Phenotypic Mixing: Mechanism of Dependent Transmission for Two Related Isolates of Barley Yellow Dwarf Virus

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Cooperative investigation of the Agricultural Research Service, U. S. Department of Agriculture, and the Cornell University Agricultural Experiment Station. Supported in part by NSF Grant PCM 8308672, and by USDA Grant NY(C) 157424.

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We are grateful to Irmgard Muller, Laura Tufford, Dawn Smith, and Forrest Sanders for excellent technical assistance, and to Dr. M. F. Dietert for critically reading the manuscript.

Portion of a thesis by the first author submitted in partial fulfillment of the requirements for the Ph.D. degree, Cornell University.

Accepted for publication 5 May 1988 (submitted for electronic processing).

ABSTRACT

Hu, J. S., Rochow, W. F., Palukaitis, P., and Dietert, R. R. 1988. Phenotypic mixing: Mechanism of dependent transmission for two related isolates of barley yellow dwarf virus. Phytopathology 78:1326-1330.

Preparations from oat plants singly or doubly infected with the MAV and PAV isolates of barley yellow dwarf virus were used to study the mechanism of dependent transmission of MAV by *Rhopalosiphum padi* in the presence of the related PAV isolate. In two-site enzyme immunosorbent assays, virions trapped by PAV-specific polyclonal antibodies reacted with MAV-specific monoclonal antibodies only in preparations made from doubly infected plants. When such virus preparations were precipitated with antibodies specific for either virus and then tested in membrane-feeding assays, the rate of MAV transmission by *R. padi* was reduced significantly. These results demonstrated that some virions in the mixed infection contain epitopes from both MAV and PAV and suggested that phenotypic mixing is a mechanism for dependent transmission of MAV together with PAV.

Additional keywords: aphid vectors.

Dependent transmission from mixed virus infections is a special feature of some aphid-virus relationships. In dependent transmission, aphids transmit one virus (dependent virus) only in the presence of a second virus (helper virus). Previous studies of the mechanism of dependent transmission for persistent viruses indicated that, during simultaneous replication of two viruses, some of the nucleic acid of the dependent virus becomes coated by the protein capsid of the helper virus (21). Such "atypical" viruses

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then can be transmitted by the vector of the helper virus because the protein capsid is similar to that of the helper virus. This general phenomenon has been referred to as heterologous encapsidation (21). There are two main kinds of heterologous encapsidations: transcapsidation and phenotypic mixing. In transcapsidation, also called genomic masking, the whole protein capsid is that of the helper virus; in phenotypic mixing, subunits of the protein capsids are those of both viruses.

Barley yellow dwarf virus (BYDV) is an isometric, single-stranded RNA virus transmitted in a persistent, circulative manner by aphids (27). Dependent transmission occurs for seven of the 15 possible interactions of five vector-specific BYDV isolates (12,22). Previous studies of MAV and RPV, which are serologically unrelated, distinct BYDV isolates, have indicated that transcapsidation is the basis for dependent transmission of these two isolates (20). However, little is known about the role of phenotypic mixing in dependent transmission for aphid-borne plant viruses. Studies with animal viruses and bacteriophages have shown that transcapsidation is common for serologically unrelated viruses, and that phenotypic mixing is more likely for related viruses in mixed infections (4).

Production of monoclonal antibodies (mAB) to BYDV (9) made it possible for us to study the mechanism of dependent transmission for two related BYDV isolates. We considered this a useful approach to understand interactions of viruses and vectors and to learn how the interactions could affect the spread of BYDV by aphids in the field.

This paper describes studies on the mechanism of dependent transmission of two related BYDV isolates, MAV and PAV, with serological and biological analyses. We investigated the possibility that phenotypic mixing is involved in the dependent transmission of MAV in the presence of PAV by *Rhopalosiphum padi* (L.). A preliminary report has been published (11).

MATERIALS AND METHODS

Two isolates of BYDV were used in this study: MAV, transmitted specifically by *Sitobion* (=*Macrosiphum*) *avenae* (F.), and PAV, transmitted by both *R. padi* and *S. avenae*. Stock colonies of the same clones of the two aphid species used in all previous work were maintained on barley as described (19). Coast Black oats (*Avena byzantina* K.) were test plants in all biological experiments. Singly or doubly infected plants were obtained by infesting 6-day-old seedlings with groups of one or two of the appropriate viruliferous aphid species. Clarified virus preparations were made by one of two methods. In one, finely chopped tissue was ground in liquid nitrogen with a mortar and pestle (23), ground again after thawing in 0.1 M potassium phosphate buffer, pH 7.0 (containing 20% sucrose), at the rate of 3 ml of buffer for 1 g of tissue, mixed with chloroform, and centrifuged at low speed to break the emulsion (23). In the second method (12), a Brinkmann polytron homogenizer (Brinkmann Instruments Co., Westbury, NY) was used to grind the plant tissue for 6 sec at high speed in 0.02 M phosphate-buffered saline (0.01 M NaCl), pH 7.4, containing 0.05% Tween 20 (PBS-T), usually at rate of 3 ml/1 g of tissue. Samples were mixed thoroughly with an equal volume of chloroform and centrifuged at low speed or allowed to settle overnight at 4 C. Purified virus and healthy control preparations were made as previously described, by chloroform clarification, differential centrifugation, and sucrose gradient centrifugation (25).

Monoclonal antibody MAV4 (mAB-MAV4), which reacts with MAV but not PAV, was used (9). A new supply of mAB-MAV4 mouse ascitic fluid was prepared, and the IgG from the ascitic fluid was purified with a protein A-Sepharose column (8). PAV-specific antibodies were made by preabsorption of anti-PAV polyclonal antibodies (26) with a 33-fold concentrated MAV preparation. Absorption was done three times at 37 C for 1 hr and at 4 C overnight. Excess MAV was inactivated by adding 1 M Tris-Cl, pH 4.5 (28). Specificity of the PAV-specific antibody was evaluated in two kinds of tests. When the PAV-specific antibody was mixed with MAV in absorption tests, no difference in enzyme immunosorbent assays (EIA) was found between MAV preparations absorbed with the PAV-specific antibody and those absorbed with control antibody (anti-healthy oat) preparations. When the PAV-specific antibody was used in the coating step of EIA, mean absorbance values from three tests for PAV, MAV, and healthy oat control were 0.152, 0.002, and 0.002, respectively.

Direct EIA and two-site EIA. Direct EIA was carried out in the two-day procedure described previously (2,24), except that mAB-MAV4 IgG (1.5 ug/ml) or PAV-specific antibodies (1:500) were used to coat plates.

In a two-site EIA, PAV-specific antibody was used as coating antibody to trap virus and mAB-MAV4 was used as a second antibody to react with trapped virus. Rabbit-anti-mouse IgG conjugated with alkaline phosphatase (Sigma, St. Louis, MO) was used to detect the mouse mAB. A series of virus dilutions were used in both direct EIA and two-site EIA.

In a double immunoabsorption test, virus preparations (MAV, PAV, or MAV-PAV made from double infections) were mixed with mAB-MAV1 (9), mAB-MAV4, or PAV-specific antibody at 37 C for 1 hr; then mixtures were tested in both direct and two-site EIA to assay unreacted virus.

Controls in all EIA tests included healthy oat preparations, buffer, and known amounts of purified virus. At least two wells were used for each sample. A reaction was considered positive only if the absorbance was at least 0.1. This threshold was much higher than the twice-background range of "healthy" controls used in such tests.

Membrane-feeding assay. Clarified or partially purified virus preparations from doubly infected plants were mixed in test tubes with mAB-MAV4, PAV-specific antibody, or mAB to healthy plants (mAB-MAV5) (9) as the control. Rabbit-anti-mouse IgG was added to mAB-treated samples to effect precipitation. Each mixture was kept at 37 C for 1 hr and stored overnight at 4 C. Mixtures were centrifuged at low speed. Supernatants were diluted with an equal volume of 40% sucrose in 0.01 M potassium phosphate buffer (unless the virus preparation had been extracted in 20% sucrose PBS-T), divided into two portions, and fed to *R. padi*. Portions of each sample also were examined in EIA. After aphids had fed through stretched Parafilm on the treated virus preparations for 24 hr at 15 C, they were moved to oat seedlings (10 aphids per seedling) for 5 days at 21 C. Plants then were fumigated with DDVP (0,0-dimethyl 2,2-dichlorovinyl phosphate) and kept in a greenhouse (19). Plants that became symptomatic during the following 4 wk were tested in EIA to determine which of the virus isolates had been transmitted from the treated virus preparations. In these EIA tests, mAB-MAV4 or PAV-specific antibodies were used to coat plates. In such experiments, mixtures of MAV and PAV from singly infected plants were used as controls.

RESULTS

Serology. The two-site EIA was used to compare preparations from oats singly or doubly infected with MAV and PAV. Virus particles trapped by PAV-specific antibody reacted with mAB-MAV4 only in preparations from doubly infected plants in each of five experiments (Fig. 1). Although the reaction of the virus preparation from doubly infected plants in the two-site EIA was weak, the reactions of preparations from singly infected plants in the two-site EIA were essentially negative (Fig. 1). The differences were clear and consistent in each of the five experiments. When the virus preparations were examined at a series of dilutions, the differences remained clear and consistent (data not shown). However, mAB-MAV4 had to be used in the two-site EIA; there was no positive reaction in the two-site EIA when mAB-MAV1 (specific to MAV) (9) was used (data not shown).

In the tests described above, virus preparations were made from polytron-extracted plant sap. Subsequently, when we found that the liquid nitrogen-extracted virus preparations worked better in bioassays than those prepared by means of the polytron, the liquid nitrogen-extracted preparations were used in the two-site EIA. The reaction of virus preparation from doubly infected plants in the two-site EIA was relatively stronger than those shown in Figure 1.

For example, in one test the absorbance value in the two-site EIA from doubly infected plants was 0.102; control values for MAV and PAV were 0.223 and 0.198, respectively. This value was about 50% that of the control, compared with a value from previous tests (Fig. 1) of about 20%.

Results from immunoabsorption tests revealed that some virions from doubly infected plants were absorbed by either MAV- or PAV-specific antibodies (Table 1). Without antibody treatment, doubly infected plants gave a positive reaction in the two-site EIA. After mAB-MAV4 or PAV-specific antibody was used in absorption tests, the two-site EIA reaction was reduced. The fivefold difference in absorbance value was consistent in two separate experiments. Immunoabsorption with another MAV-specific mAB (mAB-MAV1) removed normal MAV activity but did not reduce the reaction in the two-site EIA (Table 1).

Membrane-feeding assays. Although groups of *R. padi* transmitted MAV from MAV-PAV doubly infected plants very efficiently (20), the aphids transmitted MAV from preparations of doubly infected plants after feeding through membranes very poorly. Clarified virus preparations made with the polytron and partially purified virus preparations were tried first. In 14 tests over a 1-yr period, an average of less than 5% of diseased plants was found to be doubly infected. When we prepared virus from young, doubly infected plants by means of liquid nitrogen, however, the rate of dependent transmission increased to about 75%; these results were consistent in five tests. MAV transmission was reduced significantly by anti-MAV monoclonal and anti-PAV polyclonal antibodies (Table 2); in contrast, the effect of a control mAB (9) on transmission of MAV was insignificant. When a mixture of MAV and PAV made from singly infected plants was used in the same kind of assay, *R. padi* transmitted PAV but not MAV (Fig. 2).

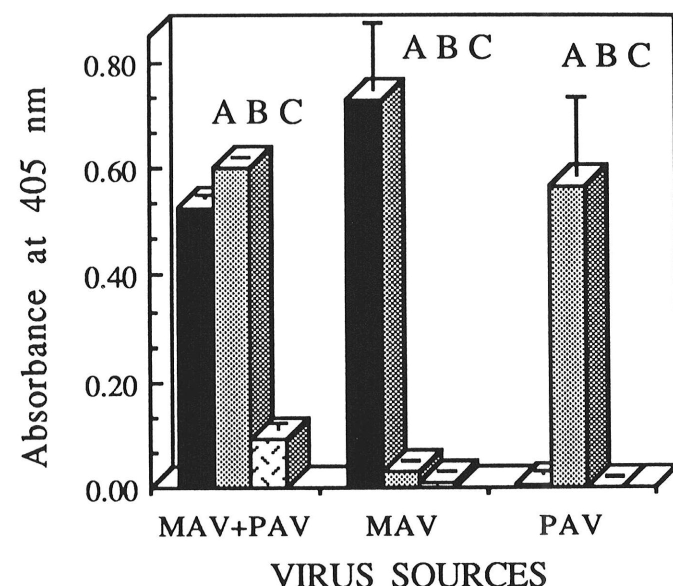

Fig. 1. Parallel detection of viruses with two enzyme immunosorbent assays (EIA). Preparations of oats singly or doubly infected with MAV and PAV isolates of barley yellow dwarf virus were examined by direct EIA and two-site EIA. Data are means of five experiments with different virus preparations. Vertical T-bars represent standard errors. **A,** A homologous direct EIA for MAV; **B,** a homologous direct EIA for PAV; **C,** a two-site EIA: coating was with PAV-specific polyclonal antibodies and detecting antibody was an MAV-specific monoclonal antibody.

TABLE 1. Parallel detection of viruses in direct and two-site enzyme immunosorbent assay (EIA) after immunoprecipitation

Virus sources[a]	Absorption antibodies[b]	Absorbance at 405 nm in EIA[c]		
		MAV EIA	PAV EIA	Two-site EIA
DI	···	0.876	0.642	0.100
DI	mAB-MAV4	0.002	0.667	0.020
DI	PAV-specific pAB	0.476	0.011	0.024
DI	mAB-MAV1	0.006	0.687	0.117
MAV	···	0.205	0.032	0.001
PAV	···	0.004	0.434	0.004
PBS-CK	···	0.001	0.002	0.000
Healthy-CK	···	0.004	0.001	0.000

[a] Virus preparations (0.8 ml), made from oats singly or doubly infected (DI) with MAV and PAV isolates of barley yellow dwarf virus, were mixed with 0.2 ml of antibody preparations (about 1 mg/ml) at 37 C for 1 hr. The mixtures then were tested in parallel in direct EIA (for MAV, PAV) and in two-site EIA (coating was with PAV-specific polyclonal antibodies and detecting antibody was MAV-specific monoclonal immunoglobulin).
[b] mAB = monoclonal antibody; pAB = polyclonal antibody.
[c] Means of two wells following 45 min reaction at room temperature.

DISCUSSION

Two major mechanisms could serve as the basis of dependent transmission for persistent plant viruses. One is transcapsidation; the other is phenotypic mixing. Both are results of viral structural interaction during replication in double infections. Much information is available on transcapsidation and phenotypic mixing for animal viruses and bacteriophages (4). One example for transcapsidation involves the interaction between single-stranded bacteriophages ϕX 172 (icosahedral) and fd (tubular); transcapsidation was detected in doubly infected cells (14). In another study, phenotypic mixing was observed for human adenoviruses (16). Particles with mixed fibers, mixed pentons, and mixed hexons were detected. That study also showed that the six protein subunits making up a hexon cluster could be any combinations of the proteins from two interacting viruses.

Previous studies of the interaction between the unrelated MAV and RPV isolates of BYDV provided evidence that transcapsidation is the kind of heterologous encapsidation that explains dependent transmission of MAV by *R. padi* from mixed infection (20). Evidence for heterologous encapsidation also exists for a system involving beet western yellow virus and lettuce speckled mottle virus (5), a system involving carrot red leaf virus and carrot mottle virus (15,29), and a system involving bean yellow vein-banding virus and pea enation mosaic virus (3). Most authors claimed that the protein capsid of the atypical virions was partially or entirely from the helper viruses, but evidence was not given to differentiate transcapsidation from phenotypic mixing. In a study using two related tobacco mosaic virus strains, Otsuki and Takebe (17) demonstrated that phenotypically mixed atypical particles were produced in tobacco mesophyll protoplasts doubly infected by the two strains.

TABLE 2. Transmission of virus by aphids (*Rhopalosiphum padi*) in membrane-feeding assays[w]

Antibodies against[x]	Number of plants (of 24 infested) that became infected with virus shown[y]	
	PAV	MAV
None	24.0 a[z]	18.6 a
Healthy oat	24.0 a	16.6 a
MAV	24.0 a	4.0 b
PAV	7.0 b	0.0 b

[w] Virus preparations from plants doubly infected with MAV and PAV were reacted with antibodies in test tubes. *R. padi* aphids were allowed to feed through a membrane on the mixtures for 1 day and feed on oat seedlings for 5 days.
[x] Antibodies were anti-healthy-oat monoclonal antibody, anti-MAV monoclonal antibody (mAB-MAV4), and preabsorbed anti-PAV polyclonal antibodies.
[y] Mean values from five experiments. Identification of isolates recovered from plants that became infected was by means of direct enzyme immunosorbent assay. None of 60 plants infested with virus-free aphids as controls became infected.
[z] Values within a column and followed by a common letter are not significantly different at $P = 0.01$, according to Duncan's multiple range test.

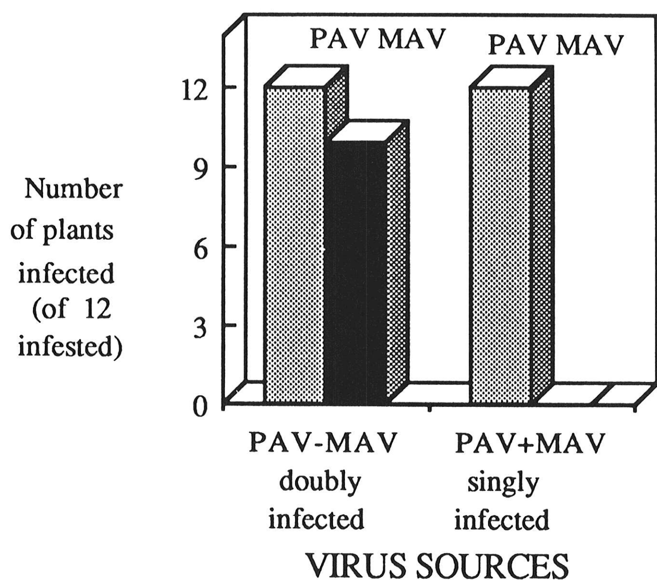

Fig. 2. Comparison of virus transmission by aphids in a membrane-feeding bioassay. *Rhopalosiphum padi* acquired virus through a stretched Parafilm membrane on preparations made from oats doubly infected with MAV and PAV isolates of barley yellow dwarf virus, or on preparations made by mixing each of the separate virus isolates. Identification of the isolates from the plants that became infected was by means of direct enzyme immunosorbent assay.

In our two-site EIA, PAV-specific epitopes were recognized by the PAV-specific antibodies and a MAV-specific epitope was recognized by the MAV-specific antibody. Therefore, only virions that had both MAV and PAV epitopes would be detected. We think that our data show the presence of phenotypically mixed atypical virions in doubly infected plants. Although the reaction of virus in preparations of doubly infected plants in the two-site EIA was weak, results were reproducible in many tests. One possible reason for the weak reaction was that the atypical virions were less stable than normal ones. Preparations made by means of liquid nitrogen were more active than those obtained by polytron extraction in both the two-site EIA and the membrane-feeding assay, a difference that could be a result of virus stability.

We observed that one anti-MAV mAB (mAB-MAV4) removed the atypical virus activity in immunoabsorption tests, but another anti-MAV mAB (mAB-MAV1) did not. This suggests that the reaction in the two-site EIA was not a nonspecific reaction to MAV. Even if the value of 0.100 represented a nonspecific reaction relative to 0.876, the value of 0.117 could not be a nonspecific reaction relative to 0.006 (Table 1). Rather, these data indicate the presence of a different kind of virus particle in doubly infected plants: a particle lacking the epitope for reaction with mAB-MAV1, but possessing that required for reaction with mAB-MAV4. We also found that mAB-MAV4 detected the atypical virions in the two-site EIA, but mAB-MAV1 did not. We think this also indicates that the epitope to which mAB-MAV1 reacts was missing on the atypical virions.

Data from the two-site EIA provided evidence for the existence of the atypical virions; results from membrane-feeding assays demonstrated that the atypical virions are responsible for dependent transmission. If transcapsidation were the only basis for the dependent transmission, MAV-specific antibody would not prevent MAV transmission because this antibody is unable to react with the PAV capsid. Our mAB-MAV4 reduced MAV transmission by *R. padi* dramatically, a result that supports the hypothesis of phenotypic mixing. These results are in sharp contrast to past studies of double infection by MAV and RPV in which only one antibody (anti-RPV) prevented dependent transmission of MAV by *R. padi* in the presence of helper virus RPV (21). In the present study, variation among virus preparations was a critical factor. Apparently, polytron extraction was too rigorous for many atypical virions to remain intact for transmission by aphids. We think that the stability of atypical virions could be a key factor in the study of dependent transmission. In our previous in vivo tests, in which aphids acquired virus from infected leaves, dependent transmission occurred in seven of 15 possible interactions of five BYDV isolates (22). In in vitro studies, in which aphids acquired virus through membranes, only the MAV-RPV system has been amenable to study. For other combinations, we have not obtained consistent dependent transmission from preparations fed upon by aphids.

Preliminary results from dot-blot hybridization experiments (10) supplemented the other lines of evidence. After virus preparations from doubly infected plants were precipitated with MAV-specific monoclonal antibody, MAV nucleic acid in supernatant samples was undetectable with an MAV-cDNA probe. The rationale for using the nucleic acid hybridization technique to study the phenotypic mixing hypothesis is as follows. If the basis of the dependent transmission is phenotypic mixing, there should be atypical virions that have both MAV and PAV epitopes in doubly infected plants; the MAV-specific monoclonal antibody should be able to remove both normal MAV virions and the atypical virions in immunoprecipitation with rabbit-anti-mouse IgG. Therefore, testing the supernatant after the immunoprecipitation by dot-blot hybridization with MAV-cDNA should give a negative result. However, if the basis for the dependent transmission is transcapsidation and atypical virions have only PAV epitopes, then the MAV-specific antibody would only react with normal MAV and the atypical virions would still be in the supernatant after immunoprecipitation. In this case, MAV nucleic acid should be detected by the dot-blot hybridization tests. The high sensitivity of the dot-blot test makes it especially applicable.

Study of dependent transmission has been a useful approach to understanding the mechanism of virus-vector specificity (20). There is a consistent vector specificity between BYDV isolates and aphid species. The current hypothesis for the mechanism of the specificity is that the interaction of epitopes on virus protein capsid and receptors on aphid accessory salivary gland membrane determines which isolate of BYDV an aphid can transmit (7). Studies have been carried out to directly test this hypothesis (6,13). Elucidation of the phenotypic mixing model provides additional evidence for the role of the virus protein capsid in the virus-vector specificity.

Dependent transmission has been reported for nonpersistent and persistent virus-aphid systems (1,18,22). The role of dependent transmission in plant virus epidemiology could be very important (21). For instance, dependent transmission could be a means of maintenance and spread of a plant virus in the field in the absence of its regular aphid vector. Although MAV is regularly transmitted only by *S. avenae*, in mixed infection with PAV (a predominant BYDV isolate in many areas), MAV also could be transmitted by *R. padi*. This relationship has been demonstrated in the greenhouse (22). Further investigation of the role of dependent transmission in virus spread in the field could be important in the understanding of plant virus epidemiology.

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