Serological Detection and Evidence for Multiplication of Maize Mosaic Virus in the Planthopper, *Peregrinus maidis*

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ABSTRACT

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Maize mosaic virus (MMV) antigens were detected by the enzyme-linked immunosorbent assay (ELISA) in individual *Peregrinus maidis* after acquisition access periods (APP) on MMV-infected maize (*Zea mays*) and after injection with either purified MMV or with sap from MMV-infected maize. MMV antigens were not detected in similarly treated *Dalbulus maidis* or in *P. maidis* that were not exposed to MMV. Individual *P. maidis* tested for MMV antigens 20 days after APP or 7 days postinjection, were 58 and 76% positive, respectively. The transmission efficiency of similarly treated insects was 42 and 85% for *P. maidis* that acquired MMV by plant acquisition or injection, respectively. In two experiments when individuals were compared for ability to transmit MMV and for the presence of MMV antigens, all transmitters were ELISA-positive. Not all of the ELISA-positive *P. maidis*, however, transmitted MMV. Injected *P. maidis* had shorter incubation periods (time between acquisition and transmission) and shorter periods between acquisition and ELISA detection than did *P. maidis* that acquired MMV by plant feeding. The concentration of injection inoculum was positively correlated with the perentage of ELISA-positive *P. maidis*, time between injection and subsequent ELISA detection, and the intensity of serological reactions (antigen titer) for injected *P. maidis*. These data further suggest that MMV multiplies in *P. maidis*.

Additional key words: ELISA, delphacid vectors, rhabdovirus, insect transmission.

Maize mosaic virus (MMV) is a plant rhabdovirus that is persistently transmitted by its vector, *Peregrinus maidis* (Ashmead). Many of the plant rhabdoviruses have been shown to infect their insect vectors as well as their plant hosts (12). Several workers have studied the multiplication of plant rhabdoviruses in their insect vectors (hosts), using electron microscopy, infectivity dilution, effects of temperature on latent period and fluorescent antibody staining (1,2,5–7,14,15,17–19). Previously, rhabdovirus particles have been observed in *P. maidis* that had fed on MMV-infected maize (10). Particles resembling those of MMV were found budding through the cellular nuclear membranes of *P. maidis* and this was interpreted as evidence for the multiplication of MMV in *P. maidis* (10). The objectives of this study were to determine if MMV antigens could be detected in *P. maidis* that had been exposed to MMV and to ascertain if MMV antigens increase over time in *P. maidis*, thereby providing further evidence for the multiplication of MMV in its vector.

MATERIALS AND METHODS

Transmission of MMV. The Florida MMV isolate and *P. maidis* were maintained using maize (*Zea mays* L. 'Guardian') as the host for both *P. maidis* and MMV (8). Acquisition access periods (AAP) on MMV-infected *Z. mays* were 72 hr. All *P. maidis* were routinely transferred to healthy maize plants every 2–3 days after exposure to MMV. This was to ensure that MMV was only acquired during the initial exposure to MMV.

Individual *P. maidis* were injected with MMV using microcapillary glass needles. Individual *P. maidis* were anesthesized with CO_2 and injected intersegmentally into the abdominal cavity with either sap from MMV-infected maize plants, or with purified MMV. Initial concentrations of purified MMV (8) were determined using the Bio-Rad microprotein assay (Bio-Rad Laboratories, Richmond, CA), and appropriate dilutions were made with 0.1 M tris, 0.01 M $MgCl_2$, 0.04 M Na_2SO_3, pH 7.5.

The MMV transmission efficiency and the median incubation period (IP_{50}) in *P. maidis* that acquired MMV by plant acquisition were compared with those that acquired MMV by injection of sap from MMV-infected *Z. mays*. Groups of 30–40 insects were used for each experiment. Individual *P. maidis* were caged on single *Z. mays* plants and insects were transferred daily to new plants until all insects died.

ELISA detection of MMV in maize and *P. maidis*. Double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) and the preparation of immunoglobulins and enzyme-immunoglobulin conjugate were as previously described (4). Immunoglobulins from antiserum to a Venezuelan MMV isolate (antiserum kindly supplied by R. Lastra, Caracas, Venezuela) were used for initial experiments. Coating and conjugated immunoglobulins were both used at 1 μg/ml. The remaining experiments were done with coating immunoglobulins purified from antiserum to the Florida MMV isolate (2.5 μg/ml) and enzyme-immunoglobulin conjugate prepared from the Venezuelan antiserum (1 μg/ml). All ELISA tests were performed in Immulon II microtiter plates (Dynatech Laboratories, Alexandria, VA). Samples of maize and *P. maidis* (from 1 to 15 insects per sample depending upon the experiment) were triturated in 1 ml of PBST (0.02 M phosphate, 0.15 M NaCl, pH 7.4 plus 0.05% Tween-20) containing 2% (w/v) polyvinylpyrrolidone 40. Two 200-μl replicates for each sample were tested. Results were assessed by measuring the absorbance for each well at 405 nm after a 1-hr incubation with a Bio-Tek (Burlington, VT) EIA reader.

The sensitivity of MMV antigen detection in *P. maidis* was determined by testing samples containing one, five, 10, and 15 *P. maidis* collected 20 days after the AAP on MMV-infected maize. This collection date was chosen to ensure that most of the insects

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were beyond their incubation periods and thus capable of transmitting MMV. Similar groups of *P. maidis* not exposed to MMV were used as controls.

Individual *P. maidis* were tested 20 days after AAP to determine the percentage of *P. maidis* that had acquired MMV by feeding on MMV-infected source plants. Similarly, *P. maidis* were individually tested by ELISA 7 days postinjection to determine the percent *P. maidis* that had acquired MMV by injection.

In two experiments, individual *P. maidis* injected with sap from MMV-infected plants also were compared both for ability to transmit MMV and for MMV-ELISA reaction. After injection, individual *P. maidis* were separately caged on individual maize seedlings. Insects were transferred to new plants every 2–3 days, and 10 days postinjection all surviving *P. maidis* were individually tested for MMV antigens by ELISA. Maize seedlings were kept an additional 15 days and visually assessed for symptom development.

Effects of acquisition method on ELISA detection of MMV antigens in *P. maidis*. The detection of MMV antigens over time was compared for *P. maidis* that had acquired MMV by plant feeding or by injection of purified MMV. Injection and plant acquisition experiments were repeated three times with 300–400 *P. maidis* per experiment. *Dalbulus maidis* DeLong and Wolcott, a nonvector of MMV, were treated similarly in one experiment. Six to 10 insects were removed from each group every other day, or daily when survivorship was high. These were stored frozen until the experiment was complete when all insects were tested by ELISA.

Effects of the concentration of injected inoculum (purified MMV) on subsequent MMV antigen detection in *P. maidis* were examined. The experiment was repeated three times. In the first experiment, concentrations of 850, 85, and 8.5 μg of MMV per milliliter were used. In the second and third trials, concentrations of 25, 2.5, and 0.25 μg of purified MMV per milliliter were used. Two hundred *P. maidis* were injected for each concentration in each experiment. Insects were collected daily and stored frozen until the end of the experiment (10–11 days postinjection) when all insects were individually tested for MMV antigens by ELISA.

RESULTS

Transmission of MMV. The average transmission efficiency for individual *P. maidis* that had acquired MMV by plant acquisition was 42% in two experiments. The IP_{50} was 13.5 days and 14.8 days for experiments 1 and 2, respectively. Minimum times between exposure to the acquisition source and subsequent transmission were 9 and 13 days, and maximum times were 28 and 16 days for experiments 1 and 2, respectively. Conversely, for *P. maidis* that had acquired MMV by injection of sap from MMV-infected plants, the IP_{50} was 5.6 days. Minimum and maximum times of transmission after injection were 3 and 8 days, respectively. Mortality was always high (20–50%) for injected *P. maidis* but of those that survived to the IP_{50} the transmission efficiency was 85%.

ELISA detection of MMV antigens in maize and *P. maidis*. MMV antigens were detected in both MMV-infected maize tissues and in viruliferous *P. maidis* by ELISA. MMV-infected maize extracts reacted positively at a dilution of 10^{-4}, and the end point for detection of purified MMV was between 640 and 64 ng (Fig. 1).

When *P. maidis* were tested by ELISA, positive reactions were obtained for samples containing individual *P. maidis* (Fig. 2). Three of the six *P. maidis* that had been given an AAP on MMV-infected maize gave intense ELISA reactions. Control samples of *P. maidis* that had not been exposed to an MMV source never gave positive ELISA reactions. Groups of 5, 10, or 15 *P. maidis* all reacted positively, but these reactions were indistinguishable, indicating that samples containing more than one positive *P. maidis* were too concentrated to allow quantitative differentiation. Therefore, only one *P. maidis* per sample was used for subsequent experiments.

The percentages of MMV-ELISA-positive *P. maidis* tested 20 days after AAP or 7 days postinjection were 58% (45/78) and 76% (82/108), respectively. In two experiments in which transmission of MMV by individual *P. maidis* was compared with the presence of MMV antigens in these same individuals, MMV was detected in several *P. maidis* that did not transmit MMV during inoculation access periods. Twenty-two of 26 (84%) and 31 of 34 (91%) of the injected *P. maidis* were MMV-positive by ELISA in experiments 1 and 2, respectively. However, only eight of 22 and 11 of the 31 ELISA-positive *P. maidis* transmitted MMV in experiments 1 and 2, respectively. The $A_{405\ nm}$ values for transmitters and ELISA-

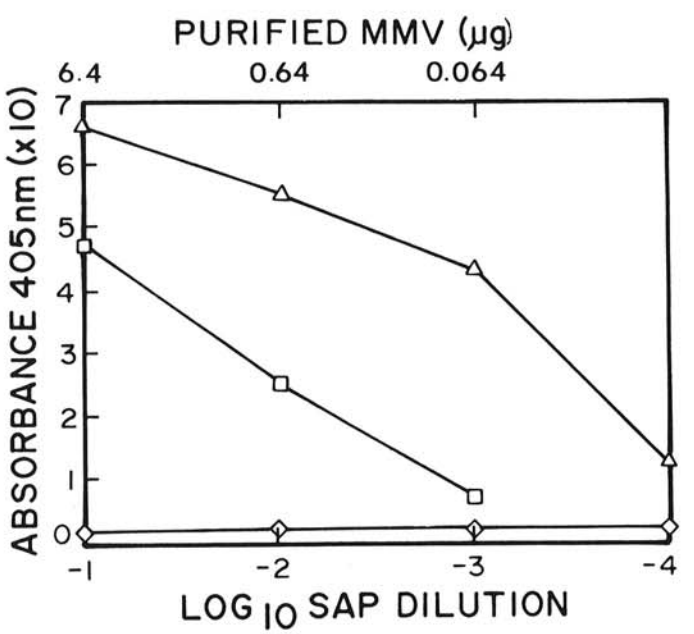

Fig. 1. Relationship of absorbance at 405 nm to the amount of maize mosaic virus (MMV) as determined by ELISA. Triangles (△——△) represent reactions for dilutions of sap from MMV-infected *Zea mays*, and squares (□——□) represent tenfold dilutions of purified MMV beginning at 6.4 μg per sample. Diamonds (◇——◇) show reactions for dilutions from healthy *Z. mays*.

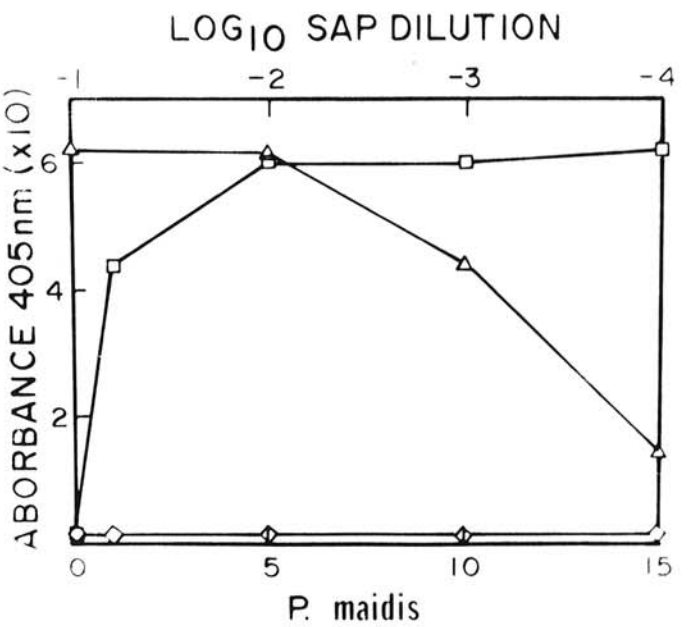

Fig. 2. Absorbance values for ELISA detection of maize mosaic virus (MMV) in *Peregrinus maidis* and MMV-infected *Zea mays*. Triangles (△——△) represent reactions for dilutions of MMV-infected *Z. mays*, squares (□——□) represent reactions for MMV-infected *P. maidis* samples (20 days after an acquisition access period on MMV-infected *Z. mays*) containing the given number of insects per sample, and diamonds (◇——◇) show reactions for healthy *P. maidis*. Values are the means for two samples at each number of insects except for the sample containing one *P. maidis* per sample which is the mean of three individual MMV-infected *P. maidis*.

positive nontransmitters were not significantly different in either experiment. The mean $A_{405\ nm}$ values for MMV transmitters were 1.38 and 0.54 in experiments 1 and 2, respectively, and the mean $A_{405\ nm}$ values for MMV ELISA-positive nontransmitters were 1.46 and 0.53 in experiments 1 and 2, respectively.

Effects of acquisition method on ELISA detection of MMV antigens in *P. maidis*. When *P. maidis* that were exposed to MMV by plant acquisition or by injection with purified MMV were subsequently tested by ELISA, MMV antigens were never detected immediately after exposure but were detected only in *P. maidis* that were collected after a suitable incubation period. MMV antigens always were detected earlier in the injected *P. maidis* than in those that acquired MMV by plant acquisition (Table 1). For three injection experiments involving over 200 *P. maidis* per experiment, the average minimum time between injection and serological detection was 4 days. For three experiments in which *P. maidis* acquired MMV by plant acquisition the average minimum time between AAP and detection was 12.3 days. The percent of MMV-positive *P. maidis* per sample showed a positive increase over time both for injected *P. maidis* and those that acquired MMV by plant feeding (Table 1). No MMV antigens were detected in *P. maidis* not allowed an AAP on MMV-infected plants, in more than 300 *D. maidis* that were individually injected with purified MMV, or in 300 *D. maidis* that were given an AAP on MMV-infected plants, even when the *D. maidis* were tested up to 24 days after exposure to MMV.

TABLE 1. Percent of MMV ELISA-positive *Peregrinus maidis* per sampling day after injection with purified MMV or plant acquisition to MMV-infected *Zea mays*

Injected[a]		AAP[b]	
Day[c]	%	Day[c]	%
0	0	0–7	0
1	0	8	3
2	...[d]	9	20
3	0	10	0
4	75	11	3
5	...	12	4.5
6	83	13	0
7	75	14	5.2
8	83	15	4
9	50	16	15
10	...	17	50
11	50	18	19
		19	25
		20	25
		21	30
		22	24
		23	40
		24	43
		25	70
		26	30
		27	30
		28	40
		29	40
		30	30

[a] *P. maidis* were individually injected with purified MMV. Six *P. maidis* were removed at each sampling date and tested for MMV antigens by ELISA. Results show the pooled mean percent from three experiments.
[b] Groups of 300–400 *P. maidis* were given a 72-hr acquisition access period (AAP) to MMV-infected *Z. mays*. Ten to 12 *P. maidis* were removed daily and were tested for MMV antigens by ELISA. Results show the pooled mean percent from three experiments.
[c] Indicates day postexposure to MMV. Day 0 is day of injection or day placed on MMV-infected plants for start of AAP.
[d] Not tested.

The concentration of purified MMV in the injection inoculum also affected detection of MMV antigens in *P. maidis*. The inoculum concentrations used in the first experiment (850, 85, and 8.5 μg/ml) were too concentrated to determine any effects due to inoculum concentration. By the second sampling date (6 days postinjection) 88% of the *P. maidis* were MMV-positive. When less concentrated inocula (25, 2.5, and 0.25 μg/ml) were used for injection, the differences in percent of MMV-positive *P. maidis* per sample, minimum time between injection and first serological detection, and the average absorbance value ($A_{405\ nm}$) for MMV-positive *P. maidis* over time were found to be dosage dependent. The total number of *P. maidis* that were positive for MMV antigens over the 11-day sampling period decreased with decreasing injection inoculum concentration (Table 2). Also, the average absorbance value (antigen concentration) for MMV-positive *P. maidis* increased with time at all concentrations (Table 2), suggesting an increase in MMV antigens over time. In all cases, the mean $A_{405\ nm}$ value for MMV-positive *P. maidis* was higher for *P. maidis* harvested 9 or 10 days after injection versus the $A_{405\ nm}$ value for MMV-positive insects harvested at days 5 and 6. Linear regression analysis showed a significant positive correlation ($P < 0.01$) between days after injection and absorbance at 405 nm for each inoculum level. The r values were 0.9, 0.86, and 0.82 for inocula at 25, 2.5, and 0.25, respectively. MMV also was detected earlier in individuals of *P. maidis* that were injected with higher inoculum concentrations.

DISCUSSION

These data demonstrate that ELISA is a sensitive method for detecting MMV antigens in individual *P. maidis* that have been

TABLE 2. Effects of maize mosaic virus (MMV) injection inoculum concentration on the subsequent ELISA detection of MMV in *Peregrinus maidis*

	25[a]			2.5			0.25		
Day	N[b]	$\bar{X}$[c]	SD[d]	N	$\bar{X}$	SD	N	$\bar{X}$	SD
3	0[e]	0	...	0[e]	0	...	0[e]	0	...
4	5	0.06	0.02	0	0	...	0	0	...
5	7	0.08	0.04	1	0.04	...	2	0.06	0.03
6	14	0.12	0.07	1	0.05	...	0	0	...
7	17	0.14	0.08	2	0.07	0.07	3	0.05	0.01
8	15	0.15	0.07	12	0.11	0.09	2	0.16	0.03
9	16	0.25	0.13	6[e]	0.08	0.04	2	0.12	0.08
10	18	0.26	0.13	9[e]	0.07	0.03	5	0.18	0.14
11	7[e]	0.18	0.12	6[e]	0.2	0.17	5[e]	0.11	0.07
	99/160[f]			36/140[f]			19/160[f]		

[a] Individual *P. maidis* were injected with freshly purified MMV at 25, 2.5, or 0.25 μg/ml. Ten *P. maidis* each were harvested for each concentration on the given day postinjection. The experiment was replicated twice. All samples were stored frozen until the experiment was completed, at which time all individuals were tested by ELISA.
[b] Shows the total number of MMV-positive *P. maidis* from both replications collected on the corresponding day postinjection.
[c] The mean absorbance value from both replications for all MMV-positive *P. maidis* on the corresponding day postinjection.
[d] The standard deviation of the absorbance values for the MMV-positive *P. maidis* in the sample shown.
[e] Indicates only 10 *P. maidis* were tested from the first experimental replication.
[f] The total MMV ELISA-positive *P. maidis* over the number sampled during the sampling period.

exposed to MMV either by plant acquisition or by injection. Previously, ELISA has been used for detecting viral antigens in the insect vectors of several plant viruses such as cucumber mosaic virus (CMV [9]), potato leafroll virus (PLRV [21]), rice ragged stunt virus (RRSV [11]), and lettuce necrotic yellows virus (LNYV [3]). In the present case, however, we have used ELISA as a quasi-quantitative assay to detect MMV antigens in individual *P. maidis*, and these data demonstrate that MMV antigen concentrations increase over time in *P. maidis* that have either fed on MMV-infected maize or were injected with either purified MMV or sap from MMV-infected maize. Similarly treated *D. maidis*, a nonvector of MMV, and healthy *P. maidis* collected from laboratory colonies did not give positive ELISA reactions for MMV antigens.

The percentage of MMV ELISA-positive *P. maidis* injected with MMV and the length of time between injection and ELISA detection were both inoculum dosage-dependent. Similar results have been obtained for sowthistle yellow vein virus (SYVV) and its aphid vector, *Hyperomyzus lactucae* (20). Also, when ELISA detection of MMV antigens and transmission of MMV by *P. maidis* were compared in similarly treated *P. maidis*, the results were in general agreement. The IP_{50} for MMV-injected *P. maidis* was shorter than that after plant acquisition and, similarly, MMV antigens were always detected by ELISA after a shorter time in injected *P. maidis* than in those that acquired MMV by plant acquisition. Whether or not these were due merely to concentration of inoculum or perhaps bypass of a transmission barrier mechanism by injection (14,16) remains to be determined.

In two experiments in which we tested individual *P. maidis* both for ability to transmit MMV and for the presence of MMV antigens, more *P. maidis* gave positive ELISA reactions than transmitted MMV to test plants. All of the *P. maidis* that transmitted MMV, however, gave positive ELISA reactions. Similar results in tests for infectivity and serological detection methods have been reported for several other plant rhabdoviruses such as SYVV (1,18), LYNV (3), and European wheat striate mosaic virus (EWSMV) (14), and for RRSV, a member of the reoviridae (13). Positive virus or viral antigen detection in the vector does not imply that the vector must transmit in a single test. Vector transmission efficiency can be affected by a variety of experimental conditions. Also, in our tests entire *P. maidis* were tested for MMV antigens. If virus transmission is determined by viral infection or accumulation in specific sites or organs (i.e., salivary glands [14]), our tests would not have detected such differences.

The characteristics shown here, such as incubation periods between acquisition and serological detection and increase of MMV antigens over time, further support the hypothesis (10) that MMV multiplies in its vector, *P. maidis*.

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