Selective Virus Transmission by Rhopalosiphum padi Exposed Sequentially to Two Barley Yellow Dwarf Viruses

W. F. Rochow

Research Plant Pathologist, Northeast Region, Agricultural Research Service, U.S. Department of Agriculture and Professor of Plant Pathology, Cornell University, Ithaca, New York 14850.

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ABSTRACT

Although *Rhopalosiphum padi* does not regularly transmit the MAV isolate of barley yellow dwarf virus from MAV-infected oats, it often transmits MAV, together with the serologically unrelated RPV isolate, from plants doubly infected by MAV and RPV. Attempts were made during a 10-year period to duplicate this dependent transmission of MAV from plants by permitting interaction of the two viruses within *R. padi*. No evidence for dependent transmission of MAV was found in any of 31 experiments when *R. padi* fed on plants infected by one virus and then was exposed to the other virus by feeding on infected leaves, by injection with concentrated virus, or by feeding through membranes on virus preparations. No evidence for dependent transmission of MAV occurred in 15 experiments based on allowing *R. padi* to feed through membranes on, or injecting the vectors with, inocula made by mixing concentrated virus preparations of each of the separate viruses. These data strengthen the conclusion based on previous indications that dependent transmission of MAV by *R. padi* results from simultaneous synthesis of the two viruses in the doubly infected plant and not from interaction of the viruses within the vector.

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Sometimes aphids are able to transmit virus from a plant only if the plant is also infected by a second virus. Eight examples of such dependent virus transmission by aphids have been discussed in a recent review (11). One example is the dependent transmission of the MAV isolate of barley yellow dwarf virus in the presence of the RPV isolate, the helper virus. *Rhopalosiphum padi* does not regularly transmit MAV from singly infected oats, but it often transmits MAV, together with the serologically unrelated RPV, from plants doubly infected by MAV and RPV. Similarly, *R. padi* readily transmits both viruses when concentrated preparations made from doubly infected plants are used as inocula in membrane-feeding or injection experiments (6, 10).

Whether the critical virus interaction occurs in the plant, in the aphid, or in both is an important aspect of any attempt to understand dependent transmission. The site of virus interaction in dependent transmission systems appears to be different for viruses that have a circulative virus-vector relationship from those that have a stylet-borne relationship. Kassanis & Govier (2, 3) recently investigated this question for two systems that involve stylet-borne viruses. They found that *Myzus persicae* (Sulzer) could transmit potato aucuba mosaic virus not only from plants also infected by a helper virus (potato virus Y), but also from plants infected by each virus alone if the aphids first fed on plants infected by the helper virus. Thus, potato virus Y served as a helper virus in sequential acquisition feedings just as it did when source plants were infected by both viruses. Similarly, potato virus C was transmitted by the aphids not only when aphids probed into plants infected by both potato viruses C and Y, but also when the aphids probed first into plants containing potato virus Y, the helper virus, before they were exposed to potato virus C.

In contrast, dependent transmission of viruses that have a circulative relationship with their aphid vectors appears to result from interaction of the viruses in the source plant. Tobacco vein-distorting virus served as a helper virus for tobacco mottle virus only if the two viruses occurred together in the source plant (13). Carrot mottle virus was transmitted only from plants also infected by carrot red-leaf virus and not when aphids had been exposed alternately to the two components of carrot motley dwarf (14).

This paper describes experiments made during a 10-year period on some aspects of dependent transmission of the MAV isolate of barley yellow dwarf virus in the presence of the RPV isolate. I studied the questions of whether dependent transmission of MAV by *R. padi* can occur following sequential acquisitions of RPV and MAV from singly infected plants, and also whether it can occur if the two viruses are given the opportunity to interact simultaneously within the vector instead of within the plant. A preliminary account of some of the work has appeared (7).

MATERIALS AND METHODS.—Stock colonies of *Rhopalosiphum padi* (Linnaeus) and *Macrosiphum avenae* (Fabricius) were maintained on barley (*Hordeum vulgare* L. 'Catskill' or 'Hudson') using

special precautions (8). The clone of each aphid species was the same as that used in previous studies, and some aphids from each group were always tested as aphid controls in every experiment. The RPV and MAV isolates of barley yellow dwarf virus were maintained by serial transmissions at 6- to 8-week intervals to oats (*Avena byzantina* K. Koch 'Coast Black'), the test plant used in most experiments. The two virus isolates, the same ones used in previous work, are differentiated on the basis of their relative vector specificity and their distinct serological properties (1, 8, 9, 12). MAV is transmitted specifically by *M. avenae*; RPV is transmitted specifically by *R. padi*. Although this vector specificity is a stable, consistent property of the viruses, it is relative; occasional transmissions of each isolate by the "nonvector" aphid species can occur, especially under certain conditions (5, 9).

Concentrated virus preparations were made by differential and sucrose-gradient centrifugation of clarified juice from infected plants (12). The aphid injection procedure was similar to that described by Muller (4), except that most needles were made by means of an automatic micropipette puller. The membrane-feeding tests were carried out as previously described by allowing starved aphids to feed through stretched Parafilm M on concentrated virus preparations containing 20% sucrose (8, 12).

In one kind of experiment, *R. padi* was first given an acquisition feeding on detached leaves or reared on plants infected by one of the viruses, and then given access to the other virus by one of several methods. The methods included feeding on infected leaves, feeding through membranes on concentrated virus preparations, and injection of virus into hemolymph of live aphids. Controls included aphids fed only on

TABLE 1. Virus transmission by *Rhopalosiphum padi* fed first on oat leaves or plants that were healthy (H), infected by RPV, or infected by MAV before given a second exposure to the other isolate of barley yellow dwarf virus by feeding on infected leaves (leaves), by feeding through membranes on concentrated inocula (membrane), or by being injected with concentrated inocula (injection)

Source for first acquisition feeding[a]	Second exposure to virus or control shown by method indicated[b]	Transmission following sequential exposure[c]	No. of infected plants tested and found to be infected by virus isolate or isolates shown[d]			
			Total tested	RPV only	MAV only	RPV & MAV
RPV	MAV Leaves	119/123	48	48	0	0
RPV	MAV Membrane	30/30	21	21	0	0
RPV	MAV Injection	36/39	21	21	0	0
RPV	H Leaves	87/96	24	24	0	0
RPV	H Membrane	30/30	3	3	0	0
RPV	H Injection	9/9	6	6	0	0
H	MAV Leaves	0/11				
H	MAV Membrane	0/30				
H	MAV Injection	0/12				
MAV	RPV Leaves	238/276	150	134	0	16
MAV	RPV Membrane	75/93	75	66	0	9
MAV	RPV Injection	44/51	37	35	0	2
MAV	H Leaves	13/225	10	0	10	0
MAV	H Membrane	4/30	4	0	4	0
MAV	H Injection	5/36	5	0	5	0
H	RPV Leaves	97/99	44	44	0	0
H	RPV Membrane	19/30	5	5	0	0
H	RPV Injection	29/30	11	11	0	0

[a] The acquisition feeding period varied among experiments from 2 days on detached leaves to rearing aphids on infected plants.

[b] When second exposure was in tests with leaves, the acquisition feeding period usually was 2 days at 15 C. Inocula used in membrane and injection experiments included extracts of viruliferous aphids and preparations from infected plants concentrated 100- to 250-fold.

[c] Numerator is number of plants that became infected; denominator is number of plants that were infested, usually for a 5-day inoculation test feeding period at the rate of about 10 *R. padi* per plant for the tests with leaves and membranes, and five aphids per plant in injection tests. None of 183 plants infested as controls in the 27 original experiments became infected.

[d] Identification of the 418 plants infected only by RPV was based on virus transmission to 1,201 of 1,278 plants by *R. padi* and to 17 of 1,277 plants by *Macrosiphum avenae*. The 19 plants infected only by MAV were identified in comparative tests in which *R. padi* transmitted virus to 3 of 63 plants and *M. avenae* transmitted virus to 62 of 63 plants. The 27 plants infected by both viruses were identified in tests in which *R. padi* transmitted virus to 162 of 162 plants and *M. avenae* transmitted virus to 154 of 162 plants. Two of 261 plants infested as controls in 43 experiments became infected.

TABLE 2. Virus transmission by *Rhopalosiphum padi* fed first on oat leaves that were healthy (H), infected by RPV, or infected by MAV before given a second acquisition feeding on one of the three kinds of leaves

First feed	Second feed	Transmission[a]	No. of plants tested and found to be infected by virus isolate or isolates shown				Transmission by aphid species shown in tests to identify virus isolates shown at left[b]	
			Total tested	RPV only	MAV only	RPV & MAV	RP	MA
RPV	MAV	60/60	60	58	0	2	178/178	7/180
RPV	H	59/60	8	8	0	0	24/24	0/24
H	MAV	10/60	10	0	10	0	1/30	30/30
MAV	RPV	60/60	60	59	0	1	179/180	5/180
MAV	H	4/60	4	0	4	0	0/12	12/12
H	RPV	56/60	8	8	0	0	24/24	0/24
H	H	0/24						

[a] Numerator is number of plants that became infected; denominator is number of plants infested for a 5-day inoculation test feeding period at the rate of 10 aphids per plant following the sequential acquisition feedings of 2 days each.

[b] Numerator is number of plants that became infected; denominator is number of plants infested with about 10 *R. padi* (RP) or *Macrosiphum avenae* (MA) for a 5-day inoculation test feeding period following acquisition feeding of 2 days on opposite halves of detached leaves. None of 48 plants infested as controls in eight experiments became infected.

healthy leaves, aphids given only the first access to virus, and aphids given only the second access. A second kind of experiment was based on use of concentrated inocula made by combining preparations of RPV and MAV made separately from singly infected plants. The combined inocula were used either in membrane feeding or injection experiments to determine whether any interaction of the two viruses within the vector could be detected.

In each experiment some or all of the plants that became infected following feeding by *R. padi* were tested in subsequent comparative transmissions by using *R. padi* and *M. avenae* (8, 9). These comparative tests permitted identification of the virus isolate or isolates transmitted following the original exposure of *R. padi* (in some cases also *M. avenae*) to both viruses. Because plants infected by both RPV and MAV usually have symptoms much more severe than comparable singly infected ones, emphasis was always placed on selecting plants with the most severe symptoms for the subsequent comparative transmission tests.

Many of the original transmission tests, and all of the subsequent comparative transmissions with the two aphid species, were based on use of opposite halves of detached leaves for a 2-day acquisition feeding period at 15 C in the dark. When acquisition feeding periods were longer than two days, aphids were caged on intact source plants either in the growth chamber or in a greenhouse compartment. The inoculation test feeding period, in a growth chamber at 21 C (8) or in an isolated section of the greenhouse, was 5 days. In most tests, where acquisition was by feeding, groups of about 10 aphids were placed on each of three seedlings in a 10-cm (4-inch) diam pot for each treatment. Usually five aphids were placed on a test plant when acquisition was by injection. Aphids were removed from test seedlings by fumigation with DDVP (1, O-dimethyl 2, 2-dichlorovinyl phosphate), and plants were placed in an isolated greenhouse under supplemental light for observation during a 4-week period.

RESULTS.—When *R. padi* had acquired RPV (the helper virus) before being exposed to MAV, only RPV was transmitted. In eight experiments involving exposure of aphids to MAV by feeding on infected leaves, by feeding through membranes on concentrated MAV preparations, or by injections of active preparations of MAV, a total of 90 infected plants were tested to determine which virus isolate or isolates had been transmitted. In all cases only RPV was detected in the plants infected by means of *R. padi* that had been exposed sequentially to RPV and then MAV (Table 1).

No evidence for dependent transmission of MAV was found in other tests based on exposure of *R. padi* to MAV before RPV. In nine of 19 experiments some transmissions of both MAV and RPV did occur, but the percentage of such doubly infected plants was not greater than that of controls where *R. padi* had fed only on MAV (Table 1). In all nine experiments the initial exposure of *R. padi* to MAV had been by rearing *R. padi* on MAV-infected plants; such long acquisition feedings are known to increase the chances for the occasional transmission of MAV by *R. padi* (9). Comparison of results from tests with aphids exposed to both viruses with results from tests with aphids exposed to MAV first and then to healthy leaves or preparations from healthy plants, not only within each of the nine experiments, but also in total, clearly shows that the sequential exposure to both viruses did not increase the probability that *R. padi* could transmit MAV. For example, the 27 plants found to be doubly infected

following sequential exposure of *R. padi* to MAV and RPV represent 6% of the 420 plants infested initially; the 22 cases of parallel MAV transmission by *R. padi* fed first on MAV-infected plants and second on virus-free material represent 8% of the 291 control plants infested (Table 1).

All of the six combinations shown in Table 1 were not necessarily included in each of the 27 experiments summarized there. Even when all six treatments were included in one experiment, the numbers of plants used for each treatment were not always the same. A final series of experiments was carried out on the sequential acquisition feedings, together with the corresponding controls, to provide parallel inoculation of 15 plants for each of the six combinations within one experiment. Results of four such experiments (Table 2) also showed that *R. padi* was no more likely to transmit MAV when exposed sequentially to both viruses than when fed only on MAV-infected plants.

Because *R. padi* readily transmits both RPV and MAV when fed on virus preparations made from doubly infected plants (6, 10), other experiments were made to determine whether *R. padi* could transmit MAV from mixed inocula made by combining preparations of each of the separate viruses. In six separate experiments concentrated inoculum made from RPV-infected plants was combined with inoculum prepared from MAV-infected plants, sucrose was added, and aphids were permitted to feed through stretched Parafilm on the mixture. In most experiments, parallel tests were made with *R. padi* and *M. avenae*. Plants that became infected were then tested to determine which isolate or isolates had been transmitted by aphids fed on the mixed inocula. Tests of 107 plants showed that *R. padi* had transmitted only RPV; *M. avenae* had transmitted only MAV (Table 3).

Mixed inocula were used in nine additional experiments based on injection of the viruses into the hemolymph of aphids, which were then permitted a 5-day feeding on test seedlings. Injected *R. padi* transmitted only RPV; injected *M. avenae* transmitted only MAV. None of 60 tests gave any indication of dependent transmission of MAV by *R. padi* (Table 3).

Since *R. padi* can transmit MAV from virus preparations made from doubly infected plants even following neutralization of the virus preparations with MAV antiserum, one of the experiments summarized in Table 3 included treatment of the mixed virus preparations with various antisera. Previous tests had been made to study the possibility that treatment with MAV antiserum might somehow alter MAV in vitro to make it transmissible by *R. padi* (10), but in those only MAV, and not mixtures of MAV and RPV, had been tested. In this experiment 1 ml of a virus preparation containing 10 μg each of MAV and RPV was mixed with 1 ml of various antisera diluted 1:2.5, kept at 37 C for 30 minutes, stored overnight at 4 C, mixed with 2 ml 40% sucrose, and used in membrane feeding and aphid injection tests. All 14 plants that became infected by means of *R. padi* that had fed on, or were injected with, the mixed preparation treated with MAV antiserum were found to be infected only by RPV. Thus, no anomalous results were obtained by incubating the mixed virus preparations with antisera.

The selective transmission of RPV by *R. padi* in these experiments is in striking contrast to the transmission of both viruses by *R. padi* fed on doubly infected plants or on inoculum made from such plants. For example, in one series of experiments carried out during the period of time when these sequential acquisitions were under study, comparative tests were made of 100 oat plants inoculated by *R. padi* that had acquired virus from doubly infected plants. From every one of the 100 plants *R. padi* transmitted both RPV and MAV. These mixed infections were identified in subsequent tests in which *R. padi* transmitted virus to 300 of 300

TABLE 3. Virus transmission by *Rhopalosiphum padi* (RP) or *Macrosiphum avenae* (MA) fed through membranes on, or injected with, inocula made by combining separate preparations of the MAV and RPV isolates of barley yellow dwarf virus

Aphid	Method[a]	No. of infected plants tested and found to be infected by virus isolate or isolates shown				No. of test plants infected following feeding by aphid shown in tests to identify virus isolates shown at left[b]	
		Total tested	RPV only	MAV only	RPV & MAV	RP	MA
RP	Membrane	48	48	0	0	152/154	6/154
RP	Injection	60	60	0	0	196/201	0/197
MA	Membrane	59	0	59	0	8/201	197/198
MA	Injection	17	0	17	0	1/75	70/71

[a] None of 121 plants infested as controls in the 15 original experiments became infected.

[b] Numerator is number of plants that became infected; denominator is number of plants that were infested with about 10 aphids for a 5-day inoculation test feeding period following acquisition feedings of 2 days on opposite halves of detached leaves. None of 96 plants infested as controls in 16 experiments became infected.

test plants and *M. avenae* also transmitted virus to each of the 300 plants in the parallel tests. None of 60 control plants, infested with *R. padi* that had no access to RPV or MAV, became infected. Similarly, *R. padi* consistently transmitted both viruses in various tests with virus preparations made from plants doubly infected by RPV and MAV. Even when such preparations had been neutralized with antiserum specific for MAV, *R. padi* transmitted both RPV and MAV to about one-third of the plants in both membrane-feeding and injection experiments (10).

Another kind of experiment was carried out to test for possible dependent transmission of MAV by *R. padi* given a series of alternate 1-day acquisition feedings on MAV- and RPV-infected tissue. Groups of *R. padi* were moved each day for a 5-day acquisition feeding period from leaves infected by RPV or by MAV to the other kind of leaves to provide three exposures to MAV and two exposures to RPV, or to provide three exposures to RPV and two feedings on MAV-infected leaves. Plants that became infected following such acquisitions were then tested to determine which virus isolates *R. padi* had transmitted. All 18 plants tested were infected only by RPV. *R. padi* transmitted virus from all 18 plants (to 54 of 54 test plants) but *M. avenae* did not transmit virus (to 0 of 54 plants). None of 15 plants infested as controls became infected.

Some other tests were directed toward the question of whether an interaction between MAV and RPV might occur at the time of inoculation of test plants. Groups of *R. padi* previously fed for 2 days on MAV-infected leaves were permitted a 3-day inoculation test feeding on oat plants inoculated 15 days previously with RPV. About 1 month later, six of the infected plants were tested in comparative tests with both aphid species. Virus was transmitted from all plants by *R. padi* (to 18 of 18 test plants) but from none of them by *M. avenae* (to 0 of 18 plants). None of six plants infested as controls became infected. In another experiment, oat seedlings were infested with groups of five *R. padi*, previously allowed a 6-day acquisition feeding on MAV-infected plants, together with five *R. padi* given a similar acquisition on RPV-infected plants. Further tests were then made on nine plants that became infected. In all cases virus was recovered by *R. padi* (to 26 of 26 plants) but not by *M. avenae* (to 0 of 27 plants). None of six controls became infected. Thus, *R. padi* had transmitted only RPV in all these tests and I found no evidence for dependent transmission of MAV.

DISCUSSION.—The main value of these data is their contribution toward an understanding of the mechanism for the dependent transmission of MAV by *R. padi*. These many failures to effect dependent transmission of MAV by establishing conditions for the possible interaction of MAV and RPV in the vector seem significant because they are based on numerous attempts during a 10-year period in 46 major experiments involving more than 7,000 test plants. Together with previous results, they underscore the idea that transmission of MAV by *R. padi* in the presence of RPV results from events that occur during simultaneous synthesis of the two viruses in the source plant. Previous studies (10, 11) using antiserum specific for the MAV and RPV isolates suggested that *R. padi* transmitted both RPV and MAV from mixed infections because of transcapsidation (genomic masking). A plausible view of the mechanism is that during simultaneous synthesis of the two viruses, some nucleic acid of MAV becomes encapsidated in particles containing RPV protein. Perhaps the "mixed" virus particles function in the aphid as RPV (because of the RPV protein capsid) but in the plant as MAV (because of the MAV nucleic acid).

These results also underscore the apparent difference between dependent transmission of stylet-borne viruses and that of viruses with a circulative aphid-virus relationship. At least two stylet-borne viruses can be dependently transmitted when aphids probe first on plants infected by the helper virus before probing on plants infected by the dependent virus (2, 3). In contrast, dependent transmission has not occurred in tests with three circulative viruses when similar sequential acquisitions were used (11). Because the basic virus-vector interactions are so different for stylet-borne and circulative viruses, it is not surprising that the mechanism for the dependent transmission of the viruses might differ.

The only possible evidence for any interaction between RPV and MAV within *R. padi* in these experiments, occurred in some of the tests in which *R. padi* had been reared on MAV-infected plants before acquiring RPV. Since the occasional transmissions of both viruses by *R. padi* given such sequential acquisitions were no more frequent than transmissions of MAV by aphids given parallel feedings only on MAV as controls, these virus transmissions are considered to be merely examples of occasional transmissions of MAV by *R. padi* (5, 8, 9). There might be some significance, as pointed out in a previous discussion (9), that these rare transmissions usually occurred in experiments when *R. padi* was exposed to MAV before RPV, and not in the opposite sequence. More likely, however, the initial exposures to MAV merely provided conditions known to increase the chances of the occasional transmission of MAV by *R. padi*. Even if the pinocytotic aspect of the theoretical model I previously proposed (9) has any validity, it is clear that the rare events involved in such sequential acquisitions are much different from the common events in dependent transmission of MAV from plants doubly infected by MAV and RPV.

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