Maize Chlorotic Dwarf Viruslike Particles Associated with the Foregut in Vector and Nonvector Leafhopper Species

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ABSTRACT

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Transmission electron microscopy was used to observe viruslike particles (VLP) in thin sections of the foreguts of leafhoppers previously fed on maize plants infected with the semipersistently transmitted maize chlorotic dwarf virus (MCDV). The VLP, of size and shape similar to that of MCDV virions, were found embedded in a semiopaque matrix attached to the cuticular intima of the pharynx, cibarium, and precibarium and occasionally to the inner surface of the maxillary food canal in three cicadellid vector species (*Graminella nigrifrons, G. sonora*, and *Amblysellus grex*) and in the nonvector cicadellid *Dalbulus maidis*; they were not found in the nonvector delphacid *Peregrinus maidis*. Matrix-embedded VLP were also observed in the esophagus of vectors and nonvectors, but they were mixed with food material and not attached to the intima. Attached matrix-embedded VLP were observed in cicadellids fixed immediately after a 3-day acquisition access period (AAP) on MCDV-infected maize and in those given a 1-hr fasting period or a 4- to 5-hr feeding period on healthy plants following AAP. Attached matrix, but not VLP, was observed in *G. nigrifrons* and *D. maidis* leafhoppers after a 4-day feeding period on healthy plants following AAP. Neither VLP nor matrix were found in *Graminella* leafhoppers not exposed to MCDV; nor were they found in *G. nigrifrons* or *D. maidis* exposed to the morphologically similar, cicadellid-transmitted, propagative maize rayado fino virus. We conclude that the matrix-embedded VLP are MCDV virions attached to putative retention sites on the cuticular intima of the foregut in vector leafhoppers. Occurrence of similar retention sites in a nonvector cicadellid (but not in a nonvector delphacid) suggests that, in addition to the attachment (or binding) of virions to the foregut cuticle, other factors may play a role in vector specificity of MCDV.

Viruses from the maize chlorotic dwarf virus group, the parsnip yellow fleck virus group (and the associated anthriscus yellows virus), and the closteroviruses are semipersistently transmitted by their homopteran vectors (13,14,15,16). Semipersistently transmitted viruses are retained in vectors for up to a few days, with a half-life measured in hours (16). Inoculative immature vectors lose their ability to transmit virus following ecdysis. Such experimental evidence suggests that transmissible virus is carried on the stylets or the cuticular lining of the foregut, both of which are lost by insects when they molt.

The in situ electron microscopic (EM) observations of the putative virions associated with the anthriscus yellows virus (AYV) and maize chlorotic dwarf virus (MCDV) provide additional evidence that transmissible virus is retained at specific sites in the foreguts of vectors. AYV-like virions were found associated with a 15- to 20-μm-long portion of the lining of the ventral wall of the pharynx, where it passes over the tentorial bar of the aphid vector (15). MCDV-like particles (VLP) were observed (by EM) adhering to the precibarium, cibarium, pharynx, and fore-esophagus of the MCDV-vector leafhopper *Graminella nigrifrons* (Forbes) but not the nonvector leafhopper *Dalbulus maidis* (DeLong & Wolcott) by Childress (7) and Childress and Harris (8). These authors suggested that the apparent absence of virus-retention sites in the nonvector *D. maidis* provides a plausible explanation for MCDV-leafhopper transmission specificity (8).

In this paper, we report putative retention sites for MCDV in three leafhopper vector species as well as in the nonvector *D. maidis*. We also provide further information on the MCDV-associated matrix in the leafhoppers foregut. Implications of these findings on MCDV transmission mechanism and vector specificity by leafhoppers are discussed. Preliminary accounts of the present work have been reported briefly (3,4,16,19).

MATERIALS AND METHODS

Viruses and insect species tested. The type isolate of MCDV used in this study was the same as that described earlier (11,17) and was maintained in maize (*Zea mays*, in inbred OH28) by serial transmission using *G. nigrifrons* vectors. An isolate of maize rayado fino virus (MRFV) (18) was used in some experiments as a control for MCDV. MRFV is another ss-RNA isometric virus similar in size to MCDV, but it is transmitted in a propagative manner by both *D. maidis* and *G. nigrifrons* (16,18).

Three species of MCDV vector leafhoppers (Cicadellidae) were used. These species, arranged in descending order by their efficiency as MCDV vectors are: *G. nigrifrons, Amblysellus grex* (Oman), and *Graminella sonora* (Ball) (17). Two hopper species that are maize pests but not vectors of MCDV were used as controls: the cicadellid leafhopper, *D. maidis*, and the delphacid planthopper, *Peregrinus maidis* Ashmead (16). Vector and nonvector species were reared on maize, except for *A. grex*, which was reared on oats and rye, as described previously (17).

Experimental procedure. Vector and nonvector young adult hoppers were exposed to MCDV-infected maize plants for a 3-day acquisition access period (AAP). Following this, exposed insects were treated in one or more of the following ways (30–50 insects per treatment per species): 1) fixed immediately for light and electron microscopy (EM), 2) fasted for 1 hr before fixation, 3) fed for 4–5 hr on healthy maize seedlings before fixation, or 4) transferred daily for 4 days on healthy maize seedlings before fixation. This 4-day period is 8 hr longer than the maximum

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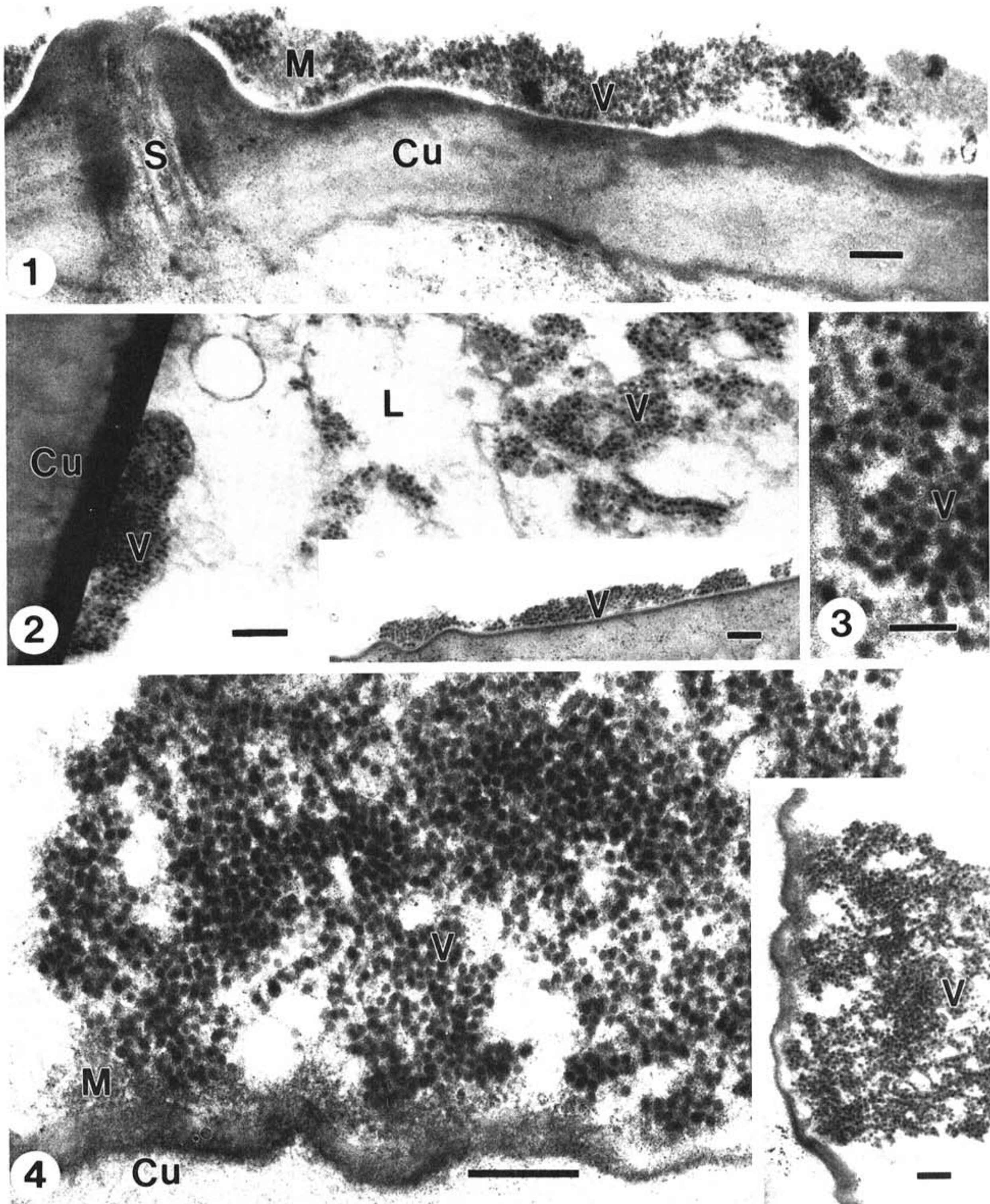
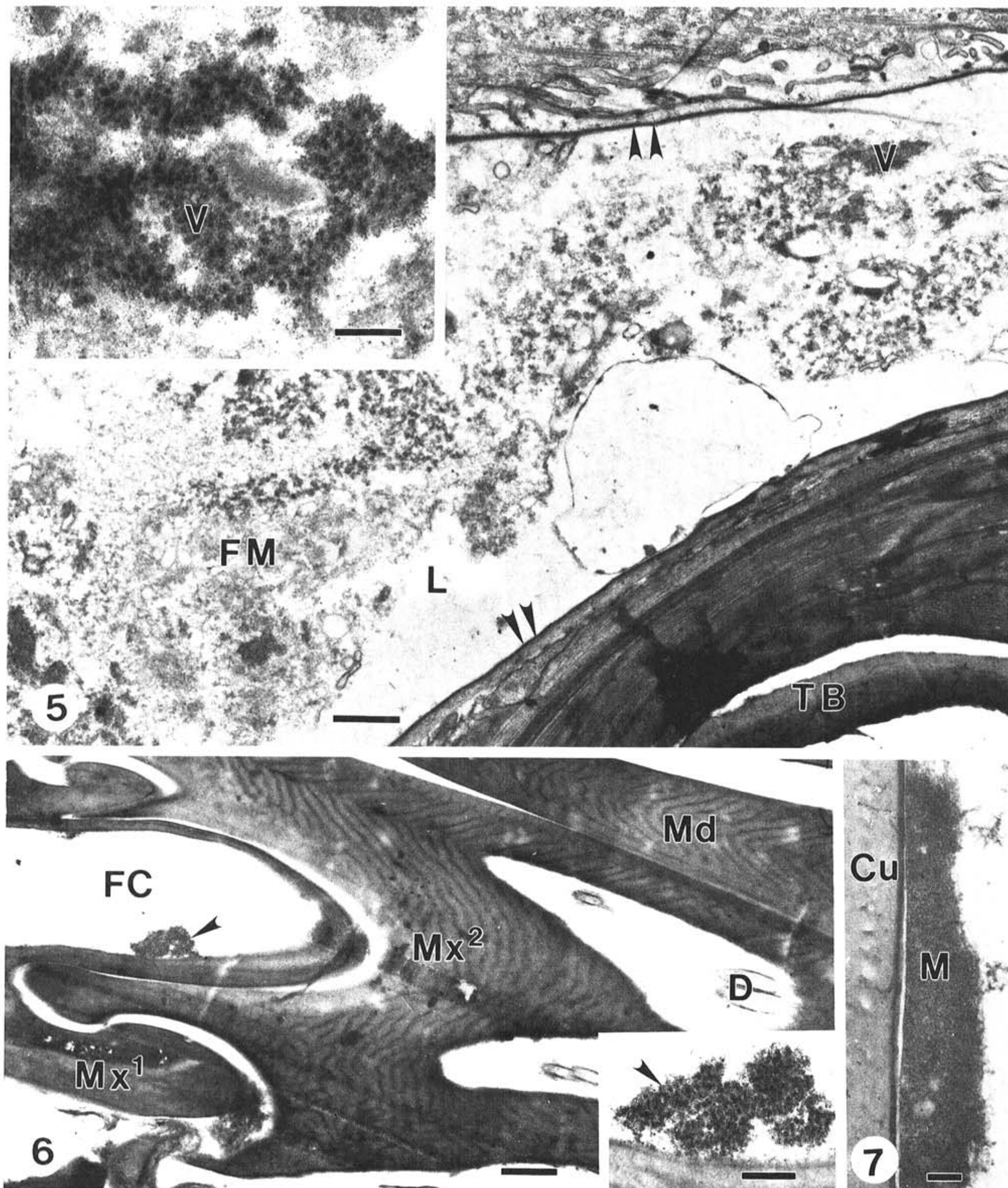
retention period reported for MCDV in *G. nigrifrons* (9). Exposed insects were usually fed on healthy maize seedlings in groups, except one experiment, in which exposed *G. nigrifrons* leafhoppers were fed singly on healthy seedlings for 4–5 hr to test their infectivity. In some experiments, *G. nigrifrons* and *D. maidis* were exposed to MRFV-infected plants for 3 days. Following AAP, these leafhoppers were fed for 4–5 hr on healthy maize seedlings before fixation. Other controls included feeding MCDV vector species *G. nigrifrons* and *G. sonora* only on healthy maize plants. All healthy seedlings on which exposed or nonexposed vector or nonvector insects had fed were sprayed with a pyrethroid insecticide and kept in the greenhouse 3–4 wk to allow for development of virus symptoms. None of the plants on which nonexposed or nonvector species were fed showed virus symptoms.

Preparation of specimens for light and electron microscopy (EM). Unlike previous studies on MCDV-retention sites in leaf-



Figs. 1–4. Aggregates of viruslike particles (VLP = V) embedded in semiopaque matrix material (M), attached to the cuticular intima (Cu) of parts of the foregut in *Graminella nigrifrons* (Figs. 1–3) or *Dalbulus maidis* (Fig. 4), previously exposed to plants infected with maize chlorotic dwarf virus, then fed on healthy maize for 4–5 hr. **1**, VLP in the precibarium, above the precibarial valve. S = sensillum. Bar = 200 nm. **2** (and inset), VLP in the cibarium, either attached to the intima (Cu) or floating in the lumen (L). Bars = 200 nm. **3**, Highly magnified, matrix-embedded, VLP (V). Bar = 90 nm. **4** (and inset), attached matrix-embedded VLP(V) in the pharynx of *D. maidis* (Bars = 200 nm).

hoppers, in which either dissected organs or whole insects were processed for EM (7,8), we fixed, embedded, and sectioned the intact foregut by processing the head and attached prothorax. This technique gave good preservation of virus particles and insect tissues (Figs. 1–7) and at the same time preserved the integrity of the leafhoppers foregut, thus allowing better identification of its parts (Fig. 1 in 16, as compared to Fig. 3B in 7). We anesthetized leafhoppers and planthoppers with CO_2 and then immersed them in 0.1 M potassium phosphate buffer (pH 7.4). With the aid of a stereomicroscope, the prothorax (with attached head) was severed from the mesothorax with a sharp blade, then immersed in 2.5% glutaraldehyde for several days, washed in buffer, post-



Figs. 5–7. **5** (and inset), Unattached, matrix-embedded, viruslike particles (VLP = V) mixed with food material (FM) in the lumen (L) of the esophagus near the tentorial bar (TB) in *Graminella nigrifrons* fed for 4–5 hr on healthy maize following exposure to plants infected with maize chlorotic dwarf virus (MCDV). Double arrowheads indicate thin cuticular intima. Bar = 900 nm, and for inset 200 nm. **6** (and inset), Matrix-embedded VLP (arrowheads) attached to the inner cuticular surface of the maxillary food canal (FC) in *G. sonora* fixed immediately following acquisition period on MCDV-infected plants. D = (maxillary) dendrites, Md = mandible, Mx1 and Mx2 = two interlocking maxillae. Bar = 1 μm, and for inset 200 nm. **7**, Semiopaque matrix (M) without VLP, attached to the cuticular intima (Cu) of the pharynx in *G. nigrifrons* fed for 4 days on healthy maize following exposure to MCDV-infected plants. Bar = 200 nm.

fixed 3 hr in 1% OsO_4, and processed further for EM as described earlier (2,3). Sagittal semithin sections (1–2 μm thick) were cut, stained with toluidine blue, and examined by light microscopy until the cibarium or pharynx was reached. Ultrathin sections were then cut, stained, and examined by EM (2,3). From each specimen (insect), 5–7 grids were examined, with an average of 10 sections per grid from the foregut region. The number of insects examined from each species-treatment combination is given in Table 1.

RESULTS

Morphology of the foregut in *Graminella* and *Dalbulus* leafhoppers. In these leafhoppers, the food canal in the maxillary stylets leads to a short, narrow precibarium, midway in which is located the precibarial valve separating two sets of chemosensilla. Similar structures have been reported for other leafhoppers (1,5). The precibarium (Fig. 1) leads to a large, cup-shaped cibarium (cibarial or sucking pump) with a very thick cuticular lining (intima) (Fig. 2), which appears rigid except for a dorsal portion that forms a flexible diaphragm attached to dilator muscles. Posterio-ventrally, the cibarium leads into a narrow pharynx lined with thick cuticular intima (Fig. 4). The pharynx widens gradually and its intima becomes thinner as it runs posterio-dorsally above the tentorial bar to connect with a much wider esophagus lined with very thin intima (Fig. 5). The esophagus leads to the microvilli-lined midgut through the esophageal (cardiac) valve. For clarification, we refer the reader to a diagramatic illustration of the *G. nigrifrons* foregut (16).

Retention sites of MCDV-like particles (VLP) in the anterior alimentary canal of vector and nonvector species. In three species of MCDV vectors and in the nonvector *D. maidis*, isometric VLP (25–30 nm in diameter) were regularly found in the anterior alimentary canal of adults previously exposed to MCDV-infected maize plants. Aggregates of VLP occurred sometimes as single rows or layers, but more commonly as small or large clusters embedded in a semiopaque granular or finely fibrillar matrix (Figs. 1–6). These matrix-embedded VLP (Fig. 3) were usually attached to the cuticular intima of the precibarium (Fig. 1), the cibarium (Fig. 2), or (more frequently) the pharynx (Fig. 4). Attached, matrix-embedded VLP were found in one or more of these putative retention sites in 17 of 20 exposed vector leafhoppers (*G. nigrifrons, G. sonora*, and *A. grex*) and in 11 of 12 exposed *D. maidis*, whether they were fixed immediately following AAP on infected plants, fasted for 1 hr following AAP, or fed 4–5 hr on healthy maize plants following AAP (Table 1). In leafhoppers fixed immediately following AAP, matrix-embedded VLP were occasionally found attached to the inner cuticular surface of the maxillary food canal (Fig. 6). In exposed leafhoppers, no VLP were found attached to the intima of the esophagus, but, in the esophageal lumen, abundant aggregates of VLP and associated matrix were frequently found mixed with food material (Fig. 5). Fasting for 1 hr post-AAP did not have much effect on the appearance of VLP aggregates, since even in insects fixed immediately following AAP, very little food material was detected by EM in the foregut, except in the esophagus and occasionally in the cibarium.

When 50 MCDV-exposed *G. nigrifrons* were tested singly by feeding them for 4–5 hr on maize test plants, 26% transmitted MCDV. Five of 6 females of these inoculative leafhoppers examined by EM had matrix-embedded VLP attached to the foregut intima. However, in *G. nigrifrons* that had been fed for 4 days on healthy maize following AAP (and thus rendered noninoculative as demonstrated by infectivity tests) only matrix without VLP (Fig. 7) was found attached to the intima of the cibarium or pharynx in 5 of 6 leafhoppers examined. In similarly treated *D. maidis*, attached matrix without VLP was found in the foreguts of 4 out of 5 leafhoppers examined. In *G. nigrifrons* and *G. sonora* exposed only to healthy plants, or in *G. nigrifrons* and *D. maidis* exposed to MRFV-infected maize, no VLP or matrix were found attached to the intima of any part of the foregut (Table 1). No attached VLP were found in the foreguts of 11 *P. maidis* exposed to MCDV-infected plants, then fixed immediately or after they had fed for 4–5 hr on healthy maize. However, in one planthopper prepared for EM immediately after AAP, some unattached VLP with associated matrix were found in the cibarium.

TABLE 1. Occurrence of matrix-embedded viruslike particles (VLP) attached to the cuticular intima of foreguts of leafhoppers and planthoppers exposed to plants infected with maize chlorotic dwarf virus (MCDV) or maize rayado fino virus (MRFV)

Virus[a]	Leafhopper or planthopper species	Post-virus-acquisition treatment[b]	Number of insects with attached matrix-embedded VLP per number observed
MCDV	*Graminella nigrifrons*	fasted 1 hr	4/5
		fed 4–5 hr	5/6[c]
		fed 4 days	0/6
	G. sonora	none	4/5
	Amblysellus grex	none	4/4
	Dalbulus maidis	none	4/4
		fasted 1 hr	2/3
		fed 4–5 hr	5/5
		fed 4 days	0/5
	Peregrinus maidis	none	0/6[d]
		fed 4–5 hr	0/5
MRFV	*G. nigrifrons*	fed 4–5 hr	0/6
	D. maidis	fed 4–5 hr	0/5
None	*G. nigrifrons*	···	0/6
	G. sonora	···	0/3

[a]Insects were exposed to virus-infected plants (MCDV or MRFV) for a 3-day acquisition access period or were never exposed to virus infected plants (none).

[b]Insects were fixed immediately following acquisition period (none), fasted 1 hr in a petri dish, or fed 4–5 hr or 4 days on healthy maize following acquisition before they were fixed in preparation for light and electron microscopy. The 4-day period rendered exposed vector leafhoppers noninoculative with MCDV.

[c]All six insects transmitted MCDV when singly placed on test plants during 4–5 hr inoculation access period before fixation.

[d]Some unattached VLP were found in the cibarium of one insect.

DISCUSSION

We interpret the VLP observed in the anterior alimentary canal of the four leafhopper species as MCDV virions. They morphologically resemble virions observed in thin sections of MCDV-infected maize or virions observed in purified preparations from MCDV-infected plants (4,6,10). Moreover, VLP were found in most leafhoppers exposed to MCDV-infected plants (including *G. nigrifrons* that transmitted virus) but were never found in leafhoppers that had previously fed on healthy or MRFV-infected plants. Furthermore, we consider the pharynx, cibarium, precibarium, and perhaps the maxillary food canal all to be potential retention sites for transmissible virus. This conclusion is supported by our findings that at 0 and 4–5 hr following exposure of leafhoppers to MCDV-infected plants, matrix-embedded VLP was observed attached to the cuticular intima of the above sites in the foregut, but at 4 days postexposure only attached matrix was observed. Four days is beyond the known maximum retention of MCDV by *G. nigrifrons* vectors (9,20). Also, this conclusion is consistent with experimental evidence demonstrating that MCDV inoculative nymphs lose ability to transmit virus following a molt (20); leafhoppers shed the cuticular intima of the foregut and stylets during ecdysis, and thus they would be expected to lose transmissible virus attached to these structures.

Except for the maxillary food canal, these putative retention sites are the same in *G. nigrifrons* as those reported previously by us (4,16) and by Childress and Harris (8). We extend these findings to two additional experimental leafhopper vectors, *G. sonora* and *A. grex*.

Contrary to Childress and Harris (8), we consistently found matrix-embedded VLP attached to the intima of the same sites in the foregut of the nonvector, *D. maidis*, as we did in vector

species. Childress and Harris (8) reported finding VLP only within food boluses in the lumen of the esophagus of MCDV-exposed *D. maidis*. From their findings they conclude that absence of sites in the foregut for virus retention and accumulation explains why *D. maidis* is not a MCDV vector (8). Our study suggests that this is the reason why the delphacid planthopper, *P. maidis*, does not transmit MCDV; however, our results lead us to a very different explanation for why *D. maidis* does not transmit MCDV. Rather than the absence of retention and accumulation sites, the failure of *D. maidis* to transmit virus more likely involves virus detachment and inoculation.

Attachment and detachment of MCDV appears to involve the matrix in which VLP are embedded. Our findings concerning the appearance of the matrix differ with those of Childress and Harris (8). They noted similarities in their electron micrographs to those of Murant et al (15), who found that anthriscus yellows VLP are associated with "M-material", which binds virus to the pharynx of its aphid vector. The M-material appears more fibrous and less electron-dense than the MCDV-associated matrix reported here. Additionally, we observed matrix only in leafhoppers exposed to MCDV-infected plants, whereas Murant et al (15) found M-material in aphids exposed to healthy or AYV-infected plants. Childress and Harris (8) reported that VLP in *G. nigrifrons* were embedded in a lightly stained matrix (M-material) or densely stained substrate (DSS), which, in turn, apparently was attached by M-material to the cuticle. From their electron micrographs, it is difficult to differentiate between M-material and DSS; it is possible that in EM a thin layer of DSS would be less opaque than a thick one and be interpreted as M-material. This explanation is consistent with the remark of Childress and Harris that "thin layer aggregates of VLP were always embedded in M-material."

It has been suggested previously that the matrix observed in MCDV-exposed leafhoppers is the same material that embeds virus particles in granular inclusions in the phloem of MCDV-infected maize plants (4,6,8,10). The matrix-embedded VLP seen in leafhoppers are probably fragments of granular inclusions ingested when leafhoppers feed on infected plants. We postulate that matrix may be the putative helper component required for leafhopper transmission of MCDV (11). Evidence for helper component was demonstrated when it was shown that purified virus from one MCDV isolate could be transmitted by *G. nigrifrons* from membranes only when leafhoppers fed first on plants infected with another isolate.

There appears to be a different rate of detachment of VLP and matrix from the foregut intima of MCDV-exposed leafhoppers. This is shown by the appearance of matrix but not VLP in the foregut 4 days after access to MCDV-infected plants. This observation could explain the results of R. Creamer, L. R. Nault, and R. E. Gingery (*unpublished*), who showed that after *G. nigrifrons* can no longer transmit one MCDV isolate acquired from a plant, it can pick up and transmit a second isolate acquired from a membrane. It is possible that residual matrix attached to the foregut intima is sufficient to trap and bind purified virus acquired from membranes. Virus that detaches from the foregut intima must find its way back through the stylets if transmission is to occur. We assume that some plant sap that enters the stylets and foregut is not ingested; for example, not all fluids pass through the coelomic valve and into the midgut. Some fluids, including virus detached from retention sites in the foregut, may be forced back through the stylets and into the plant by extravasation (12). The ability of leafhoppers to extravasate may explain vector specificity. Wayadande and Nault (21, *unpublished data*) have shown that specific phloem-probing behaviors are associated with transmission of MCDV. Electronically monitored, inoculative *G. nigrifrons* transmit MCDV only when they produce x-waveform patterns; there is a positive correlation between duration of x-waveforms and transmission rate of MCDV. Moreover, it has been shown that *G. nigrifrons* and other leafhopper vectors (17) have similar x-waveform patterns. In contrast, *D. maidis* and other leafhoppers that do not transmit MCDV (17) have very different, less complex waveforms. One of the behaviors represented by the x-waveform in vectors is extravasation. This may well be a behavior missing in the repertoire of *D. maidis*. This reason rather than the failure of virus to attach to and accumulate on the cuticular intima of the foregut is a more plausible explanation for why *D. maidis* does not transmit MCDV.

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