabbits contained a single antibody population against NT-PEMV (cross-reactive with T-PEMV) but two antibody populations were produced against T-PEMV (one cross-reactive with NT-PEMV and one specific for T-PEMV). A soluble protein concentrated from the high-speed centrifugation supernatant fraction obtained during purification of T-PEMV reacted specifically with T-PEMV antiserum and not with NT-PEMV antiserum. No reactive soluble protein was detected in similar extractions from NT-PEMV infected plants.

Additional key words: aphid transmission, vectors, pea aphid, *Acyrthosiphon pisum*.

Several plant viruses with a persistent insect vector relationship have lost their vector-transmissibility after prolonged propagation within host plants without passage through the insect vectors (2, 4, 5, 16, 23, 25, 26). This phenomenon also has been reported for several plant viruses with a nonpersistent vector relationship (1, 7, 10, 13, 15, 20, 21). Research on these anomalous isolates generally has not proceeded beyond demonstration of the vectorless quality of the newly selected isolate. Thus, our knowledge of possible biochemical differences between these isolates is scant.

Tsai and Bath (23) reported that a previously aphid-transmissible California isolate of pea enation mosaic virus (T-PEMV) became aphid-nontransmissible (NT-PEMV) following prolonged perpetuations by sap-inoculation. The objective of this research was to compare these two isolates, NT-PEMV and T-PEMV, biologically and serologically to learn of differences which might explain the loss of aphid-transmissibility.

MATERIALS AND METHODS

Aphid colonies and virus source plants.—The pea aphid, *Acyrthosiphon pisum* (Harris), was reared on broad bean, *Vicia faba* L., at 20 C, 60-70% relative humidity, and a 14-hr photophase. Garden pea, *Pisum sativum* L. 'Midfreezer' served as the virus source plant and as the transmission test plant for both isolates. Pea seedlings were grown and transplanted at the cotyledon stage as described by Bath and Tsai (3).

Virus isolates and perpetuation.—An aphid-transmissible (T-PEMV) isolate and an aphid-nontransmissible (NT-PEMV) isolate (23) of pea enation mosaic virus were used. Isolate NT-PEMV was perpetuated using inoculum prepared by grinding 10-day-old, virus-infected leaf tissue in tap water with a sterile mortar and pestle and then rubbing the inoculum on Carborundum-dusted (about 22-μm particle size) healthy pea seedlings with a finger. These seedlings then were rinsed and placed in a greenhouse.

Isolate T-PEMV was perpetuated using second-stage aphid nymphs given a 12-hr acquisition access period (AAP) on 10-day-old, virus-infected pea plants. Aphids were transferred to healthy seedlings (three to four nymphs per plant) for a 3-day inoculation access period (IAP). Both the AAP's and the IAP's were completed at 20 C, 60-70% relative humidity, and a 14-hr photophase. Plants were fumigated with naled insecticide before being placed in a greenhouse.

Purification of isolates.—Isolates were partially purified from 10-day-old, virus-infected pea plants as previously described (22). Virus pellets were resuspended in 0.1 M pH 7.0 potassium phosphate buffer. This partially purified virus was stored in 1-ml lots at −20 C until use. Freezing had little detectable effect on the biological properties of the isolates.

Rate-zonal density gradient centrifugation was used to further purify and concentrate the isolates. One-ml samples of partially purified isolate preparations were layered on linear 10-40% sucrose gradients in either 0.05 M pH 7.0 potassium phosphate buffer (T-PEMV) or 0.1

M pH 6.0 sodium acetate buffer (NT-PEMV). These were centrifuged for 2 hr at 24,000 rpm (4 C) in the SW 27.1 rotor of the Model L Beckman ultracentrifuge. Tubes were monitored during virus-zone collection with a UA-2 Ultraviolet Analyzer (ISCO, Lincoln, NB 68505) coupled to an ISCO Density Gradient Fractionator Model D. Virus was concentrated from virus zones collected from several tubes by diluting the samples 1:1 with gradient buffer and centrifuging them at about 145,000 *g* for 90 min at 4 C. Virus pellets were resuspended in either 0.05 M pH 7.0 potassium phosphate buffer (T-PEMV) or 0.1 M pH 6.0 sodium acetate buffer (NT-PEMV). These gradient-purified virus solutions were clarified by low-speed centrifugation and viral concentrations were measured in a Beckman DB Spectrophotometer ($\lambda = 260$ nm and an extinction coefficient of 7.2) (9).

Bioassay.—Sap from virus-infected peas, partially-purified, and gradient-purified virus preparations were assayed for infectivity on the local lesion host, *Chenopodium amaranticolor* Coste and Reyn, and on pea seedlings. Aphid-transmissibility of the isolates from infected pea plants was tested as described earlier (3). Aphid-transmissibility of partially-purified virus preparations was assayed by feeding aphids on the samples across an artificial membrane (22) and by injection of virus into aphids (6).

Antisera preparation.—One-ml doses of each gradient-purified isolate (2-3 mg/ml) emulsified in an equal volume of Freund's incomplete adjuvant were injected intramuscularly (IM) into the hind leg muscles of several New Zealand White rabbits. Four injections were given at 1-wk intervals for each isolate with the final injection of the four being an intravenous injection (IV) 7-10 days before bleeding the particular rabbit. The IV injection consisted of a 0.5-ml dose of gradient-purified virus (2-3 mg/ml) in buffer only. All rabbits were bled from the ear and the serum was separated by low-speed centrifugation and stored in (0.1-1.0 ml) lots at −20 C.

TABLE 1. Transmission of aphid-transmissible pea enation mosaic virus (T-PEMV) and aphid-nontransmissible pea enation mosaic virus (NT-PEMV) isolates by the pea aphid, *Acyrthosiphon pisum* (Harris) using three virus acquisition methods

Virus	Virus acquisition method	Mean transmission[a]
T-PEMV	Plant[b]	94 ± 6.0
	Membrane[c]	52 ± 7.6
	Injection[c]	78 ± 27.5
NT-PEMV	Plant	0
	Membrane	0
	Injection	0

[a]Means and standard deviations of three replications of each virus acquisition method using 20 aphids per replicate. Aphids were placed singly on test pea seedlings after a 3-day inoculation access period.

[b]Second-stage numphs given a 4-hr acquisition access period (AAP) on PEMV-infected pea plants.

[c]Partially purified virus was used in both the membrane and injection acquisition methods. The second-stage nymphs were given a 6-hr AAP on partially purified virus solutions in the case of the membrane test.

Antiserum was produced against pea protein by using 10-day-old healthy pea plants and processing them using the standard purification schedule (22). The soluble pea protein fraction which was concentrated from the high-speed supernatant liquid by ammonium sulfate precipitation was dialyzed against 0.15 M NaCl and freeze-dried. The injection (0.5-ml dose of about 5 mg/ml pea protein) and bleeding schedule was the same as that used for the virus-injected rabbits.

Test antigens for serology.—Sap from virus-infected and healthy pea plants was collected by grinding 1-2 g of pea leaf tissue in a sterile mortar and pestle and expressing the sap through a cheese cloth. Expressed sap was given a low-speed centrifugation and used immediately in serological tests. Gradient-purified virus was adjusted to 0.2 - 0.8 mg/ml for agar gel diffusion tests and to 50-80 μg/ml for precipitin ring tests. Pea protein was adjusted to 0.5 - 2.0 mg/ml for agar gel diffusion tests.

Serological test procedure.—Agar gel diffusion tests were conducted in plastic petri dishes (9 cm in diameter), each containing 20 ml of 1% Ionagar No. 2S (Wilson Diagnostic, Inc., 3 Science Rd., Glenwood, IL 60425) in [Phosphate buffered saline (PBS), 0.1 M p H 6.0 potassium phosphate, 0.15 M NaCl] with about 0.01% sodium azide. Antigen and antiserum wells were both 6 mm in diameter and 6 mm apart. Plates were held in a moist chamber at room temperature and observed for precipitin band formation for a 2- to 5-day period. They were then washed overnight in PBS and photographed without staining. Precipitin ring tests (24) were conducted using twofold antiserum dilutions made in 10% glycerine saline (0.15 M NaCl). Reactions were observed for 90 min, and the highest dilution forming a visible precipitin line at the antigen-antiserum interface was recorded as the antiserum titer.

Antisera absorption.—Isolate T-PEMV antiserum (1:5) was absorbed with an equal volume of NT-PEMV (0.8 mg/ml) and incubated at 35 C for 30 min. After low-speed centrifugation, the resultant supernatant liquid (1:10) was again mixed with an equal volume of NT-PEMV (0.8 mg/ml) with the above procedure repeated. The resultant absorbed T-PEMV antiserum (1:20) was reacted against purified samples of both isolates in agar gel diffusion tests.

Soluble protein extraction.—Virus from 10-day-old

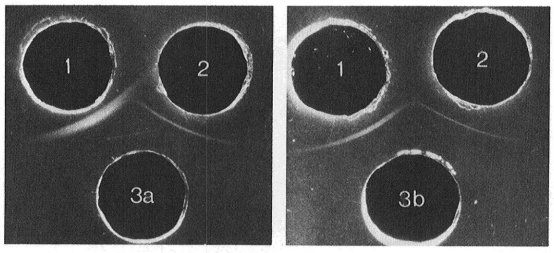

Fig. 1. Agar gel serology of aphid-transmissible pea enation mosaic virus (T-PEMV) (well 3a) and aphid-nontransmissible pea enation mosaic virus (NT-PEMV) (well 3b) isolate antisera (1:5 and 1:10, respectively) against: T-PEMV (well 1) and NT-PEMV (well 2) expressed from 10-day-old virus-infected pea plants.

infected T- and NT-PEMV pea plants was partially purified. The supernatant liquids from the high-speed centrifugation step were collected and given a second high-speed centrifugation for 90 min at 145,000 *g* (4 C). The supernatant liquid from this second high-speed centrifugation was added to an equal volume of saturated ammonium sulfate and allowed to react for 30 min over ice. The precipitated protein was pelleted by centrifugation at about 90,000 *g* for 15 min (4 C) and resuspended in PBS. Precipitated protein was then dialyzed overnight against PBS to remove excess ammonium sulfate.

RESULTS

Symptomatology.—Virus isolates, T- and NT-PEMV, induced identical symptoms on pea and on *C. amaranticolor*. Typical PEMV symptoms on pea of vein clearing, stunting of growth, chlorotic spots, and enations along the leaf veins were produced by both isolates. Symptoms began to appear 5-6 days after inoculation with either isolate.

Transmission characteristics.—Both isolates were readily sap-transmissible to pea seedlings. Partially-purified and gradient-purified virus preparations of either isolate were infective when rubbed onto pea seedlings and *C. amaranticolor*.

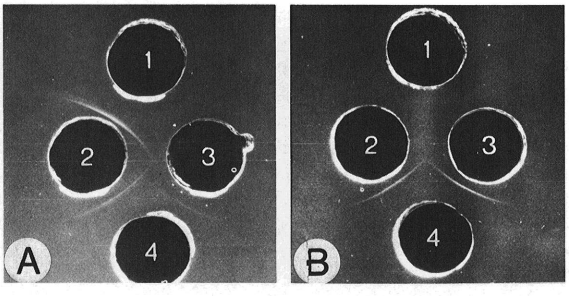

Fig. 2-(A, B). Agar gel serology of aphid-transmissible pea enation mosaic virus (T-PEMV) and aphid-nontransmissible pea enation mosaic virus (NT-PEMV) antisera. **A)** Agar gel serology of: (well 1) = T-PEMV antiserum (1:20) absorbed with gradient-purified NT-PEMV antigen; (well 2) = purified T-PEMV (0.2 mg/ml); (well 3) = purified NT-PEMV (0.2 mg/ml); and (well 4) = T-PEMV antiserum unabsorbed (1:20). **B)** Same as A) except that (well 1) = NT-PEMV antiserum (1:20) was absorbed with gradient-purified T-PEMV antigen and (well 4) = NT-PEMV antiserum (1:20) unabsorbed.

Aphid-transmissibility of the isolates was tested using three virus acquisition methods (Table 1). Isolate NT-PEMV was not transmitted by the pea aphid using any of the acquisition procedures. Previously, the green peach aphid, *Myzus persicae* (Sulz.), was shown not to transmit NT-PEMV (24). Even when 0.2 - 1.0 mg/ml concentrations of NT-PEMV were injected directly into the hemocoel of, or fed directly to, second-stage pea aphid nymphs no transmission of NT-PEMV resulted. Isolate T-PEMV was readily transmitted by all three methods. Pea aphids transmitted gradient-purified T-PEMV with high efficiency (approximately 95%) following injection of virus at 0.2 - 0.3 mg/ml. This indicated that, in addition to sap-transmissibility, aphid-transmissibility was maintained during purification.

Antisera testing.—During initial antibody production, NT-PEMV was purified in the same manner as T-PEMV in phosphate buffer. Antibody titers of NT-PEMV, however, were not of the same level as those produced by T-PEMV (1/1,280); in one rabbit a titer of 1/64 was achieved, whereas only 1/32 was achieved in two other rabbits. Isolate NT-PEMV subsequently was gradient-purified and resuspended in 0.1 M pH 6.0 sodium acetate buffer and found to induce an antibody titer of 1/2,560, comparable to that of T-PEMV. To confirm the serological results and to eliminate any possible effects of buffer type on antibody production, antiserum against T-PEMV also was produced in acetate buffer.

Antibody produced against pea protein reacted specifically (1/64) against its pea protein antigen (0.5 - 2.0 mg/ml). No precipitin band formation was observed in agar gel diffusion tests with anti-pea protein antiserum against either T- or NT-PEMV purified virus preparations. This indicated that our purified preparations were relatively free from contaminating pea proteins. In reciprocal experiments, no visible precipitin band formation was detected when either T- or NT-PEMV antiserum was reacted in agar gel diffusion tests with concentrated pea protein. Also, no reaction occurred when these antisera were tested against sap from healthy pea plants. Thus, if present at all, nonspecific antibodies were too low in concentration to be detected by our test procedures.

Serological relationship of isolates.—In all agar gel diffusion tests in which both isolates were reacted simultaneously with one of the antisera, a coalescent

TABLE 2. Homologous, heterologous, and absorbed titers of antisera produced in rabbits against aphid-transmissible pea enation mosaic virus (T-PEMV) and aphid-nontransmissible pea enation mosaic virus (NT-PEMV) isolates

Virus antiserum	Buffer system[a]	Antiserum titer[b]		
		Homologous	Heterologous	Absorbed[c]
NT-PEMV	Acetate	2,560	2,560	0
T-PEMV	Phosphate	640	160	320
	Phosphate	1,280	1,280	1,280
	Acetate	640	40	320

[a]In 0.1 M pH 6.0 sodium acetate and 0.05 M pH 7.0 potassium phosphate buffers.

[b]Expressed as reciprocal of highest antiserum dilution which formed a visible precipitin line with the viral antigen in a precipitin ring test (24).

[c]Antiserum absorbed with heterologous viral antigen and then reacted with its homologous antigen in a precipitin ring test.

(confluent) precipitin band pattern formed with NT-PEMV antiserum and a spur pattern with T-PEMV antiserum (Fig. 1). These precipitin band formations were observable in 12-24 hr and were identical when either expressed sap, partially purified, or gradient-purified isolates were tested.

Numerous tests were performed to confirm the repeatability of these patterns under different experimental conditions. Spur and confluent patterns were produced in agar gels prepared at both pH 6.0 and 7.0, but at pH 8.0 no visible precipitin bands were formed. Similarly, the above patterns were duplicated in agar concentrations of 1, 1.5, and 2%. The patterns were constant over the range of gradient-purified virus concentrations that were tested (0.2 - 1.8 mg/ml). Antiserum produced against T-PEMV gradient-purified in sodium acetate buffer also produced the spur pattern.

Origin of spur.—Absorbed T-PEMV antiserum reacted visibly with T-PEMV antigen, but not with NT-PEMV antigen (Fig. 2). This indicated that a second antibody population was present which had not reacted with NT-PEMV antigen during absorption. This second antibody reacts specifically with sites on T-PEMV protein which are apparently lacking on NT-PEMV. Antiserum to NT-PEMV absorbed with T-PEMV antigen in the same manner described for T-PEMV antiserum did not react against either viral antigen (Fig. 2). This showed the presence of a single cross-reactive antibody population in NT-PEMV antiserum.

Homologous, heterologous, and absorbed PEMV antisera titers from several rabbits were determined using the precipitin ring test (Table 2). The heterologous titer represents the relative concentration of cross-reactive antibody and the absorbed antiserum titer represents the relative concentration of specific antibody. The titers of the specific antibody varied (1/320 - 1/1,280) among the three rabbits injected with T-PEMV. Homologous and heterologous titers for antisera from the rabbit injected with NT-PEMV were equal, as would be expected with a single cross-reactive antibody population.

Neutralization of transmissibility.—Because PEMV is a two-component virus (8, 9, 14), the specific antibody may be produced against one of the viral components, possibly an aphid-transmissible component. Two transmission neutralization assays were completed by treating gradient-purified T-PEMV (0.8 mg/ml) with an equal volume of phosphate buffer, preimmunization serum (1:20), absorbed T-PEMV (1:20), or NT-PEMV antisera (1:20). Each solution was incubated for 30 min at 35 C and given a low-speed centrifugation. When the four resulting supernantant liquids were each injected into a group of 12 second-stage pea aphids, no transmission resulted from the aphids injected with T-PEMV that had been incubated with antisera. Virus transmission did occur from the pre-immunization serum (12 of 12) and buffer-treated (6 to 12) controls. Apparently, the antigenic determinants of T-PEMV coat protein which stimulated the production of the two antibody populations are both on the aphid-transmissible component or components.

Detection of soluble protein antigens.—The presence of low-molecular-weight protein antigens in expressed sap from PEMV-infected pea plants has been reported (14, 17). No such antigens could be detected in expressed sap from plants infected with our T- and NT-PEMV isolates; apparently they are present at a concentration below the threshold of our test procedure.

When precipitated soluble-protein fractions from both virus isolates were tested in agar gel diffusion tests against T- and NT-PEMV antisera, only the soluble-protein fraction from T-PEMV-infected pea plants reacted (Fig. 3). This indicated that a soluble-protein antigen was extracted from T-PEMV infected plants and not from NT-PEMV infected plants grown under identical conditions. The soluble-protein antigen reacted with the specific antibody component of the T-PEMV antisera. This was confirmed by tests using absorbed T-PEMV antiserum and by the fact that the T-PEMV soluble-protein antigen failed to react with NT-PEMV antiserum known to be cross-reactive with T-PEMV.

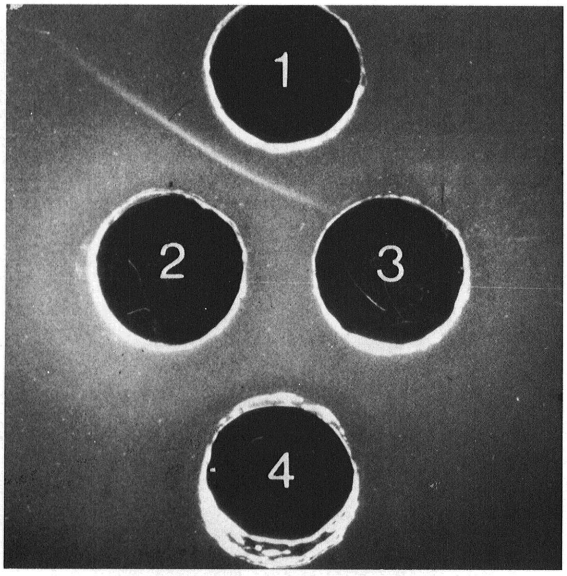

Fig. 3. Agar gel serology of aphid-transmissible pea enation mosaic virus (T-PEMV) and aphid-nontransmissible pea enation mosaic virus (NT-PEMV) antisera and soluble pea protein fractions: (well 1) = T-PEMV antiserum (1:40); (well 2) = T-PEMV soluble protein fraction; (well 3) = NT-PEMV soluble protein fraction; and (well 4) = NT-PEMV antiserum (1:80).

DISCUSSION

The loss of insect-transmissibility of several plant viruses has been associated with either elimination of the insect vector from the virus transmission cycle by relying on repeated sap inoculations for virus perpetuation (1, 7, 15, 20, 21, 23) or prolonged maintenance of the virus in a perennial host (4, 5, 16, 24, 25, 26). In all the reported cases, insect-transmissibility was lost before the researchers realized what was occurring. Disturbance of the natural transmission cycle possibly resulted in the selection of an insect-nontransmissible portion of the virus population or in the selection of a recent mutant. In either case, the result was insect-nontransmissibility of the original isolate. Researchers need to remain cognizant

of this possibility if artificial transmission procedures are adopted for experimentation.

The detection of a specific antibody against T-PEMV protein indicated that NT-PEMV viral protein had become altered. The exact nature and extent of this change is not known. Further work is needed to determine precisely the nature of this alteration.

The presence of a soluble protein in T-PEMV infected peas was one of the major differences detected between the two viruses. This protein may be a key factor in the expression of aphid-transmissibility in T-PEMV. At least three origins on this protein are possible. First, it could be an additional host protein produced in peas in response to viral infection by T-PEMV. The host protein possibility would imply that it was carried through the purification procedure as a contaminant. Host-plant proteins present in noninfected peas, and undoubtedly in infected peas, were not detected in our gradient-purified preparations used to inject rabbits. If the soluble protein is a host protein contaminant, it must have special properties of its own or affinity for T-PEMV which allows it to remain at a significant concentration through the various purification steps. A second possibility is that the soluble protein represents degraded viral protein. The cross-reactive antibody did not react with this protein. This would indicate that dissociated protein from the T-PEMV particles has antigenic determinants that differ from the available sites on intact particles. If this is true, NT-PEMV protein does not behave in a similar fashion.

Although PEMV has been reported previously to have a single structural protein (12), a third possibility may be that a second structural protein is present in T-PEMV and not in NT-PEMV. This second protein characteristically may become dissociated from a portion of the virus population during purification. Recent reports (18, 27, 28) indicate the presence of a second structural protein in several spherical plant viruses similar to PEMV.

A change (either structural or conformational) in PEMV viral protein (NT-PEMV) may account for the lack of insect-transmissibility. It has been suggested (19) that protein compatability between insect membrane systems and viral proteins may be an important element determining virus-vector specificity. Our results provide additional supportive evidence. Alteration of the protein in normally pea aphid-transmissible PEMV, resulting in NT-PEMV, may hinder passage of virus particles through membrane systems within the vector. The salivary gland is the vector membrane system most likely to be selective for PEMV in the pea aphid because bypassing of the intestinal tract by injection of NT-PEMV into the hemocoel of pea aphids did not permit virus transmission in this study and Harris et al. (11) found virions of PEMV in salivary glands of aphids injected with T-PEMV but not in those of aphids injected with NT-PEMV. Further comparative studies of T- and NT-PEMV will provide more information about this specific virus-vector combination.

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