Vector Specificity of Barley Yellow Dwarf Virus (BYDV) Transmission: Identification of Potential Cellular Receptors Binding BYDV-MAV in the Aphid, *Sitobion avenae*

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Two proteins (SaM35 and SaM50) isolated from head tissues of the aphid vector, *Sitobion avenae,* were identified as potential receptors for barley yellow dwarf virus MAV isolate (*Luteoviridae*) based on MAV virus overlay assays and immunoblots of urea SDS 2-D gels. An anti-idiotypic antibody (MAV4 anti-ID) that mimics an epitope on MAV virions and competes with MAV in antibody binding assays also bound to SaM50 and SaM35 and to six additional proteins including a GroEL homolog. No MAV-binding proteins were detected from the nonvector aphid, *Rhopalosiphum maidis,* although MAV4 anti-ID did react with four proteins from *R. maidis.* It is hypothesized that SaM35 and SaM50 may be MAV receptors involved in MAV transmission based on their high affinity for MAV and their unique association with the vector, *S. avenae.* The additional aphid proteins binding the MAV4 anti-ID may represent less specific virus-binding proteins facilitating transmission through different aphid tissues. © 2001 Academic Press

Key Words: Sitobion avenae; Rhopalosiphum maidis; Luteoviridae; anti-idiotypic antibody; anti-idiotypes; virus receptor; virus overlay assays.

INTRODUCTION

Barley yellow dwarf disease of cereals is caused by several different viruses currently classified in two genera, *Luteovirus* and *Polerovirus*, of the plant virus family *Luteoviridae* (Mayo and D'Arcy, 1999). Viruses in the *Luteoviridae* are single-stranded, positive-sense RNA viruses that infect only the phloem tissues of specific host plants and are transmitted by aphids in a circulative, nonpropagative manner. To be transmitted, the virus must penetrate the aphid gut lining via receptor-mediated transport, circulate in the hemocoel, traverse the salivary gland via receptor-mediated transport, and then be secreted with saliva when the aphid vector feeds on plants (Gildow, 1999). Each member of the *Luteoviridae* is transmitted by specific species of aphids. For example, the luteovirus *Barley yellow dwarf virus* (BYDV) MAV isolate is transmitted most efficiently by the aphid *Sitobion avenae,* but is rarely transmitted by the aphids *Rhopalosiphum padi* or *R. maidis.*

Three barriers regulating MAV transmission vector specificity have been identified in aphids: apical plasma membrane (plasmalemma) lining the hindgut and the basal lamina and basal plasma membrane surrounding the accessory salivary gland (ASG) (Gildow, 1999). Few virions of MAV are able to penetrate the ASG basal lamina of poor or nonvector aphid species, and preincubation of MAV virions with MAV4 monoclonal antibody

(MAb) Fab fragments prior to injection into vector aphids prevented MAV attachment to the ASG basal lamina (Gildow and Gray, 1993). When the ASG of vector aphids, *S. avenae,* or nonvector aphids, *R. maidis,* are incubated *in vitro* with MAV, virions attached only to the ASG basal lamina of *S. avenae* and not to *R. maidis* (Peiffer *et al.,* 1997). It was hypothesized that the ASG basal lamina might act as a barrier to transmission by preventing virus attachment or impeding penetration of virus into the ASG, thus reducing transmission efficiency. The ASG cell membrane facing the hemocoel presents the second distinct selection barrier regulating virus recognition at the ASG. Some viruses of the *Luteoviridae* are able to penetrate the ASG basal lamina of some aphid species, but they are unable to pass the ASG basal plasma membrane (Gildow and Gray, 1993; Peiffer *et al.,* 1997). Recent studies of the differential penetration of ASG basal lamina and plasma membrane by different strains of soybean dwarf luteoviruses in different aphid species also supported the idea that different sites in different aphid species may determine vector specificity (Gildow *et al.,* 2000a). A virus-recognizing receptor(s) determining transmission specificity at the ASG basal lamina and basal plasma membrane might interact with structural proteins of certain members of *Luteoviridae,* allowing specific viruses to pass those barriers. Inability (or reduced ability) to transmit virus may be due to lack of or mutation of only one of several different receptor proteins located in the transcellular transport process. In addition to vector-specificity receptors, other aphid virus-binding proteins that do not determine transmission

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specificity also might be involved in the process of virus transmission, as suggested by ultrastructural studies (Gildow, 1993; Gildow and Gray, 1993). Plasma membrane invaginations initiating coated pits leading to development of coated vesicles and tubular transport vesicles have been observed to be associated with endocytotic and exocytotic pathways for virus transcellular transport through aphids. Luteoviruses acquired into aphids by traversing the gut lining are transported sequentially from coated pits to coated vesicles and then to tubular vesicles that finally release the virus particles through the basal plasma membrane into the aphid hemocoel (Garret *et al.,* 1993; Gildow, 1993). A similar pathway in the reverse direction appears to function at the aphid salivary gland (Gildow, 1982, 1999). Given the multiple sites and cellular processes involved, we would expect to identify more than one aphid protein capable of binding to MAV. Only some of these proteins might function as MAV vector-specificity receptors in vector aphids. It is possible that vector and nonvector aphids for a specific luteovirus could share some of the same virus binding proteins, if those proteins had a conserved role in aspects of virus transport through the gut or salivary gland that did not determine transmission specificity. A conservation of transmission-related proteins is to be expected because aphid species unable to vector MAV can acquire and transmit other members of *Luteoviridae.* An example of a conserved virus-binding protein is a 63-kDa GroEL homologue protein found in most aphids that binds to the polerovirus potato leafroll virus (PLRV) and is hypothesized to function in protecting members of *Luteoviridae* from degradation in the aphid hemocoel (van den Heuvel *et al.,* 1997). In addition, ultrastructural evidence suggested that some aphid species may utilize a common virus receptor at the hindgut to acquire several different luteoviruses and poleroviruses into the hemocoel even though the acquired viruses are not transmitted by these aphid species (Gildow, 1993). This hypothesis for receptor-mediated virus acquisition through the aphid gut was supported by recent findings that PLRV-14.2, an isolate with several amino acid substitutions in a structural protein, was acquired by aphids much less efficiently than other PLRV isolates with different amino acid sequences (Rouze-Jouen *et al.,* 2001). It was suggested that altered protein sequence may have resulted in reduced affinity of PLRV-14.2 for putative receptors on the aphid gut.

A common strategy to study ligand–receptor interaction is to replace the ligand with an anti-idiotypic antibody (anti-ID) mimicking the ligand (Sege and Peterson, 1978). This strategy has been successfully applied in the study of virus–receptor interactions. Anti-IDs prepared to MAbs that specifically recognized two membrane glycoproteins, GP1 and GP2, of tomato spotted wilt virus (TSWV) specifically labeled a 50-kDa thrips protein that may be a potential cellular receptor for TSWV (Bandla *et al.,* 1998). Monoclonal anti-IDs made against mouse
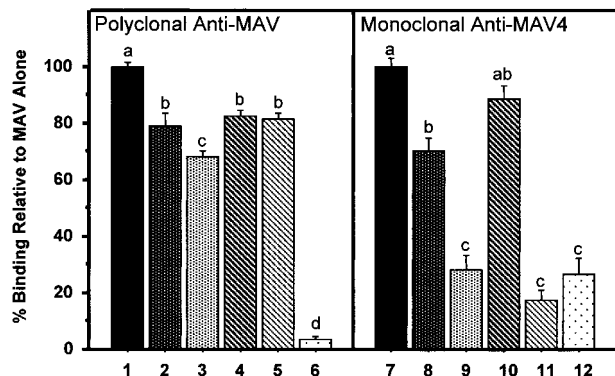

FIG. 1. Competition-binding assays between purified MAV virions and MAV4 anti-idiotypic antibodies for MAV-specific polyclonal or monoclonal antibody. Results for each treatment are expressed as the percentage of the mean value of the absorbance at 405 nm relative to treatment 1, the positive control. Treatments were (1 and 7) maximum binding of MAV suspended in ELISA sample buffer at 50 ng MAV/well expressed as 100%, (2 and 8) preliminary incubation of wells 1 h with preimmune serum followed by a second incubation with MAV to test for nonspecific inhibition or steric hindrance by rabbit IgG, (3 and 9) preliminary 1-h incubation of MAV4 anti-idiotype IgG followed by a second incubation with MAV4 virions to test for competition, (4 and 10) simultaneous incubation of preimmune serum and MAV to test for nonspecific inhibition, (5 and 11) simultaneous incubation of a mixture of MAV4 anti-idiotypic IgG and MAV virions to test for competition, (6 and 12) incubation of MAV4 anti-idiotypic IgG only as a negative control. Preimmune serum and MAV4 anti-idiotypic IgG were loaded at 100 μg/well. Standard deviations are indicated by bars. Values with a different letter are significantly different ($P < 0.05$) based on Tukey's pairwise comparison.

MAbs that neutralize the infectivity of the tick-borne encephalitis virus (TBEV) bound to virus-susceptible porcine cells and also inhibited the TBEV infectivity (Kopecky *et al.,* 1999). The anti-IDs recognized a 35-kDa protein on the cell surface that could also bind to TBEV.

The transmission-specificity of BYDV-MAV is determined at the ASG (Gildow, 1999). Therefore, a MAV binding protein(s) identified from protein extract from the head region of the vector aphid *S. avenae,* but not identified in the nonvector aphid *R. maidis,* may function as a potential vector-specific receptor(s) for MAV transmission. In this report we describe the identification of two MAV-binding proteins from *S. avenae* that may serve as potential receptors for MAV.

RESULTS

Binding competition between MAV virions and MAV4 anti-idiotypic antibody

Competitive binding of MAV and MAV4 anti-ID to polyclonal and monoclonal IgG specific for MAV was done to determine whether MAV4 anti-ID mimicked MAV epitopes. In the presence of 100 μg/well MAV4 anti-ID, significantly less MAV was bound to microtiter plates coated with polyclonal antibody specific to MAV (Fig. 1, treatments 3 and 5). This result may be partially due to steric inhibition of nonspecific antibodies as indicated by
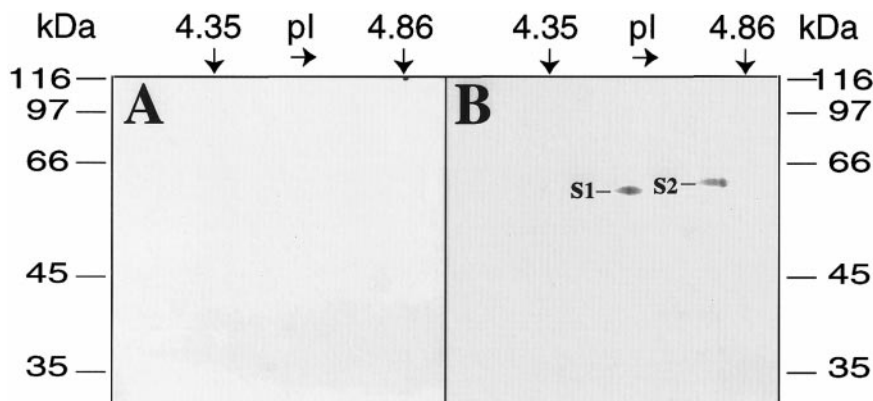
FIG. 2. Detection of GroEL proteins on Western blots of *S. avenae* head proteins separated by urea/SDS 2-D gels. Sample loading site (p*I* 4.86) is indicated by arrow at the top. (A) Preimmune serum at a concentration of 0.1 μg/ml did not bind any proteins from 100 head equivalents per lane (24 μg protein/lane), indicating that there was no nonspecific binding of antibody. (B) Anti-GroEL antibody (Epicentre Technologies, Madison, WI) detected two GroEL protein homologs, symbionin-like protein S1 (MW 63 kDa, p*I* 4.53) and symbionin-like protein S2 (MW 65 kDa, p*I* 4.86), in samples loaded at 25 head equivalents per lane (6 μg protein/lane). Sample loading site (p*I* 4.86) and p*I* distribution are marked at the top. Relative molecular weights are indicated on the sides.

treatment 4 (Fig. 1). However, steric hindrance could not account for the observed significant decrease in MAV binding seen when the MAV4 anti-ID was reacted in the plate first, followed by MAV (treatment 3), compared to reaction with preimmune serum first and then MAV (treatment 2). This indicated that the MAV4 anti-ID could compete specifically with the MAV virions for binding to MAV-specific polyclonal antibodies. Similarly, significantly less MAV was bound to plates coated with the monoclonal anti-MAV4 IgG in the presence of anti-idiotype (Fig. 1, treatments 9 and 11). The binding inhibition was dependent on concentration of anti-idiotype (results not shown). The presence of preimmune serum did not significantly inhibit the binding between MAV virions and the MAV4 MAb coating antibody when they were mixed together (treatment 10, $P > 0.05$). However, there was a significant decrease in MAV virions bound to the plate if preimmune serum was added prior to the MAV (treatment 8), indicating that a detectable level of nonspecific inhibition did occur. Combined results of the two binding competition assays using both polyclonal and monoclonal antibodies indicate that MAV4 anti-ID is an anti-idiotypic antibody that mimics an epitope on MAV virions recognized by MAV4 monoclonal antibody, and this similarity allows the MAV4 anti-ID to either compete with or interfere with MAV virions for attachment to antigen binding sites on MAV4 MAb.

Development of blocking solution and the negative control blots

Obvious binding between native aphid proteins and rabbit preimmune serum was observed on isoelectric focusing (IEF) gel blots following blocking procedures used by van den Heuvel *et al.* (1994) (results not shown). Aphid proteins were also found to bind to preimmune serum on SDS–PAGE blots if the gels were equilibrated

in transfer buffer for less than 30 min. If equilibration lasted for over 60 min, nonspecific reactions between aphid proteins and preimmune serum almost completely disappeared probably due to loss of aphid proteins. Using the blocking methods described in this study (see Gel overlay assays under Materials and Methods), much weaker reactions between preimmune serum and aphid proteins were observed even if the gel was equilibrated in transfer buffer for less than 15 min. To reduce the loss of proteins that may function as potential receptors during gel equilibration, we applied this blocking solution to all the reported blots. On 2-D gel immunoblots, almost no aphid proteins were bound by preimmune serum except one spot at the sample loading site when the extensive blocking method was used (Fig. 2A).

Two GroEL proteins were identified from *S. avenae*

A 63-kDa GroEL homologue from vector aphids has been shown to interact with certain members of *Luteoviridae in vitro* (van den Heuvel *et al.,* 1994; Filichkin *et al.,* 1997). In our study, two GroEL proteins, S1 (symbionin-like protein-1), with MW of 63 kDa and p*I* of 4.53, and S2 (MW 65 kDa, p*I* 4.86), were identified from *S. avenae* based on their reactions to anti-GroEL antibody specific to prokaryotic GroEL (Fig. 2B). However, neither S1 nor S2 reacted with MAV virions (Fig. 3A), and only S1 was detected by MAV4 anti-ID (Fig. 3B, spot 6, and Table 1, SaM63). This work was independently replicated twice.

MAV overlay assays showed relatively high affinity to two proteins from *S. avenae*

Two proteins having relatively high affinity for binding to MAV virions (Fig. 3A) were identified from *S. avenae* head extract by MAV overlay assays, SaM50 (*S. avenae* protein binding MAV), with apparent MW of 50 kDa and p*I* of 4.51, and SaM35 (MW 35 kDa, p*I* 4.35). In addition to
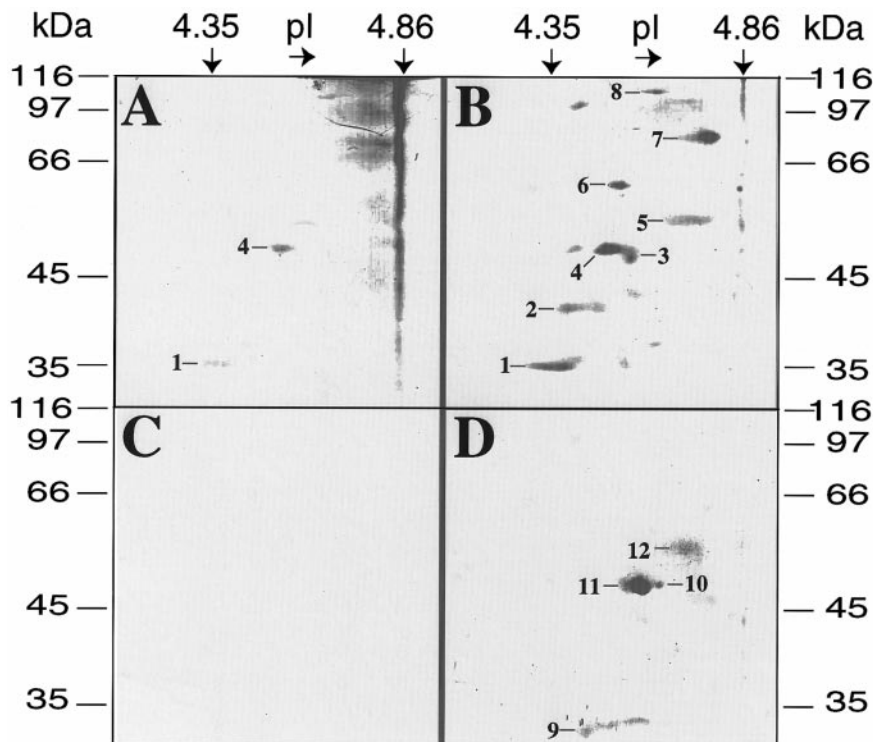
FIG. 3. Reaction of aphid head proteins with MAV virions or MAV4 anti-idiotypic IgG following separation by urea SDS 2-D gel electrophoresis. (A) MAV overlay assays of *S. avenae* proteins detected two proteins, SaM35 and SaM50 (Table 1), that bound MAV. (B) MAV4 anti-ID overlays of *S. avenae* proteins detected eight proteins binding the anti-idiotypic IgG. (C) MAV overlay assay of *R. maidis* head proteins did not detect MAV-binding proteins in the nonvector species. (D) MAV4 anti-ID overlays of *R. maidis* proteins detected four proteins binding the anti-idiotypic IgG. None of the four *R. maidis* proteins corresponded to the two MAV-binding proteins or GroEL proteins in *S. avenae*. Proteins were loaded at 24 μg/lane (100 aphid head equivalents) in gels for blots A, C, and D and at 6 μg/lane for blot B. Sample loading site (p*I* 4.86) and p*I* distribution are marked by arrows at top. Relative molecular weights are indicated on the sides.

TABLE 1

MAV-Binding and MAV4-Anti-ID-Binding Proteins Identified in Head Extracts of *Sitobion avenae* and *Rhopalosiphum maidis* Using Virus Overlay Assays and Immunoblots

Aphid	Label No.[a]	kDa	p*I*	Designation
S. avenae	1	35	4.35	SaM35[b]
S. avenae	2	40	4.40	SaM40
S. avenae	3	49	4.56	SaM49
S. avenae	4	50	4.51	SaM50[b]
S. avenae	5	56	4.72	SaM56
S. avenae	6	63	4.53	SaM63[c]
S. avenae	7	82	4.79	SaM82
S. avenae	8	97	4.43	SaM97
R. maidis	9	32	4.48	RmM32
R. maidis	10	49	4.60	RmM49a
R. maidis	11	49	4.56	RmM49b
R. maidis	12	56	4.70	RmM56

[a] As indicated on Fig. 3.

[b] Virus-binding proteins that bound specifically to purified MAV. All other proteins were detected only by MAV4-anti-ID binding.

[c] Protein SaM63 is a GroEL chaperonin protein identified by immunoblotting with anti-GroEL antiserum (Epicentre Technologies, Madison, WI).

these two proteins, other proteins that were not resolved along the sample loading site also showed high affinity to MAV virions (Fig. 3A). This result was independently replicated three times.

MAV4 anti-ID identified eight major spots from *S. avenae*

The MAV4 anti-ID that mimics an epitope on MAV virions bound to eight major proteins immobilized on nitrocellulose membrane (Table 1 and Fig. 3B). Among these eight were SaM50 and SaM35, which also bound to MAV virions. Reactions between aphid head proteins and MAV4 anti-ID were independently replicated six times with similar results.

MAV and MAV4 anti-ID overlay assays in the nonvector *R. maidis*

To confirm that binding between MAV virions and SaM50 and SaM35 was vector-specific, MAV overlay assays were performed on blots containing separated proteins from head extract of *R. maidis,* an aphid species that does not transmit MAV. No obvious reactions between MAV virions and the *R. maidis* aphid proteins (Fig. 3C) were observed under the same reaction conditions as used for *S. avenae* (Fig. 3A). This result was indepen-

dently replicated twice. Immunoblots of *R. maidis* head proteins utilizing MAV4 anti-ID did not detect proteins of 50 or 35 kDa, further confirming the lack of proteins similar to the MAV-binding SaM50 and SaM35 in the nonvector aphids. However, four proteins binding only to MAV 4 anti-ID were detected in *R. maidis* (Fig. 3D). One of these proteins, RmM49b (Fig. 3D, protein 11), was identical in molecular weight (49 kDa) and isoelectric point (4.56) to a similar anti-idiotype-binding protein identified in *S. avenae,* SaM49 (Fig. 3B, protein 3). Note that proteins in the gel for blot D (Fig. 3) were loaded at 24 μg/lane, compared to 6 μg/lane in blot B. This difference in protein loading concentration was confirmed on Coomassie blue-stained gels (not shown). Therefore, protein RmM49b appears more intense than SaM49, but it is likely that both proteins occur at similar concentrations in both aphid species. No reaction between these two proteins and purified MAV was apparent on MAV overlay assays (Figs. 3A and 3C).

DISCUSSION

Transmission by aphids is one of the most conserved features of viruses in the *Luteoviridae* (Herrbach, 1999). Luteoviruses have a very high level of vector specificity and each luteovirus is efficiently transmitted by only one or a few aphid species. For instance, the BYDV-MAV isolate used in this study is transmitted efficiently by *S. avenae,* but not by *R. padi.* However, *R. padi* does transmit BYDV-PAV, a luteovirus serologically closely related to MAV. Evidence suggests an intimate association between a luteovirus and its vector in which interactions on cell membranes or basal lamina of specific tissues in the aphid regulate virus transmission (Gildow, 1999). Gildow and Rochow (1980a,b) have demonstrated the importance of viral capsid proteins in vector transmission of barley yellow dwarf virus. Transmission interference of PAV in the presence of MAV was thought to result from receptor-binding competition between these two closely serologically related luteoviruses when both viruses occurred simultaneously in *S. avenae.* Interference of PAV transmission in the presence of MAV ceased when MAV was treated by UV irradiation that serologically altered the capsid of MAV virions. Based on this observation, it was hypothesized that MAV and PAV shared common receptors for transmission and that MAV binding affinity was reduced in structurally altered particles. Genetic studies imply that the readthrough protein encoded by open reading frame 5 (ORF5) of *Luteoviridae* is involved in the transmission process. Infectious clones of beet western yellows luteovirus lacking portions of ORF5 were infectious by agroinoculation, but were not aphid transmissible (Brault *et al.,* 1995). Similarly, PAV transcripts lacking the central portion of the readthrough protein could not be transmitted by aphids (Chay *et al.,* 1996). Amino acid substitutions at only 2 sites in the C-terminal region of the readthrough protein of PLRV-V

was found to reduce aphid transmissibility (Jolly and Mayo, 1994). A naturally occurring PLRV isolate (PLRV 14.2), with a very low ability to be aphid transmitted, was found to differ in amino acid sequence at 2 sites in the coat protein and 11 sites in the readthrough structural protein compared to efficiently transmitted PLRV isolates (Rouze-Jouan *et al.,* 2001). These amino acid substitutions reduced the ability of PLRV 14.2 to be acquired through the aphid gut during feeding, but did not affect the ability of the virus to move through the salivary glands when injected into vector aphids. Other results suggest that the major coat protein component of PLRV particles encoded by ORF3 may carry determinants that interact with cellular receptors in aphids to allow transmission (Gildow *et al.,* 2000b). The potato leafroll virus-like particles produced in a baculovirus–insect cell system and lacking the P5 readthrough protein were acquired through the midgut and passed through the ASG of *Myzus persicae.* The molecular details on virus recognition sites and on the role of coat protein and readthrough proteins have yet to be determined. Although multiple domains of both the coat protein and the readthrough protein of *Luteoviridae* appear to be involved in virus transmission, little is known about aphid contributions to transmission specificity. Knowledge of the aphid components involved in the virus–aphid interactions that determine virus transmission phenotype is derived mainly from ultrastructural observations (Gildow, 1999). To be transmitted, luteoviruses must first be acquired through the lining of the gut and circulate in the aphid hemocoel. Acquired luteoviruses then may accumulate at the ASG where virus-specific selectivity occurs to regulate transmission. Only transmissible luteoviruses are able to penetrate through the ASG basal plasma membrane and be transported to the salivary duct. After penetrating the ASG basal lamina, the luteovirus is concentrated in basal plasma membrane invaginations (coated pits) assumed to be associated with receptor-mediated endocytosis. Virions are then packaged in tubular vesicles which generate virus-containing coated transport vesicles. Finally, the virions are exocytosed through the apical plasma membrane and released into the canal lumen (Gildow and Gray, 1993). We hypothesize that virus-binding proteins at each of these locations may mediate the transmission of specific luteovirus, thus facilitating transmission. However, some of these virus-binding proteins may bind and transport luteoviruses indiscriminately by recognizing virion recognition sites common to a range of related luteoviruses, while possibly only one or a few virus-binding proteins might recognize more specific recognition sites common to only one or two luteoviruses and thus determine vector specificity.

We have identified two proteins (SaM35 and SaM50) in the vector aphid, *S. avenae,* that bind specifically to purified MAV particles and also bind to an anti-idiotypic antibody that may mimic a receptor-binding domain on

the MAV virion associated with the ASG. The facts that these two proteins are detected only in head extracts from vector aphids and not from the nonvector aphids and that they are not GroEL homologues suggested that they may be potential receptors for MAV virions associated with the ASG. Similar results were reported by Wang and Zhou (personal communication and the 7th International Congress of Plant Pathology, Edinburgh, UK, Abstr. 1.13.16), who described aphid proteins from two vector species, *S. avenae* and *Schizaphis graminum,* that could immobilize purified luteoviruses in virus overlay assays. Two proteins (31 and 50 kDa, respectively) from both *S. avenae* and *Sc. graminum* displayed strong affinity to the BYDV-GAV isolate. The GAV isolate studied shares 97.5% coat protein nucleotide sequence similarity with the MAV isolate used in our research (personal communication). Based on the similarity of coat proteins of both viruses, it is possible that the 50-kDa proteins in both studies are similar or identical proteins that interact with the same domain on both viruses.

Conservation of the 49-kDa MAV4 anti-ID-binding protein in both vector (SaM49) and nonvector (RmM49) aphids was not surprising. Although *R. maidis* does not vector MAV, it vectors other members of *Luteoviridae,* such as RMV, which share certain amino acid sequences with MAV (46% homology). Thus RmM49 and SaM49 might represent a class of nonspecific luteovirus-binding proteins that function in intracellular trafficking of luteoviruses.

A GroEL homologue (symbionin) has been hypothesized to protect PLRV from proteolytic degradation in aphid hemolymph (van den Heuvel *et al.,* 1997). However, this protein does not appear to determine transmission specificity based on the interactions between virions and a nonvector aphid GroEL homologue (Filichkin *et al.,* 1997). In our study, one of the two GroEL proteins reacted with MAV4 anti-ID, whereas neither of them reacted with purified MAV virions. We assume the difference between our results and those of van den Heuvel *et al.* may result from: (1) lower affinity between purified MAV virions and electrophoretically isolated GroEL protein or (2) a very limited amount of GroEL protein on the blots resulting from the dissection technique and tissue isolation method used in our study. Dissection of aphid head tissue in buffer for protein extraction would reduce the occurrence of proteins normally suspended in the hemolymph, such as GroEL, and would minimize the possibility of recovering gut proteins associated with BYDV acquisition or GroEL proteins localized in mycetocytes in the abdomen.

In addition to GroEL, four other proteins with relative MW of 84, 78, 50, and 49 kDa, from *M. persicae* have been reported to bind to PLRV in virus overlay assays (van den Heuvel *et al.,* 1994). It is interesting that three of the four *M. persicae* proteins have molecular weights similar to those of proteins identified in *S. avenae* in this study. Unfortunately, we cannot compare the p*I's* of these

proteins to our results (Table 1) because Van den Heuvel *et al.* used urea denaturing IEF gels and we used native IEF gels. Therefore, the calculated p*I's* are not directly comparable. It is interesting to note that the anti-idiotypic antibody AiAB#5 did bind to the GroEL homologue from *M. persicae* in the van den Heuvel study, but that none of the four non-GroEL PLRV-binding proteins from *M. persicae* reacted with the AiAB#5. The AiAB#5 is an anti-idiotype made against the monoclonal AiAB#5, which specifically inhibited PLRV transmission by *M. persicae.* In our study, MAV4 anti-ID also bound to one GroEL homologue, to the two MAV-binding proteins, and to several other aphid proteins not detected by MAV binding. These results indicate that several different luteovirus-binding proteins are probably involved in movement of viruses through aphid tissues.

In summary, two MAV-binding proteins were identified as potential vector-specific receptors based on the fact that they occurred only in the vector species and not in the nonvector aphid, and both had high affinity for MAV virions and an anti-idiotypic antibody mimicking an epitope of MAV virions. The work reported here is another step toward identifying virus-binding proteins from aphids that are candidates for vector-specific receptors for members of *Luteoviridae.* This information and additional studies on this luteovirus–vector system will help us to understand the cellular mechanisms controlling virus transmission and the evolution of *Luteoviridae* and their vector aphids.

MATERIALS AND METHODS

Aphid species and virus isolates

Virus-free New York biotypes of *S. avenae* (Fabricus) and *R. maidis* (Fitch) (Hemiptera, Aphididae) were maintained as previously described (Rochow, 1969). The New York BYDV-MAV isolate was maintained as described by Peiffer *et al.* (1997) and purified as described by Webby and Lister (1992). The identity of purified virus was checked by double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) (Rochow and Carmichael, 1979) and immunosorbent electron microscopy (Roberts, 1986).

Preimmune serum, MAV4 monoclonal antibody, MAV4 anti-ID, and anti-GroEL antibody

Production of MAV4 monoclonal antibody was described by Hsu *et al.* (1984). Anti-ID antibody to MAb MAV4 was produced in New Zealand White rabbits. The rabbit received an initial injection of 100 μg IgG in Freud's complete adjuvant followed by two booster injections of 250 μg IgG in Freud's incomplete adjuvant 4 and 6 weeks later. A test bleed was done at the time of the final booster and 4 weeks later. The rabbit was exsanguinated 16 weeks after the initial injection. The immunoglobulin fraction was obtained by ammonium

TABLE 2

Treatments for Competitive Binding Assays Comparing the Ability of Purified Virions of MAV and of Purified MAV4 Anti-idiotypic Antibody IgG to Bind to Microtiter Wells Coated with Polyclonal (Treatments 1 to 6) or Monoclonal (Treatments 7 to 12) Anti-MAV IgG in Double Antibody Sandwich Enzyme-Linked Immunosorbent Assay Tests

Treatment	Description[a]	Function
1 and 7	MAV only	Positive control
2 and 8	Preimmune serum → MAV	Test for nonspecific binding inhibition or steric hindrance
3 and 9	MAV 4 anti-ID → MAV	Test for competitive binding
4 and 10	Preimmune serum + MAV simultaneously	Test for nonspecific binding inhibition or steric hindrance
5 and 11	MAV4 anti-ID + MAV simultaneously	Test for competitive binding
6 and 12	MAV4 anti-ID only	Negative control

[a] Concentration of MAV virions was 50 ng/well, preimmune serum was 100 μg/well, and MAV4 anti-ID was 100 μg/well, in all treatments where used.

sulfate precipitation and cross absorbed twice with normal mouse serum and mouse IgG, and the remaining IgG fraction was purified using a MAPII kit (Bio-Rad, Hercules, CA) as per the manufacturer's instructions. Preimmune serum was collected from the rabbit just prior to the initial injection of MAV4 IgG. Anti-GroEL polyclonal antibodies were purchased from Epicentre Technologies.

Competition between MAV virions and MAV4 anti-ID

Treatments to test for competitive binding or binding interference between MAV and MAV4 anti-ID antibody on anti-MAV IgG-coated microtiter plates are listed in Table 2. Microtiter plates (Nalge Nunc International, Naperville, IL) were coated with MAV4 MAb (1:100) or MAV-specific polyclonal antibodies (1:100), incubated at 4°C overnight, and washed three times in phosphate-buffered saline (PBS) containing 0.05% Tween 20. Treatments 1, 2, 4, and 6 were incubated for 1 h at room temperature. Preimmune serum (Treatment 3) and MAV4 anti-ID (Treatment 5) were loaded and incubated 1 h before rinsing and loading MAV into the microtiter plate for a 1-h incubation. MAV virions in all treatments received the same 1-h incubation time with the coating antibody. The binding between coating antibodies and MAV virions was detected by alkaline phosphate-conjugated polyclonal antibodies specific to MAV virions. Reaction between p-nitrophenyl phosphate (Sigma, St. Louis, MO) and alkaline phosphatase was monitored at 405 nm at 10-min intervals. The data were statistically analyzed by ANOVA and Tukey's pairwise comparison method in Statview 5.0 (SAS Institute, Inc., Cary, NC).

Sample preparation

Live aphids were collected and frozen at −80°C. To isolate head tissues for protein extraction, frozen aphids were submerged in Grace's medium (Cat. No. 117-048-100; Quality Biological, Inc., Gaithersburg, MD) containing 1 mM ethylene diaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzimide hy-

drochloride, and a protease inhibitor cocktail (1 mM each pepstatin A, chymostatin, antipain, aprotinin, and leupeptin), and the heads were removed with a sterile surgical blade. During the dissection process, the aphid heads were stored in microcentrifuge tubes on ice. The aphid heads were freeze/thawed at −80°C five times to disrupt cell membranes and then homogenized on ice in 36 mM Tris–HCl (pH 8.4) extraction buffer containing 0.2 mM PMSF and protease inhibitor cocktail using a Teflon pestle. The homogenate was centrifuged at 14,000 g for 3 min and the protein concentration in the homogenate was quantified using a BCA kit (Pierce, Rockford, IL) as described by the manufacturer using bovine serum albumin (BSA) as the standard.

Gel overlay assays

Aphid proteins were separated on a pH 4–6 native IEF gel made of 3.25 g acrylamide, 0.25 g bis-acrylamide, 3.00 ml Ampholine ampholytes, pH 4–6.5 (LKB–Pharmacia, Uppsala, Sweden), 0.3 ml Ampholine ampholytes pH 3.5–10 (LKB–Pharmacia), and 59 ml Milli-Q water. A 12-μl aliquot of *S. avenae* protein containing either 24 μg (or 100 aphid heads)/lane for virus overlay assays or 6 μg (or 25 aphid heads)/lane for immunoblots was loaded onto the center of a slab IEF gel. For *R. maidis* protein, 24 μg (or 100 aphid heads)/lane was loaded on each gel. After completion of isoelectric focusing, the pH distribution on the IEF gel was measured as described in Rosenberg (1996). The IEF gel lanes were separated and equilibrated in buffer for a total of 40 min as described by Cox and Willis (1987) and stored dry separately at −20°C in sealed sterile 15-ml plastic tubes.

Urea SDS 2-D electrophoretic gels were prepared and run according to Cox and Willis (1987). Briefly, the resolving gel contained 6.4 M urea, 11% acrylamide, 0.003% SDS, 0.675 M Tris–HCl (pH 8.65); the stacking gel contained 8 M urea, 3% acrylamide, 0.125 M Tris–HCl (pH 6.8). Molecular weight standards (broad range SDS–PAGE standard and biotinylated SDS–PAGE standard; Bio-Rad) were loaded on both sides of the gel. After

completion of 2-D gel electrophoresis, the proteins were either electrophoretically blotted onto 0.1-μm nitrocellulose membrane (Schleicher & Schuell, Keene, NH) or stained with Coomassie Brilliant Blue. Stained gels were enhanced using the Gelcode Silver Stain Kit (Pierce) according to the manufacturer's protocol. Gels used in blotting experiments were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol) for 15 min without shaking. The aphid proteins were then electrophoretically transferred onto nitrocellulose membranes at 4°C, at 110 V for 20 h (Towbin, 1979). After being fixed in 0.1% glutaraldehyde for 15 min at room temperature without shaking, the blots were rinsed in 0.01 M PBS (pH 7.4) containing 0.5% fish gelatin and 0.05% Tween 20 (PBSTG). To further reduce nonspecific reaction, the blots were incubated overnight in solution containing 5% nonfat milk, 2% normal goat serum, 5% fish gelatin, and 1% Tween 20, in ELISA sample buffer (2% polyvinyl pyrrolidone-10, 0.2% BSA, 0.05% Tween 20, 0.01% sodium azide, in 0.01 M PBS (pH 7.4)). The blots were then rinsed once in PBSTG and further blocked in avidin/biotin blocking solution (Vector Laboratories, Burlingame, CA) prepared in PBSTG according to the manufacturer's instructions. After three rinses in PBSTG (pH 7.0 for virus overlay assays or 7.4 for immunoblots), the blots were reacted with purified MAV virions (10 μg/ml) in 20 ml diluted blocking solution (1.67% nonfat milk, 0.67% normal goat serum, 1.67% fish gelatin, and 0.33% Tween 20, in ELISA sample buffer (pH 7.0)) for 3 h or reacted with preimmune serum or MAV4 anti-ID (0.1 μg/ml) or anti-GroEL antibody (1:2000) in 20 ml PBSTG (pH 7.4) for 1 h. After three rinses in PBSTG (pH 7.0), the virus overlay assay blots were incubated at room temperature for 1 h with anti-MAV polyclonal antibodies (0.125 μg/ml) in 20 ml PBSTG (pH 7.0), whereas the immunoblots were directly reacted with biotinylated secondary antibodies. After three rinses in PBSTG (pH 7.4), the virus overlay assay blots were also reacted with biotinylated anti-rabbit antibody (Vector Laboratories) in PBSTG (pH 7.4) for 1 h. After three rinses in PBSTG (pH 7.4), the blots were reacted with ABC reagents containing avidin and biotinylated horseradish peroxidase to amplify the reactions (Vector Laboratories) according to the manufacturer's suggestion. After reaction with the ABC reagents, the blots were then rinsed three times in PBS/2% fish gelatin (pH 7.4) followed by a quick rinse in 0.1 M Tris–HCl (pH 7.6). The final reaction used DAB peroxidase substrate (Sigma, Cat. No. D4418) enhanced by NiCl$_2$ as described by the manufacturer. The blots were rinsed twice with Milli-Q water to stop the reaction. The blots were then scanned and photographed.

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