Plant Host Range and Leafhopper Transmission of *Maize fine streak virus*

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Accepted for publication 23 June 2010.

ABSTRACT

Todd, J. C., Ammar, E.-D., Redinbaugh, M. G., Hoy, C., and Hogenhout, S. A. 2010. Plant host range and leafhopper transmission of *Maize fine streak virus*. *Phytopathology* 100:1138–1145.

Maize fine streak virus (MFSV), an emerging *Rhabdovirus* sp. in the genus *Nucleorhabdovirus*, is persistently transmitted by the black-faced leafhopper, *Graminella nigrifrons* (Forbes). MFSV was transmitted to maize, wheat, oat, rye, barley, foxtail, annual ryegrass, and quackgrass by *G. nigrifrons*. Parameters affecting efficiency of MFSV acquisition (infection) and transmission (inoculation) to maize were evaluated using single-leafhopper inoculations and enzyme-linked immunosorbent assay. MFSV was detected in $\approx 20\%$ of leafhoppers that fed on infected plants but $<10\%$ of insects transmitted the virus. Nymphs became infected earlier and supported higher viral titers than adults but developmental

stage at acquisition did not affect the rate of MFSV transmission. Viral titer and transmission also increased with longer post-first access to diseased periods (PADPs) (the sum of the intervals from the beginning of the acquisition access period to the end of the inoculation access period). Length of the acquisition access period was more important for virus accumulation in adults, whereas length of the interval between acquisition access and inoculation access was more important in nymphs. A threshold viral titer was needed for transmission but no transmission occurred, irrespective of titer, with a PADP of <4 weeks. MFSV was first detected by immunofluorescence confocal laser scanning microscopy at 2-week PADPs in midgut cells, hemocytes, and neural tissues; 3-week PADPs in tracheal cells; and 4-week PADPs in salivary glands, coinciding with the time of transmission to plants.

At least 90 tentative species of plant-infecting rhabdoviruses have been identified (6,29,39). All known rhabdoviruses are persistently and propagatively transmitted by hemipteran insects, particularly leafhoppers, planthoppers, and aphids. Rhabdoviruses replicate within the insect vector; therefore, the vector is also a virus host (27). Plant *Rhabdovirus* spp. are horizontally transmitted from plant to plant by vectors but are not transmitted vertically; therefore, the disease cycle requires inoculation into a plant host, which is typically a preferred food host of the vector (5,36). Persistence of a virus species in the environment and its associated ability to cause disease in annual crops is enhanced if an overwintering plant host or reservoir is present. Thus, an understanding of plant host range is important for understanding disease epidemiology. In addition, plant host range of a rhabdovirus can be used for diagnostic purposes (51).

Rhabdoviruses transmission by insect vectors has high species specificity that is reflected in the number, relative efficiency, and relatedness of vector species for each virus (5,49). For example, *Maize mosaic virus* (MMV) and *Maize Iranian mosaic virus* (MIMV) are primarily vectored by *Peregrinus maidis* and *Ribautodelphax notabilis*, respectively (26,28,33). However, *P. maidis* can transmit MIMV, albeit at a much lower rate than it

transmits MMV (2). Similarly, transmission of *Wheat American striate virus* (WASMV) by its primary vector, the leafhopper *Endria inimica*, was much more efficient than virus transmission by another leafhopper, *Elymana virescens* (42,44).

Rhabdoviruses systemically infect insect hosts, replicating in nearly all organs, muscles, and hemolymph, but are especially neurotropic (3,4,6,13,19). The route of infection is hypothesized to begin with virus entry into the midgut, the first barrier to acquisition (3). To be transmitted, rhabdoviruses must reach salivary glands via the hemolymph, nervous system, or other route (3,27). Successful rhabdovirus transmission ultimately depends on virus exiting salivary glands (3,6,27). Viral titer within the vector is correlated with transmission efficiency (7,38). Longer acquisition access periods (AAP) and longer intervals between first acquisition and inoculation feeding periods increase rates of rhabdovirus transmission (12,43,44). Life stage of the vector during acquisition is also important, with nymphs being generally more susceptible to infection than adults (9,22).

Maize fine streak virus (MFSV) is an emerging nucleorhabdovirus infecting maize in southwestern Georgia (40). Virus purified from infected plants consists of bullet-shaped particles of ≈ 70 by 230 nm. *Graminella nigrifrons* (Forbes) (Hemiptera: Cicadellidae), the black-faced leafhopper, was identified as the only MFSV vector among several leafhopper and planthopper species tested. The complete viral genome sequence and expression of viral genes in plant hosts has been reported (50). To better understand factors that affect the dissemination of MFSV, we have investigated plant host range and properties of vector transmission for MFSV.

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doi:10.1094/PHYTO-05-10-0144

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MATERIALS AND METHODS

Maintenance of leafhoppers and virus cultures. Laboratory colonies of *G. nigrifrons* were derived from field collections taken near Wooster, OH and identified by R. J. Anderson (United States Department of Agriculture–Agricultural Research Service [USDA-ARS], retired). Colonies were maintained on oats (*Avena sativa* cv. Armor) or sweet corn (*Zea mays* L. hybrid ‘Early Sunglow’) (Schlessman Seed Co. Milan, OH) in greenhouse and growth chamber rearing facilities as previously described (18,24,31). MFSV was maintained in maize by serial transfer using *G. nigrifrons* (40). Inoculated plants were used as source plants 2 weeks after MFSV symptoms became visible as fine, short streaks on the tertiary leaf veins. AAPs and inoculation access periods (IAPs) for leafhoppers were carried out in a growth chamber with periods of 27°C for 15 h of light and 22°C for 9 h of darkness.

Seed sources. Seed of *Bromus inermis* Leyss., *Dactylis glomerata* L., *Elymus repens* L., *Lolium multiflorum* Lam., *Phleum pratense* L., *Poa pratensis* L., *Setaria faberi* Herrm., *S. viridis* (L.) P. Beauv., and *Sorghum halepense* (L.) Pers. were obtained from L. R. Nault (The Ohio State University). *S. bicolor* (L.) Moench seed of cvs. FCI4210 and Hegari were obtained from the GRIN Germplasm Center. *S. bicolor* ‘Atlas’, ‘Rio’, and ‘Sart’; *Zea mays* (L.) inbred Oh28; *Z. diploperennis* Iltis, Doebley, & Guzman; and *Oryza sativa* L. cv. Sabine were from stocks maintained by the USDA-ARS Corn and Soybean Research Unit. *Cynodon dactylon* (L.) Pers. cvs. Common, Riviera, and Wrangler were from the Johnston Seed Co. (Enid, OK). *Hordeum vulgare* L. ‘Bowers’, *Secale cereale* L., and *Triticum aestivum* L. ‘Freedom’ were from stocks maintained by the Department of Plant Pathology, The Ohio State University. In all, 3 to 10 seeds of each species were sown into 10-cm-diameter pots of sterilized soil, and plants were grown to a height of 5 to 10 cm in a greenhouse before being used for experiments.

Inoculation of host range test plants. *G. nigrifrons* was introduced to MFSV-infected maize as first instar nymphs and allowed a 3-week AAP. Following the AAP, at least 200 leafhoppers were transferred into each cage containing healthy test plants. Cages were 38 cm³, aluminum framed, and Dacron/organza sheathed, with a Plexiglas door and aluminum floor (31). In these “choice tests,” up to nine pots containing different grass species and three pots of the maize Early Sunglow were arranged randomly in each cage. After a 1-week IAP, the plants were removed from the cages, fumigated with 1100 Pyrethrum TR (Whitmire Micro-Gen, St. Louis), and moved to an insect-proof greenhouse for symptom development. Another set of test plants was then introduced into the cage for inoculation by the same leafhoppers for another 1-week IAP. Sequential inoculations continued until the number of surviving leafhoppers in the cage was <100. Each plant species was tested in a minimum of three independent trials. In “no choice” trials for *Sorghum* spp., 50 to 100 inoculative leafhoppers were contained in a tube cage placed over individual plants for 1 week. Inoculated test plants were monitored for symptom development for 6 weeks; then, leaf tissue was sampled for enzyme-linked immunosorbent assay (ELISA). F_{(ab)²} ELISA (32) or protein-A sandwich (PAS)-ELISA (14) were done using antisera specific for MFSV (40) at a dilution of 1:800. Plants were considered infected with MFSV if the change in absorbance at 405 nm (A₄₀₅) per minute was more than twice that of samples from plants of the same species that were not exposed to *G. nigrifrons*.

Detection and titer of MFSV in individual insects by ELISA. Individual leafhoppers were placed in 1.2-ml polypropylene cluster tubes (Costar, Corning, NY) containing 150 µl of phosphate-buffered saline with Tween (PBS-T) (20 mM Na-P_i, pH 7.4; 150 mM NaCl; and 0.02% Tween-20) with a 4.76-mm stainless steel ball bearing (McMaster-Carr Supply Co., Los

Angeles) and macerated at room temperature for two 4-min periods with a KLECO Pulverizer (Kinetic Laboratory Equipment Co., Visalia, CA). Samples were centrifuged at 475 × g for 1 min and the supernatant was used for ELISA. Leaf tissue ground in PBS-T (1:4 wt/vol) from symptomatic MFSV-infected and healthy control plants served as positive and negative controls, respectively. Virions, purified as previously described (40), were used to calibrate the PAS-ELISA. PAS-ELISA was carried out as for the plant host range tests, except that reagent volumes were reduced to 100 µl per well and MFSV antisera was used at a 1:400 dilution. The A₄₀₅ was determined at 0, 1, 2, 3, and 13 min after addition of substrate. Samples for which the increase in absorbance was not linear ($R^2 < 0.95$) were removed from further analysis.

To verify that leafhopper colonies were uninfected with MFSV and to assess background in the ELISA assay, one-half of the insect samples on each ELISA plate were insects that had been raised on healthy maize. Individual insects were considered to be positive for MFSV if the increase in A₄₀₅/min was greater than the cutoff value, C (16,48). For each 96-well plate, C was calculated from the mean of slopes for insects reared on healthy maize plus six standard deviations. Change in A₄₀₅/min was used as an estimate of MFSV titer in individual *G. nigrifrons*. Titer estimates for each leafhopper were compared across experiments after standard normal transformation of individual slope values was used to minimize differences among plates.

Acquisition and transmission of MFSV by *G. nigrifrons* nymphs and adults. To test for acquisition by young nymphs, oviposition was synchronized by caging ≈200 gravid females, reared on healthy maize, onto 10 to 15 MFSV-infected plants in a 38-cm³ rearing cage for 2 days. Insects were then removed and plants were monitored for emergence of nymphs from hatching eggs. Because nymphs emerged over several days, the start of acquisition (day 0) was designated as 3 days after the first nymph was observed, which was consistently 14 days after the start of oviposition. Thus, a 1-week AAP for nymphs was 7 to 10 days and a 3-week AAP was 21 to 24 days. For acquisition by adults, ≈500 newly enclosed (within 3 days of final molt) adult leafhoppers of both sexes were caged on 30 to 35 MFSV-infected plants, with time of first exposure designated as $t = 0$. For experiments in which a post-acquisition holding period (HP) was used, leafhoppers were caged on healthy maize and moved to new healthy plants weekly, to prevent reinfection. For the IAP, single leafhoppers were placed on healthy maize seedlings at the coleoptile (VE) stage within butyrate tube cages for 1 week (18). Four treatments were used: (i) a 1-week AAP followed immediately by a 1-week IAP, (ii) a 1-week AAP followed by a 3-week HP and a 1-week IAP, (iii) a 3-week AAP followed by a 1-week IAP, and (iv) a 3-week AAP followed by a 3-week HP and a 1-week IAP. The sum of the intervals from the first day of the AAP to the last day of the IAP is referred to as the post-first access to disease period (PADP) (3). To test the effect of AAP duration on MFSV acquisition and transmission, first- and second-instar nymphs were allowed to feed on MFSV-infected maize for an AAP of 1, 8, 24, or 48 h, followed by a 3-week HP on healthy oat plants and a 1-week IAP on single maize seedlings.

At the end of the IAP, surviving leafhoppers were harvested and stored individually at –80°C for PAS-ELISA and test plants were moved to the greenhouse for MFSV symptom development. Each insect was given an identification number that corresponded to a test plant. To determine the effect of gender on acquisition and transmission of MFSV by leafhoppers, leafhopper sex was determined prior to ELISA by noting presence or absence of an ovipositor (45).

Data analysis. Means separation was performed with analysis of variance (PROC GLM, LSM, SAS Enterprise Guide 4; Statistical Analysis Systems, Cary NC). Percentages of infected or inoculative individuals were calculated from ELISA-positive in-

sects and MFSV-infected plants, respectively, within treatments. Percentages of infected and inoculative insects were transformed by the arcsine square root (17) before means separation using LSM. Comparison of difference in infection and infection by gender was done by χ^2 analysis.

MFSV distribution in leafhopper organs and tissues. A study of the infection of MFSV in *G. nigrifrons* over time using immunofluorescence confocal laser-scanning microscopy (iCLSM) with antisera to MFSV (40) was initiated by placing 500 first- and second-instar nymphs on MFSV-infected maize for either a 1- or a 3-week AAP, followed by a 0- to 3-week HP and a 0- or 1-week IAP. At the end of each treatment, 30 to 50 leafhoppers were collected and starved for 3 to 4 h prior to dissection to help clear the gut contents. Where a 1-week IAP is indicated, insects were caged singly on test plants prior to dissection, so that transmitting insects could be identified.

Dissection and preparation of whole-mount organs and hemocyte smears for iCLSM were performed as described previously (1,3). MFSV antiserum (diluted 1:300) was used as the primary antibody and was incubated with organs for 2 to 3 h and with hemocyte smears for 1 h. Subsequently, organs were incubated for 1 h and hemocyte smears for 30 min in a 1:600 dilution of goat anti-rabbit immunoglobulin G labeled with AlexaFluor 488 (Invitrogen, OR), followed by a 5-min incubation in 3 nM propidium iodide. In some cases, organs were also incubated for 1 h in 6.6 μ M AlexaFluor 633 Phalloidin (Molecular Probes; Invitrogen) to stain actin filaments. Processed organs and hemocyte smears were mounted on glass slides in 50 to 80% glycerol and examined with a confocal microscope (Leica TCS SP).

RESULTS

Plant host range of MFSV. Susceptibility to MFSV was tested in 17 grass species by examining symptom appearance on plants after transmission of MFSV by inoculative *G. nigrifrons* (Table 1). *Sorghum* spp. were tested using no-choice trials and the remaining species were tested in choice trials. Virus infection in test plants was confirmed using ELISA. In all cases tested, sympto-

matic plants were positive for MFSV in ELISA and asymptomatic plants were negative. To ensure that virus transmission was occurring under experimental conditions, MFSV-susceptible maize plants were included in test cages as positive controls. Mean percentages of symptomatic maize cvs. Seneca Chief, Spirit, Early Sunglow, and Oh28 used as controls were 92, 88, 91, and 84%, respectively, with a range of 23 to 96%. MFSV symptoms on infected test plants were similar to those previously described for maize (40), and included stunting and chlorotic streaking of primary, secondary, tertiary, or small leaf veins. In addition to maize, susceptible grains included barley (83% of test plants symptomatic), oat (70%), rye (67%), and wheat (92%). MFSV-susceptible annual grasses and weeds included two species of foxtail (*Setaria faberi* and *S. viridis*) and annual ryegrass (*L. multiflorum*). Neither johnsongrass (*Sorghum halepense*) nor the sorghum (*S. bicolor*) cvs. Atlas, Rio, Sart, PI721-4201, or Hegari became infected. None of three cultivars (Common, Riviera, or Wrangler) of bermudagrass (*C. dactylon*) became infected. Of the eight perennial species tested, only quackgrass (*E. repens*) became infected with MFSV.

Detection of MFSV in *G. nigrifrons* using ELISA. An ELISA protocol was developed to assess infection in individual leafhoppers. Titration of the MFSV PAS-ELISA using purified virions indicated the expected logarithmic response to antigen ($y = 0.0105\ln(x) + 0.1484$; $R^2 = 0.93731$) with a threshold of detection of MFSV protein at ≈ 4.6 ng/ml. The ELISA response (A_{405}/min) for 295 *G. nigrifrons* individuals fed exclusively on healthy maize in three independent experiments was 0.00161 ± 0.00005 (mean $\pm$ standard error). Although the majority of *G. nigrifrons* individuals fed on MFSV-infected maize also had ELISA responses in the same range as insects fed on healthy maize, $\approx 3.4\%$ had responses more than six standard deviations greater than the mean of insects fed on healthy plants (data not shown), and these individuals were considered to be MFSV infected.

Infection and inoculativity of *G. nigrifrons*. First-instar nymphs or newly enclosed adults of *G. nigrifrons* were allowed a 1- or 3-week AAP on MFSV-infected maize, moved to healthy maize for the indicated HP, and, finally, individually caged on

TABLE 1. Host plant range of *Maize fine streak virus*

Species	Common name	SF ^u	Habit ^v	H ^w	Number of		
					Symptomatic/tested ^x	ELISA positive/tested ^y	Trials
Susceptible							
<i>Avena sativa</i>	Oats	Po	A	H	35/44	3/3	6
<i>Elymus repens</i>	Quackgrass	Po	P	ND	1/7	1/4	4
<i>Hordeum vulgare</i>	Barley	Po	A	H	17/19	3/3	4
<i>Lolium multiflorum</i>	Annual ryegrass	Po	A	ND	9/18	3/3	5
<i>Secale cereale</i>	Rye	Po	A	H	10/17	1/1	5
<i>Setaria faberi</i>	Giant foxtail	Pn	A	H	4/6	2/2	4
<i>Setaria viridis</i>	Green foxtail	Pn	A	H	7/8	4/4	4
<i>Triticum aestivum</i>	Wheat	Po	A	H	25/35	5/5	8
<i>Zea mays</i>	Maize, corn	Pn	A	H	171/192	2/2	8
Unsusceptible ^z							
<i>Bromus inermis</i>	Smooth brome	Po	P	U	0/9	nt	3
<i>Cynodon dactylon</i>	Bermudagrass	C	P	ND	0/9	0/9	3
<i>Dactylis glomerata</i>	Orchardgrass	Po	P	H	0/12	0/2	6
<i>Oryza sativa</i>	Rice	O	A	H	0/3	0/3	3
<i>Phleum pratense</i>	Timothy	Po	P	H	0/6	nt	3
<i>Poa pratensis</i>	Kentucky bluegrass	Po	P	U	0/16	nt	3
<i>Sorghum bicolor</i>	Sorghum	Pn	A	H	0/11	0/1	8
<i>Sorghum halepense</i>	Johnsongrass	Pn	P	H	0/10	0/1	4
<i>Zea diploperennis</i>	...	Pn	P	H	0/3	nt	3

^u Subfamily within the family *Poaceae*: C, *Chloridoideae*; O, *Oryzoideae*; Po, *Pooideae*; Pn, *Panicoideae*.

^v Growth habit: A, annual; P, perennial.

^w Status as a host plant for *Graminella nigrifrons*: U, unacceptable; H, food and reproductive host; ND, no data (47).

^x Symptoms of MFSV are fine chlorotic striping on the primary, secondary, tertiary, and minor veins, and stunting.

^y Symptomatic plants, if present, were selected for enzyme-linked immunosorbent assay (ELISA); if not, random samples were taken. Plants of the same species that had not been exposed to insects were used as negative controls; nt = not tested.

^z Species were considered unsuitable if no plants had symptoms in three independent trials.

healthy maize plants for a 1-week IAP (Table 2). Significant effects on MFSV infection (acquisition) and inoculativity (transmission) were detected for developmental stage at acquisition, and for length of the IAP, HP, and PADP. Although the AAP for nymphs included a period of up to 3 days for hatching and, therefore, was not exactly comparable with the AAP for adults, more nymphs than adults became infected with MFSV after a 1-week AAP. Developmental stage at acquisition had no effect with a 3-week AAP. Transmission at the two developmental stages was similar for each PADP, indicating that PADP had a greater effect on inoculativity than developmental stage. Neither nymphs nor adults transmitted MFSV until the 4-week PADP. Once transmission began, the proportion of inoculative insects increased with increasing PADP but remained less than half (13 to 33%) of the proportion of infected insects (Table 2).

To determine the minimum time required for *G. nigrifrons* to acquire MFSV, the effect of shorter AAP on virus acquisition and transmission was tested by allowing first- and second-instar nymphs to feed on MFSV-infected maize for 1, 8, 24, or 48 h. After a 3-week HP, individual leafhoppers were allowed a 1-week IAP on single maize seedlings (Table 3). The proportion of infected insects was lower with a shorter AAP but even a brief AAP was sufficient for acquisition by some individuals. Following a 1- and 48-h AAP, 0.7 and 5% of *G. nigrifrons*, respectively, became infected. Inoculative leafhoppers were not detected after a 1-h AAP but the proportion of inoculative leafhoppers increased with increasing AAP, from 0.4% after 8 h and 2.2% after 48 h.

Effect of virus titer in leafhoppers on MFSV transmission. MFSV titer in individual leafhoppers was estimated by ELISA (Table 4). Within each treatment, MFSV titer was significantly higher in inoculative compared with infected but non-

inoculative leafhoppers. Virus titer tended to increase with longer PADP; however, developmental stage at acquisition and relative length of AAP and HP also influenced titer. Within a PADP, virus titer was higher in leafhoppers that acquired MFSV as nymphs than adults. In leafhoppers that acquired MFSV as nymphs, virus titer was higher in insects given a 1-week AAP and 3-week HP (33.7) than those given a 3-week AAP with no HP (22.5) (Table 4).

Effect of gender on MFSV acquisition and transmission by *G. nigrifrons*. There were no significant differences in the percentage of infected or inoculative insects between males and females ($P < 0.05$, χ^2 test) (data not shown).

Distribution of MFSV in organs or tissues of *G. nigrifrons*. Dissected organs and hemocyte smears taken from leafhoppers collected after a 1- to 3-week AAP, a 0- to 3-week HP, and a 0- or 1-week IAP were immunolabeled and examined with iCLSM to detect MFSV accumulation (Table 5; Fig. 1). No MFSV was detected in the organs of leafhoppers after a 1-week PADP but, by a 2-week PADP, MFSV was detected in cells of the midgut and filter chamber, neural tissues, visceral muscles, and hemocytes in 1 or 2 of 33 *G. nigrifrons* tested (Fig. 1; Table 5). After a 3- to 4-week PADP, MFSV infection was detected further in muscle tissues, tracheal cells, hindgut, and male accessory glands (Fig. 1G and H), accumulating around nuclei of cells (Fig. 1F). In salivary glands at a 4-week PADP, MFSV was detected in both nuclear and cytoplasmic locations (Fig. 1D), the periphery of secretory lobes (acini), and neural tissues. The proportion of MFSV-infected leafhoppers increased with time following the 2-week AAP, with the percentage of MFSV-positive midguts increasing to 27% by the 6-week PADP. Inoculative insects were detected at the same time that MFSV was detected in the salivary glands at the 4-week PADP (Table 5).

TABLE 2. Influence of the acquisition access period (AAP) and holding period (HP) on transmission of *Maize fine streak virus* (MFSV) by *Graminella nigrifrons*

Stage ¹	AAP (weeks)	HP (weeks) ^u	PADP (weeks) ^v	No. of infected/no. tested ^w	Percentage infected ^x	No. inoculative/no. tested ^y	Percentage inoculative ^x	No. ^z
N	1	0	2	22/259	8.5 c	0/259	0.0 a	3
N	1	3	5	24/219	10.5 cd	8/219	3.7 b	3
N	3	0	4	63/384	16.4 d	14/384	3.6 b	3
N	3	3	7	51/235	21.7 e	17/235	7.2 c	3
A	1	0	2	16/332	4.8 b	0/332	0.0 a	3
A	1	3	5	15/337	4.4 b	2/337	0.6 ab	3
A	3	0	4	60/546	11.0 cd	14/543	2.6 b	4
A	3	3	7	100/505	20.0 e	29/505	5.7 c	4

¹ Developmental stage of *G. nigrifrons* at acquisition. N, first instar nymph; A, adult.

^u All insects were subsequently given a 1-week inoculation access period (IAP).

^v Post-first access to disease period, from the beginning of the AAP to conclusion of the IAP.

^w An insect was considered to be infected with MFSV if the increase in absorbance at 405 nm per minute in the enzyme-linked immunosorbent assay was greater than that of the mean of the negative controls on the same plate plus six standard deviations.

^x Means followed by different letters in the same column are significantly different (LSM, $P < 0.05$). Means separation was applied after arcsine-square root transformation but data shown are untransformed.

^y Insects were considered to be inoculative if, after a 1-week IAP as individuals on single maize seedlings, the plants they fed upon expressed symptoms at 6 weeks postinoculation.

^z Number of independent experiments, each starting with 100 to 150 individuals.

TABLE 3. Effect of acquisition access period (AAP) on *Maize fine streak virus* (MFSV) infection of and transmission by *Graminella nigrifrons*.

AAP (h) ^v	No. infected/no. tested ^w	Percentage infected ^x	No. inoculative/no. tested ^y	Percentage inoculative	No. ^z
1	2/286	0.70 $\pm$ 0.12	0/286	0	2
8	11/528	2.08 $\pm$ 0.14	2/528	0.38 $\pm$ 0.00	3
24	13/576	2.26 $\pm$ 0.12	5/576	0.87 $\pm$ 0.07	3
48	20/416	4.81 $\pm$ 0.24	9/416	2.16 $\pm$ 0.11	3

^v First- and second-instar nymphs were allowed access to MFSV-infected plants for the indicated AAP. Subsequently, insects were held for 3 weeks on healthy oats, then caged individually on maize seedlings for a 1-week inoculation access period (IAP).

^w An insect was considered to be infected with MFSV if the increase in absorbance at 405 nm per minute in the enzyme-linked immunosorbent assay was greater than that of the mean of the negative controls on the same plate plus six standard deviations.

^x Percentage of infected individuals $\pm$ standard error.

^y Insects were considered to be inoculative if, after a 1-week IAP as individuals on single maize seedlings, the plants they fed upon expressed symptoms of infection at 6 weeks postinoculation.

^z Number of independent experiments.

DISCUSSION

Newly identified crop plant experimental hosts of MFSV include oat, barley, rye, and wheat (Table 1). Quackgrass, annual ryegrass, and two annual foxtail species also served as hosts. Because *G. nigrifrons* was used to transmit MFSV in host-range experiments, and because the experimental design allowed leafhoppers to move freely between plants, the resulting experimental host range likely reflects vector feeding preferences as well as susceptibility of test plant species (5,36). For example, neither smooth brome nor Kentucky bluegrass is a host of *G. nigrifrons* (47), and neither became infected with MFSV in this study. None of the limited number of asymptomatic plants tested was positive for MFSV by ELISA but the possibility of asymptomatic infection in plants listed as unsusceptible cannot be ruled out without further study. Although spread of MFSV beyond southern Georgia is not known, the ability of the virus to infect quackgrass, a widespread perennial weed, provides a potential virus reservoir, and its ability to infect several important cereal crops suggests that emergence of MFSV as an important agronomic pathogen is possible.

MFSV has a unique host range among North American maize-infecting rhabdoviruses. MFSV, MMV, WASMV, and SSMV are difficult to distinguish by early symptoms (9,34,40,44). However,

in contrast to MFSV, MMV does not infect wheat, SSMV does not infect barley or oats, and WASMV does not infect *E. repens*. SSMV had some cross-reactivity with MFSV on Western blots, indicating that the viruses are serologically related (40). *G. sonora* (Ball), the SSMV leafhopper vector, and *G. nigrifrons* are also related, and sorghum is a host for both *Graminella* spp. However, MFSV did not infect any of five sorghum cultivars or johnsongrass in no-choice experiments, clearly distinguishing these two viruses.

In previous studies, *G. nigrifrons* was the only vector of MFSV identified among several leafhopper and planthopper species tested (40). Transmission characteristics for MFSV and temporal increases in virus titer in infected insects found in this study are consistent with persistent propagative transmission of MFSV, as is the case for other rhabdoviruses (29,39). The proportion of infected and inoculative insects increased with increasing PADP, and no transmission was detected with a PADP shorter than 4 weeks (Tables 2 and 3), the time when MFSV was detected in salivary glands (Table 5; Fig. 1). Virus titer also increased with increasing PADP (Table 4), as did in the extent of MFSV infection of *G. nigrifrons* organs. MFSV was detected first in gut, nerve, and visceral muscle cells, and hematocytes of a small percentage of *G. nigrifrons*, then progressed to all organs except

TABLE 4. Maize fine streak virus (MFSV) titer in uninfected, infected noninoculative, and infected inoculative *Graminella nigrifrons* insects

Stage ^s	AAP (weeks) ^t	HP (weeks) ^u	PADP (weeks) ^v	Insects ^f			
				Uninfected ^w	All infected ^x	Noninoculative infected ^y	Inoculative ^z
N	1	0	2	0.1 a	16.2 c	16.2 c	...
N	1	3	5	0.2 a	33.7 e	23.9 d	53.3 f
N	3	0	4	0.2 a	22.5 d	19.1 c	35.3 e
N	3	3	7	0.5 a	33.9 e	25.8 d	50.0 f
A	1	0	2	-0.3 a	6.0 b	6.0 b	...
A	1	3	5	0.5 a	9.0 b	8.2 b	14.2 bc
A	3	0	4	0.2 a	12.7 bc	7.8 b	28.9 de
A	3	3	7	-0.3 a	17.8 c	14.7 c	25.6 d

^r Means followed by different letters were statistically different (LSM, $P < 0.05$).

^s Developmental stage at first exposure to MFSV: N, first instar nymph; A, adult.

^t Acquisition access period.

^u Holding period.

^v Post-first access to disease period.

^w Mean titer estimates (standard normal transformation of individual reaction slopes) of insects that did not meet the definition of infected (the increase in absorbance at 405 nm per minute in the enzyme-linked immunosorbent assay was greater than that of the mean of the negative controls on the same plate plus six standard deviations).

^x Mean titer estimates of all insects that met the infected criterion.

^y Mean titer estimates of only those infected insects that were noninoculative (insects were considered to be inoculative if, after a 1-week inoculation access period as individuals on single maize seedlings, the plants they fed upon expressed symptoms of infection at 6 weeks postinoculation).

^z Mean titer estimates of those infected insects that were also inoculative; ... indicates no inoculative insects found in the treatment.

TABLE 5. Maize fine streak virus (MFSV) accumulation in *Graminella nigrifrons* organs

Exp ^w	Treatment period (weeks) ^x				Organ, tissue ^y						Inoculativity ^z
	AAP	HP	IAP	PADP	Midgut	Nerve	Muscles	Hemocytes	Tracheae	Glands	
1	1	0	0	1	0/40	0/40	0/40	0/40	0/40	0/40	nt
3	2	0	0	2	2/33	1/33	1/33	1/33	0/33	0/33	nt
3	2	0	1	3	5/31	3/31	3/31	2/31	2/31	0/31	0/40
3	3	0	0	3	8/37	2/37	2/37	1/37	2/37	0/37	nt
3	2	1	1	4	7/37	6/37	5/37	3/37	6/37	2/37	2/49
3	3	0	1	4	6/34	2/34	3/34	1/34	2/34	2/34	1/49
2	3	1	0	4	12/28	8/28	3/28	nt	7/28	3/28	nt
3	2	2	1	5	9/37	7/37	6/37	3/37	3/37	3/37	2/49
3	3	1	1	5	4/25	2/25	2/25	1/25	2/25	2/25	3/28
3	2	3	1	6	12/43	10/43	7/43	3/43	4/43	5/43	4/49
2	3	2	1	6	7/26	5/26	5/26	3/26	3/26	3/26	13/127

^w Experiment number.

^x *G. nigrifrons* nymphs were given the indicated 1-, 2-, or 3-week acquisition access period (AAP) on MFSV-infected maize followed by a 0- to 3-week holding period (HP) on healthy maize plants and a 0- or 1-week inoculation access period (IAP) on single maize seedlings. The post-first access to disease period (PADP) in weeks is also indicated.

^y Organs and hemocyte smears were immunolabeled with Alexa-Fluor (green) and examined with iCLSM for detection of MFSV as shown in Figure 1. Midgut, midgut or filter chamber; Nerve, nerve tissue; Muscle, visceral muscles; Glands, salivary glands; nt, not tested.

^z Subsample of insects was tested for individual transmission of MFSV to maize; nt, not tested.

salivary glands by the 3-week PADP. For MMV in *Peregrinus maidis*, virus was detected in gut tissues after a 1-week PADP and, subsequently, detected in other organs between 2- and 3-week PADPs (3). MMV was detected in salivary glands after a

3-week PADP, at the same time that inoculative insects were detected. Thus, patterns of MSFV and MMV infection of their respective vectors are similar but MFSV appears to move more slowly than MMV.

Fig. 1. Maize fine streak virus (MFSV) accumulation in *Graminella nigrifrons* organs and tissues. Confocal micrographs showing progress of MFSV infection in *G. nigrifrons* **A to C, G, and H**, gut; **D**, muscle and nerve tissues; **E**, hemocytes; and **F**, salivary glands. Leafhoppers were either **A**, reared on healthy plants or allowed to feed on MFSV-infected plants for 2 weeks, then dissected either **B and D**, immediately or **C and E**, after a 1- or 3-week post-first access to diseased period (PADP) or **F to H**, 2-week holding period (4-week PADP) on healthy plants. **A to D and F to H**, Dissected organs or **E**, hemocyte smears were immunochemically stained for MFSV (green), stained with propidium iodide to visualize nuclei (red), and stained with Phalloidin (except for **E** and **F**) to visualize actin (blue). Anterior midgut (amg), compound ganglion (cg), esophagus (es), filter chamber (fc), hindgut (hg), male accessory gland (mag), Malpighian tubules (mt), muscles (mu), nerve (ne), nerve cord (nc), posterior midgut (pmg), salivary glands (sg), and tracheae (tr) are identified on the panels. Arrows in **E** indicate infected hemocytes and red dots show nuclei of uninfected hemocytes. In each panel, the bar represents 50 μ m.

There were only minor differences in proportions of *G. nigrifrons* nymphs and adults that became infected with or transmitted MFSV (Table 2). Furthermore, developmental stage at acquisition did not affect the length of PADP required for individuals to become inoculative. The somewhat higher percentage of infected nymphs relative to adults after a 1-week AAP may have been influenced by the fact that nymphs hatched from eggs laid on infected plants for up to 3 days prior to start of the AAP. However, nymphs had significantly higher virus titer than adults at each PADP, with titer in nymphs after a 2-week PADP being similar to that in adults after a 5- to 7-week PADP (Table 4).

MFSV was transmitted by *G. nigrifrons* at a much lower efficiency compared with several other rhabdovirus–vector systems. Even with a 3-week AAP, the percentages of MFSV-infected and -inoculative leafhoppers never exceeded 22 and 7.2%, respectively. In contrast, *P. maidis* transmitted MMV with 16% efficiency after a 1-week AAP (3) and 42% efficiency after a 3-day AAP and 9- to 13-day HP (15). *G. sonora* transmitted SSMV at 100% efficiency after a 6-h AAP and 10- to 12-day HP (12), and *Endria inimica* transmitted WASMV at 90% after a 2-day AAP and a 14-day HP (44). Other rhabdoviruses, including *Rice transitory yellowing virus*, *MIMV*, *Barley yellow striate mosaic virus*, and *Cereal chlorotic mottle virus*, were transmitted by 16 to 50% of their primary vectors (10,11,23,28). Lower transmission efficiency was found for secondary vectors, with WASMV and MIMV being transmitted by *E. virescens* and *P. maidis* at 3 and 1.6%, respectively (2,42). Thus, transmission rates observed for MFSV by *G. nigrifrons* in this study are more similar to a secondary vector rather than a primary vector. Although it is possible that *G. nigrifrons* is a secondary vector of MFSV, isolated occurrences of the virus in the field suggest that highly efficient vectors of MFSV are rare or not present. It is also possible that the *G. nigrifrons* population in our laboratory colony has drifted genetically from the wild population and differs in ability to transmit MFSV.

Consistent proportions of uninfected, infected, and inoculative *G. nigrifrons* found in this study suggest qualitative or quantitative genetic differences among individuals. We found 3.0 to 3.5 times as many MFSV positive as inoculative insects at a 7-week PADP. Similarly, Falk and Tsai (15) reported 2.3 to 2.6 times as many MMV-positive *P. maidis* as inoculative insects, and Creamer (12) reported 1.9- to 3.9-fold more SSMV-positive *G. sonora* as inoculative insects. Qualitative and quantitative differences in ability of individuals to acquire and transmit have been identified among other virus families and their vectors (8,20, 21,30,35,37,52).

Internal barriers to MFSV infection of *G. nigrifrons* were hypothesized for rhabdoviruses by Hogenhout et al. (27) and later confirmed for MMV in *P. maidis* (3). At a 6- to 7-week PADP after a 3-week AAP, the proportion of infected midguts detected by iCLSM (27%) (Table 5) and infected leafhoppers detected by ELISA (21%) (Table 2) were similar and significantly higher than the proportion of inoculative insects, implicating the midgut as one of these barriers. With the exception of nervous tissue, which was infected at rates similar to midgut tissues, the proportion of infected midguts was higher than other tissues, indicating the presence of barriers in the midgut or nervous tissue. Appearance of infected salivary glands concurrently with inoculative insects supports the importance of salivary gland infection for successful transmission. Overall, proportions of inoculative individuals and those with infected salivary glands were quite similar, indicating invasion of this organ as a major barrier to transmission. Similarly, MMV infection of salivary glands and virus transmission rates were similar in *P. maidis* after a 3-week AAP, and both were lower than the rate of MMV infection of gut tissues (3). Thus, multiple barriers to rhabdovirus infection in vectors may be a common property of rhabdovirus interactions with insect vectors.

There was a strong correlation between higher MFSV titer in *G. nigrifrons* and successful inoculation into maize, and MFSV titer in inoculative insects was higher than in noninoculative but infected insects (Table 4). The correlation between virus titer and inoculativeness supports the hypothesis that there is a minimum titer threshold in the vector that is required for successful inoculation of plants, and this threshold titer represents the viral population needed to overcome plant host defenses and establish infection (25). However, virus titer in rhabdovirus vectors is not always associated with transmission (46), and it is possible that higher virus titer in *G. nigrifrons* is required for the virus to invade the salivary glands. Virus titer was higher in leafhoppers that acquired MFSV as nymphs than as adults but the differences in titer did not translate directly into differences in inoculativeness.

We did not detect a difference between sexes in ability to either acquire or transmit MFSV. Although transmission of *Tomato spotted wilt virus* was similar to that of MFSV, in that it was correlated with virus titer in thrips, male thrips carried less virus but transmitted the virus more efficiently than female thrips (41). For semipersistent transmission of *Maize chlorotic dwarf virus* by *G. nigrifrons*, females ($\approx 60\%$) are more efficient vectors than males ($\approx 35\%$), and differences in gender behavior on plants were correlated with transmission (18).

The intimate relationship between rhabdoviruses and insect vectors is fascinating biologically and evolutionarily; however, currently, there is little detailed information available on genes or other factors governing transmission. Although the MFSV–*G. nigrifrons* system has great potential for developing such information, low rates of virus transmission and long periods required for transmission and symptom development may impede experimental design. Although the MFSV genome is fully sequenced, there is no genomic sequence data for *Graminella* or related leafhopper species. However, *G. nigrifrons* is not difficult to rear and, if the hypothesis that MFSV acquisition and transmission are under genetic control is correct, it should be possible to develop populations with both a high and a low percentage of vectors to facilitate investigations of the molecular and cellular bases of MFSV acquisition and transmission.

ACKNOWLEDGMENTS

Salaries and research support were provided by state and federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University. S. A. Hogenhout is supported by The John Innes Centre and The Gatsby Charitable Foundation. The John Innes Centre is grant-aided by the Biotechnology and Biological Sciences Research Council (BBSRC). We thank K. Willie and J. Abt for expert technical assistance, R. Anderson for experimental materials and assistance with rearing insects, and W. Styer and L. R. Nault for providing seed and valuable advice.

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