Molecular interactions between Pea enation mosaic virus and its pea aphid vector

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Molecular interactions between *Pea enation mosaic virus* and its pea aphid vector

by

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ABSTRACT

Insect transmission of plant viruses results in tremendous economic loss within the agricultural sector worldwide. Aphids account for nearly half of insect-borne plant virus transmission. Viruses in the family Luteoviridae are transmitted by aphids in a persistent-circulative manner that requires specific molecular interactions between the aphid and virus. Ingested virions cross the aphid gut and salivary gland epithelial barriers using receptors that have not been identified. We assessed the binding of a model luteovirid, Pea enation mosaic virus (PEMV), to brush border membrane vesicles (BBMV) of the pea aphid, Acyrthosiphon pisum using a two-dimensional far-western blot method. Pea aphid membrane alanyl aminopeptidase N (APN) was identified by mass spectrometry following specific binding to PEMV virions and to a PEMV coat protein-eGFP fusion peptide (CP-P-eGFP). The binding of PEMV to APN was confirmed by multiple methods including a pull-down assay, surface plasmon resonance (SPR) analysis, and by increased binding of CP-P-eGFP to baculovirus-expressed pea aphid APN in Sf9 cells. We also show that a peptide (GBP3.1) that was previously shown to impede uptake of PEMV into the pea aphid also binds to APN. Based on these results, we conclude that APN is a putative gut receptor for PEMV in the pea aphid and if confirmed would be the first insect receptor identified for a plant virus. Interestingly, PEMV appears to bind to a different, as yet unidentified, receptor in a second vector, Myzus persicae, suggesting that different gut receptors may be used by luteoviruses in different vector species.

Luteoviruses are acquired when aphids ingest the phloem sap of an infected plant. Phloem proteins have been shown to associate with luteovirus particles and facilitate aphid transmission in in vitro feeding assays. We showed an increase of virus in the hemocoel of
aphids fed on artificial diet containing purified PEMV with bovine serum albumin (BSA) compared to aphids fed on virus in the absence of BSA. Interestingly, BSA reduced the amount of a mutant virus lacking the minor structural protein readthrough domain (RTD) detected in the aphid hemocoel. We also demonstrated that the PEMV RTD binds to multiple aphid proteins. SPR analysis indicated that the CP and RTD both bind to BSA. Based on these data, models are presented to account for the role of the RTD and mechanism by which BSA and plant proteins facilitate virus entry into the aphid hemocoel.

Little is known about the role of glycans in mediating luteovirus-aphid interactions. We used the lectins Concanavalin A (ConA) and *Galanthus nivalis* agglutinin (GNA) for lectin blot analysis of BBMV and confirmed that pea aphid proteins are glycosylated with mannose and glucose moieties. APN, the PEMV gut receptor, is glycosylated with mannose residues. However, we did not detect any binding of PEMV to a synthesized tri-mannose glycan that is common in insects using both isothermal titration calorimetry or a carbohydrate microarray. These results suggest that mannose by itself is not involved in PEMV-APN binding. ConA bound to PEMV indicating that viral structural proteins are glycosylated. The potential role of virus glycosylation in aphid transmission of luteoviruses is discussed. Taken together, our increased understanding of luteovirus-aphid vector interaction will facilitate research into other plant virus-insect vector systems, and the development of mitigation strategies.
CHAPTER 1

Introduction

Aphids as crop pests

Aphids can inhabit up to 25% of plant species in temperate climates and are responsible for widespread crop loss worldwide. These insects cause plant damage in multiple ways including i) extraction of essential nutrients from the plant phloem, ii) injection of potentially toxic saliva, iii) transmission of 275 of the 600 insect-borne plant viruses, iv) and production of honeydew leading to growth of sooty molds that can inhibit photosynthesis (1). Aphids are so pervasive that estimating agricultural losses caused by aphids is difficult. Outbreaks are hard to predict with large variation in aphid populations from year to year. Aphids are estimated to cause yield losses of 7% in tomato, 22% in potatoes, 27% in cotton and 100% in rapeseed in the absence of control measures (2).

Estimated economic losses from aphid transmitted viruses include sugar beet yields in Europe reduced up to 49% by aphid-transmitted Beet yellows virus (BYV) and Beet mild yellowing virus (BMVY) (3). Barley yellow dwarf virus (BYDV) is transmitted by multiple aphid species and is the most important and widespread viral disease of cereal crops worldwide. In the U.S., yield losses of up to 5% are estimated from BYDV each year (4).

*Aphid transmission of plant viruses.* Aphids are efficient vectors of plant viruses, capable of transmitting 275 of the 600 insect-borne plant viruses that have been described to date (5). Aphids and other hemipteran insects including whiteflies, leafhoppers, and planthoppers, have modified mouthparts called stylets designed for feeding on either the phloem, xylem, or mesophyll cells or a combination of tissues. The aphid stylets are capable of navigating
intercellularly through the layers of plant cells and extracting phloem sap (6, 7). Plant viruses can be acquired during brief sampling of plant tissues or from prolonged feeding on the phloem sap (8). Plant virus transmission by insects is categorized as “nonpersistent”, “semipersistent”, and “persistent” based on virus acquisition and retention time in the aphid (9). Nonpersistent or “stylet-borne” viruses have a brief acquisition period (seconds to minutes) and are retained in the insects mouthparts for a short time, typically minutes (10). Semipersistent viruses have slightly longer retention times (hours) and are proposed to associate with the foregut and thus referred to as “foregut-borne” (11). However, *Cauliflower mosaic virus* (CaMV), originally classified as semipersistent, has been shown to bind to the tip of the aphid stylet (12) suggesting the distinction between nonpersistent and semipersistent viruses may not be as clear cut (13). Nonpersistent and semipersistent viruses are lost upon insect molting when the cuticle (and associated plant viruses) is shed and both groups are categorized as noncirculative. Persistent viruses are also referred to as “circulative” and have a retention time in the vector of days to weeks (10). Virions are taken up into the insect hemocoel (body cavity) and therefore are not lost during molting. Persistent-circulative viruses are further classified as “propagative” or “nonpropagative” relating to whether replication of the virus occurs in the vector. Propagative transmission is more common for animal viruses, with few examples for plant viruses (10).

*Noncirculative transmission.* Noncirculatively transmitted plant viruses bind to aphid receptors in the chitin-lined stylet (nonpersistent) or foregut (semipersistent) and do not enter the aphid hemocoel. There are two mechanisms for virion attachment to aphid stylets: the “capsid” and “helper” strategies (13). *Cucumber mosaic virus* (CMV, *Cucumovirus*, *Bromoviridae*) is the best studied for the capsid strategy. CMV has a broad host range
reportedly infecting 1,241 species within 101 plant families (14). In vitro experiments involving recombination of CMV RNAs with coat proteins (CP) of different virus isolates demonstrated that the CP alone was the sole determinant of transmission efficiency (15-17).

In contrast to the capsid strategy, the helper strategy requires an extra virus encoded “helper component” (HC) that is separate from the virion that forms a link between the virus and the aphid cuticle (18). This phenomenon was realized by the observation that virions lost transmissibility after purification (19, 20), suggesting the capsid alone was not sufficient for transmission. The most well known viruses using this “bridge” strategy are from the genera *Potyvirus* and *Caulimovirus*. Potyviruses are filamentous particles that encode a positive sense RNA of about 10 kb (21). The genome encodes a polyprotein that is cleaved into multiple products including the helper component called HC-Pro because of its autocatalytic protease activity (13). Along with being required for aphid transmission, HC-Pro plays a role in systemic movement in plants, replication of the genome, and suppressing gene silencing (22, 23). The domains of HC-Pro involved in forming the bridge between the virus and aphid cuticle have been determined (24).

The helper strategy for *Caulimoviruses* differs slightly compared to the *Potyviruses*. The type member for the *Caulimovirus* genus is CaMV which forms isometric particles and has a circular double stranded DNA genome. CaMV requires two viral encoded proteins that must interact to form the bridge between the virus particle and aphid stylet. The first protein, P3, interacts with the virion and the second protein, P2, connects the virion-P3 complex to the receptor on the aphid stylet (25). This method differs from the *Potyviruses* but still fits with the bridge hypothesis. In the infected plant the P2 and P3 proteins are separated in inclusion bodies and are acquired in a sequential fashion. The vector first acquires the P2
protein which binds to the receptor in the aphid stylet. P3 undergoes a confirmation change upon binding virions, which allows for interaction with P2 (25, 26). The advantage of this type of helper strategy is not well understood (13).

*Circulative transmission.* Circulative viruses are taken up into the body cavity of the insect vector. Ingested virions pass from the gut lumen into the hemolymph and eventually enter the salivary glands from which virions are secreted with the saliva when the aphid feeds (10, 27). Circulative viruses have longer acquisition and inoculation access periods, but virions will remain in the vector for the duration of the insect’s life and are not lost during molting (10). Although a majority of the circulative plant viruses are nonpropagative, there are few enveloped viruses from the family *Rhabdoviridae* that can replicate in their aphid vectors (13). In addition to rhabdoviruses, leafhoppers and planthoppers can also transmit propagative viruses from the *Reoviridae* and *Tymoviridae* (10). Rhabdoviruses acquired from an infected plant replicate first in gut cells before spreading to multiple insect organs (28). Rhabdoviruses can infect the reproductive organs and a low level of transovarial transmission has been observed (29). To be introduced with saliva into healthy plants virions transmitted in a propagative manner must travel through the hemocoel and enter the principal salivary glands (PSG). This is in contrast to nonpropagative viruses that enter the assessory salivary glands (ASG). Virions can infect the nervous tissue which may provide an additional route for virions to reach the PSG, although this has not been conclusively shown in aphids (13, 30).

**Luteoviridae**

Viruses in the *Luteoviridae* have single stranded, positive sense RNA genomes of 5.5 to 6 Kb and form iscosahedral particles of 25-30 nm in diameter (27). The first records of
plants with symptoms resulting from luteovirus infection go back to the end of the 18th century, however luteoviruses were first recognized as a group in 1976 (31). The Luteoviridae is comprised of three genera, Luteovirus, Polerovirus, and Enamovirus categorized primarily on genome characteristics (32). Luteoviruses share common properties including: transmission by one or a few aphid species in a persistent-nonpropagative manner, multiplication in the phloem of the host plant, and characteristic symptoms of plant infection including yellowing, reddening, and/or rolling of infected leaf tissue (31).

**Genome organization and gene expression.** The 5’ genome organization differs between the genera Luteovirus and Polerovirus (Figure 1). Pea enation mosaic virus is the sole member of the genus Enamovirus and resembles poleroviruses. Viruses of the genus Luteovirus have two 5’ open reading frames (ORFs) whereas species of the genus Polerovirus have 3 ORFs. ORFs 1 and 2 overlap significantly for Poleroviruses, but only by a few base pairs for the Luteoviruses. ORFs 3, 4 and 5 are similar among Luteoviridae, but the genus Luteovirus contains a 6th ORF (33). The genus Luteovirus lacks a 5’ cap or genome linked protein (VPg). There is a VPg at the 5’ end of the genome in the genera Polerovirus and Enamovirus (34). The VPg is thought to be involved in genome replication (34). ORFs 0, 1, and 2 are expressed by translation of the genomic RNA by a cap independent translation mechanism. (34). ORF 2 is translated by a ribosome frameshift resulting in a gene product which is a fusion of ORFs 1 and 2 (33). The function of the product of ORF 0 (P0) is not fully understood but has been shown to act as a suppressor of host plant RNA silencing for some poleroviruses and for the Enamovirus Pea enation mosaic virus (35, 36). P1 contains the VPg in the case of poleroviruses which is excised by proteolytic cleavage. P2 functions as the RNA-dependent RNA polymerase (RDRP). ORFs 3-5 are translated from subgenomic
RNA generated from a negative strand intermediate (34). P3 is the major coat protein (CP) and P5 is the minor coat protein translated by in-frame readthrough of the ORF 3 stop codon. Although highly variable, it is estimated that 1-5% of the translated CP contains the RTD (37). The P4 protein is the predicted movement protein required for systemic movement of the virus in plants (38).

Figure 1. Genome organization of Barley yellow dwarf virus (BYDV: Luteovirus), Potato leafroll virus (PLRV: Polerovirus), and PEMV-1 (Enamovirus). PEMV-1 resembles Polerovirus, but lacks the P4 movement protein (MP). POL, polymerase; CP, coat protein; RTD, readthrough domain expressed by translational suppression of CP stop codon; VPg, genome-linked protein; P, proteinase motif; V, VPg coding region.

Pea enation mosaic virus. The systemic infection of plants by Pea enation mosaic virus requires the presence of two virus species, PEMV-1 and PEMV-2. PEMV-1 is the sole member of the genus Enamovirus with genome organization similar to that of the Poleroviruses, while PEMV-2 belongs to the genus Umbravirus (39). Both genomes are encapsidated separately by structural proteins encoded by PEMV-1. While only positive sense RNA was thought to be encapsidated, we detected negative strand PEMV1 at low levels in purified virus preparations (Appendix 1). PEMV-2 is translated in a similar fashion to PEMV-1, with ORFs 3 and 4 translated from subgenomic RNA. ORF 4 of PEMV-2
encodes a putative movement protein, which is lacking in PEMV-1. This protein is necessary for systemic infection in plants (40). Hence, PEMV-1 requires PEMV-2 for movement in the plant, while PEMV-2 requires PEMV-1 for encapsidation. Both RNAs can replicate independently of each other in the plant (41). As a result of its association with PEMV-2, PEMV-1 is the only luteovirus that is not phloem limited, and can be mechanically transmitted to plants. Interestingly, co-inoculation of Potato leaf roll virus (PLRV) with an umbravirus such as PEMV-2 can result in systemic infection with PLRV (42).

Virus infection of the host plant. With the exception of PEMV, luteoviruses must be introduced into the phloem of the plant either by aphids, by agroinoculation, or by grafting (36, 43, 44). The plant phloem consists of parenchyma and companion cells that border the sieve elements (SE). Mature SE lack a nucleus and translational machinery and rely on a close association with the companion cells for normal cellular functions (45). Connecting sieve elements form sieve tubes through which the phloem sap containing sugars, amino acids, nucleic acids, and protein, moves over long distances in the plant (46-48). Luteoviruses replicate in the phloem parenchyma and companion cells, move through branched plasmodesmata to the SE (49, 50), and travel in the sieve tubes over long distances in the plant (51). Although luteoviruses can replicate in epidermal or mesophyll cells, virions will not spread systemically from these tissues (52). Luteoviruses are thought to move as intact virions throughout the plant (53). PEMV is an exception as it can move from epidermal or mesophyll cells to the vascular tissue and does not require intact CP for systemic movement of the virus (39, 54). This is likely attributed to the movement protein provided by co-infection with the umbravirus, PEMV-2 (54).
In the plant, the RTD is involved in systemic movement and accumulation of virus in infected tissues and also may be responsible for the restriction of luteovirus replication to the phloem (53, 55, 56). Mutations to the C-terminal region of PLRV RTD allows for infection of multiple cell types. Interestingly, the RTD of PEMV is truncated in comparison to that of other luteoviruses and coincidently PEMV is the only luteovirid that is not phloem limited (57). The restriction and concentration of luteoviruses to the phloem is favorable for transmission by phloem-feeding aphids. Another proposed explanation for phloem limitation is that luteoviruses are unable to suppress plant defenses in tissues other than the phloem (36, 58). PEMV replication occurs in double membrane bound replication complexes associated with the nucleus of the cell (57).

*Aphid transmission.* Aphids are the sole vectors of luteoviruses. Aphids feed using piercing, sucking mouthparts to probe intercellularly through the epidermis and mesophyll cells before eventually penetrating the phloem sieve elements and companion cells. The aphid digestive tract consists of four different parts: a chitin lined foregut, the stomach or anterior midgut, the posterior midgut, and the hindgut (27). Ingested virions are recognized by specific receptors in either the midgut or hindgut depending on the virus (59). Viruses in the genus *Luteovirus* tend to be acquired through the hindgut, whereas viruses in *Polerovirus* genus use either the mid- or hindgut, and PEMV uses the midgut (59). The site of uptake for each virus is based on visualizing virions in aphid gut tissues by transmission electron microscopy (TEM). Regardless of the site of acquisition, virions rely on clathrin-mediated endocytosis and cross epithelial cells in tubular vesicles (60). After release into the hemocoel virions diffuse through the hemolymph. Some of the virions then undergo another receptor-mediated endocytosis event at the assessor salivary glands (ASG). To enter the lumen of the ASG
virions must cross the basal lamina, the basal plasmalemma, and finally the apical plasmalemma, i.e. the opposite direction to virus uptake from the gut into the hemocoel. Luteovirids have never been observed in the principal salivary glands (60). Virions are transported across the cell in coated pits and are released into the salivary gland lumen. From here virions move with the salivary secretions and are introduced into the phloem of the plant when the aphid feeds.

*Luteovirus structural proteins.* The viral coat protein (CP, 22 kDa) and readthrough domain (RTD 33-55 kDa) are the sole determinants of the specificity of luteovirus transmission by aphid vectors (61). The CP is sufficient for virion formation and transcytosis of the aphid gut epithelium (38, 60, 62, 63). Icosahedral particles are formed by 180 copies of the CP and a minor amount of the RTD which protrudes from the surface of the virion (27). Both the CP and the RTD are required for aphid transmission (56, 64, 65) although the exact role of the RTD in the aphid vector is not fully understood. In the aphid, the RTD could determine transmission specificity through interactions with the ASG and also determine tropism for the mid- or hindgut (62, 66). Although not required for transcytosis of the gut epithelium, the RTD may enhance the efficiency of this process (62). The RTD appears to be necessary for recognition and transcytosis of the ASG (38, 62), although baculovirus-expressed particles of PLRV lacking the RTD were observed in the ASG by electron microscopy (67).

The crystal structure for a luteovirus coat protein has not been determined, but a model for the PLRV CP has been designed by comparison to the crystal structure of *Rice yellow mottle virus* (RYMV, genus *Sobemovirus*) (68). A CP epitope on the surface of the PLRV CP (68, 69) is involved in virion assembly, systemic movement, and aphid transmission (70). This epitope, the βG-βH loop, has the sequence ‘HDSSEDQ’ and is
predicted to be an acidic surface loop. There is a similar motif, ‘GPSSDCQ’, in the PEMV CP. Another PLRV surface loop, βB-βC, is conserved among luteoviruses (68, 69). Sequence conservation of these surface loops suggests that these regions could be important for virus interaction with aphid receptors.

*Role of plant proteins in aphid transmission.* Plant phloem proteins associated with ingested virions may be involved in aphid transmission of luteoviruses (71, 72). *Cucurbit aphid borne yellows virus* (CABYV, genus *Polerovirus*) binds plant phloem proteins and the addition of these proteins to virus solutions artificially fed to aphids increased virus transmission to host plants (71). The number of viral genomes detected in whole insects increased when these phloem proteins were added to the diet. Interestingly, the addition of some (but not all) non-phloem proteins including bovine serum albumin (BSA) and casein to artificial diet also increased aphid transmission, suggesting the observed effect was not specific to phloem proteins (71). In a separate study, treatment of *Cereal yellow dwarf virus* (CYDV, genus *Polerovirus*) virus particles with sodium sulfite to remove associated plant proteins prevented uptake of virions into the hemocoel of aphids fed on artificial diet (72). Also, treated virions were not transmitted by aphids when injected directly into the hemocoel suggesting the inability of virions to cross the ASG (72). These results for CYDV are contrary to the idea that the CP and CP-RTD are the sole determinants mediating interactions between virus and aphid receptors (27, 60).

*Plant virus receptors in insects.* Identification of receptors for plant viruses in the insect vector has not been successful. The receptor for CaMV was localized to the tip of the maxillary stylets using the CaMV P2 protein fused to green fluorescent protein (GFP) (12). The identity of this protein has not been determined, but the receptor is a non-glycosylated
protein embedded deep in the chitin-lining of the sytlet. The association of receptors with the chitin matrix hinders identification (12) and may explain the lack of progress in characterizing aphid receptors for nonpersistently transmitted viruses. The CaMV receptor is the first and currently only confirmation for the existence of specific receptors for a non-circulative plant virus.

Receptors for circulatively transmitted viruses in the insect vector have not been identified. However, several insect proteins that bind plant viruses in vitro have been described, although the biological relevance, if any of these interactions, is unclear (73-79). A common method used to identify vector proteins that bind virus particles in vitro is far-western blotting, also known as a virus overlay binding assay (73-75, 77). Two proteins, SaM35 and SaM50, from the head of the English grain aphid, *Sitobion avenae*, bound to *Barley yellow dwarf virus*-MAV (BYDV-MAV) particles in far-western blots. These proteins were immunolocalized to the ASG and are hypothesized to be receptors at the ASG (73). In the aphid *Myzus persicae*, Rack-1, GAPDH3, and actin were shown to bind to *Beet western yellows virus* (75). Rack-1 and GAPDH3, along with three ribosomal proteins from the small brown planthopper, (*Laodelphax striatellus*), also bind to *Rice stripe virus* (genus *Tenuivirus*) (77). *Rice stripe virus* is transmitted by the planthopper in a circulative-propagative fashion. Due to the location of these proteins in the cell, the authors suggest that Rack-1, GAPDH3, and actin function in endocytosis and intracellular transport, but are not actual virus receptors.

A 32 kDa membrane protein from the rice brown planthopper (*Nilaparvata lugens*) was bound by a viral spike protein of *Rice ragged stunt oryzavirus* (RRSV, family *Reoviridae*) (79). This viral spike protein also inhibits virus transmission when fed to the
planthopper prior to feeding on an infected plant suggesting the spike protein interacts with the receptor in the insect (79). Two proteins from a thrips vector, Frankliniella occidentalis, were shown to bind Tomato spotted wilt virus (TSWV, genus Tosposvirus). The first is a 50 kDa protein localized to the brush border gut membrane of thrips and shown to bind the viral glycoproteins (78). TSWV has two glycoproteins, G_N and G_C, on the surface of the virion that mediate virus interactions with the insect vector. Recombinant G_N reduces transmission of TSWV when fed to thrips suggesting that this glycoprotein is responsible for receptor binding (80). The amino acid sequence for this 50 kDa thrips protein has not been determined (81). An important factor in TSWV transmission is that thrips must acquire the virus during larval stages as transmission efficiency is reduced as the vector ages (81). The role of the 50 kDa thrips protein as a receptor is supported by the fact that the protein is more abundant in larvae than in adults (78). A second thrips protein of 94 kDa also binds TSWV (74). This protein was not found in the larval midgut and therefore is not a gut receptor but could be involved in virus movement through the insect. Although all of these proteins are capable of binding viruses under in vitro conditions, they may not be receptor proteins but rather may function in virion transport through their respective vectors.

Role of carbohydrates in insect-virus interactions. Animal viruses are known to use glycans in host cell recognition (82-84). In contrast to many animal viruses, plant viruses are primarily non-enveloped and the vast majority of non-enveloped viruses do not contain glycans (85). There is little known about the glycosylation of plant viruses. Although Potato virus X and the aphid transmitted Plum pox virus and Lettuce necrotic yellow virus have been reported to be glycosylated (86-89), the role of these sugars has not been determined. The structural protein sequences of luteoviruses contain predicted glycosylation sites that vary in
number and location (85). PEMV structural proteins contain four predicted N-glycosylation sites and four predicted O-glycosylation sites based on sequence analysis. *Turnip yellows virus* (TuYV, formerly named *Beet western yellows virus*) was reported to contain α-D-galactose residues on capsid proteins that when altered by N-glycosidase or α-D-galactosidase disrupted aphid transmission (90). There was no evidence that the structural proteins of TuYV and a related polerovirus, *Cucurbit aphid-borne yellows virus* (CABYV) are glycosylated and consequently argued against a role of virus glycosylation in aphid transmission (85). The role of virus glycosylation in other luteovirus-aphid systems is unknown.

*Aphid genetics and transmission.* Aphids within a population can differ in their ability to transmit luteoviruses and the transmission phenotype is a heritable trait that can be regulated by a major gene or group of associated genes (91, 92). Yang et al. (76) used a combined genetic and proteomic approach using Two-Dimensional DIGE comparisons of proteins from spring grain aphids, *Schizaphis graminum*, differing in their ability to transmit *Cereal yellow dwarf virus*-RPV. Luciferase and cyclophilin were both differentially expressed and confirmed to bind virions using co-immunoprecipitation assays (76). These proteins may function in endocytosis and trafficking of molecules in the cell (93, 94) which may relate to virion transport across epithelial barriers. Studies on luteovirus transmission-competent or -refractive populations of spring grain aphids demonstrated that different genes control the ability of viruses to cross the gut or ASG (91). Using a proteomics approach, Cilia et al. (95) identified proteins predicted to be involved at different transmission barriers based on differential protein expression between aphids with varying transmission phenotypes. Twelve proteins were predicted to be involved in the hindgut while six proteins were
predicted to be associated with the hemolymph or ASG (95). Comparing differential gene expression between competent and refractive aphid genotypes may provide clues as to what proteins are involved in aphid transmission, but interpretation of these results are complicated. Differential expression of a protein does not guarantee direct interaction with virions or ensure any involvement in transmission. Also, the ability of aphid proteins to bind virions does not directly correlate with a role in virus transmission. More work is needed to identify aphid proteins required for circulative transmission, particularly the receptors for virus recognition and movement across aphid gut and salivary gland cells.

*Insect receptors of animal viruses.* Arboviruses (arthropod-borne) are animal viruses transmitted primarily by mosquitoes, but also by biting flies, midges, or ticks (96). Arbovirus transmission is circulative-propagative and has similarities to the circulative transmission of plant viruses. When mosquitoes ingest a blood meal from an infected host, the virus must attach and penetrate the midgut epithelial cells. The virus replicates and escapes from midgut, disseminates in the hemocoel, and must infect the salivary glands in order to enter the saliva and be transmitted to the next host (97). Although more is known about entry of these viruses into vertebrate cells, information about recognition and uptake of arboviruses into insect cells is increasing (97). For Sindbis virus (genus *Alphavirus*), a natural resistance-associated macrophage protein is required for infection of both insect and mammalian cells (98). Dengue virus (DENV, genus *Flavivirus*) is considered the most important viral disease transmitted by mosquitoes worldwide (99). Multiple proteins from mosquito or mosquito cells lines have been shown to bind DENV using far-western blot methods (97), but only prohibitin has been well characterized as a receptor required for entry of DENV into mosquito cells (100, 101). Prohibitin is a highly conserved protein ubiquitously expressed in
eukaryotes with multiple functions in the cell (97, 101). Interestingly, prohibitin was also shown to be the receptor for Chikungunya virus (CHIK, genus Alphavirus) in mammalian cells (102). Whether prohibitin is the receptor for CHIKV in mosquitoes is unknown, although some uncharacterized mosquito proteins have been shown to bind CHIKV (103). Comparing insect receptor proteins for plant and animal viruses may not be relevant given the differences between the viruses: Plant viruses are predominantly nonenveloped and lack replication in the insect vector, whereas animal viruses are usually enveloped and replicate in both the insect and mammalian hosts. In addition, mechanisms for uptake and intracellular transport of these viruses through insects may differ, particularly with respect to endocytosis into versus transcytosis across the insect gut epithelium.

**Aminopeptidase N.** In this thesis, we demonstrate that aminopeptidase N (APN) is the receptor for PEMV in the pea aphid vector (Chapter 2). APN is an exopeptidase found in insect guts from multiple insect orders (104). APN is primarily membrane associated (105) and is involved in digestion of proteins in the insect gut by cleaving neutral amino acids from the N-terminus of polypeptides (104).

Insect APNs contain an N-terminal signal sequence that guides the protein to the outer membrane surface where the protein attaches by a glycosylphosphatidylinositol (GPI) anchor (104). APNs are classified as zinc-binding metalloproteases containing a HEXXH domain with a downstream glutamic acid as well as a GAMEN domain. These domains are involved in zinc binding and important in the active site for enzyme catalysis (105, 106). In the pea aphid, *Acyrthosiphon pisum*, APN is estimated to account for 15.6% of the total gut protein (105). The pea aphid genome encodes at least six full length isoforms of APN (107). Pea aphid APN is heavily glycosylated with at least 16% of the molecular mass attributed to
carbohydrates (105). APN was determined to be the most abundant glycoprotein in the pea aphid gut (105) and subsequently shown to be the target of aphidicidal mannose binding garlic lectins (108). Sucrase was identified as another lectin receptor in the pea aphid (108).

APN is a major receptor for entry of group 1 coronaviruses (109-112). Viruses in the family Coronaviridae are enveloped positive sense RNA viruses that cause disease in mammals and birds, (111) primarily causing respiratory and enteric infections and occasionally neuronal diseases (113). Coronavirus binding to APN receptors seems to be fairly host-specific (111). Monoclonal antibodies against the catalytic site of APN prevent uptake of human coronavirus HCV-229E, suggesting an overlap between the virus binding and catalytic sites of APN (110). Additional exopeptidases have been reported as receptors for other coronaviruses (114). The use of APN and other functionally related proteins for coronavirus recognition and cell entry sets a precedent for the potential use of these proteins as virus receptors in other systems. In addition to the role of APN in digestion, APN also functions as a receptor for Bacillus thuringiensis (Bt) δ- endotoxins (115-117). Bacillus thuringiensis is a gram positive bacterium found in the soil that produces insecticidal toxins during sporulation. Bt toxins (Cry or Cyt proteins) form pores in the insect midgut leading to cell leakage and eventual insect death (118), and have been widely adopted for insect pest management (119, 120). However, Bt toxins have only a low level of toxicity against hemipteran pests (121-123). In addition to APN, insect receptors for Bt toxins include alkaline phosphatases and cadherin-like receptors (104).

**Management of plant viruses and aphid vectors**

*Management of aphids.* Aphids are primarily managed by the use of chemical insecticides that can be environmentally damaging (124). However, aphids are notorious for developing
resistance to insecticides (125). Biological control of aphid vectors with entomopathogenic fungi, natural enemies, and parasitoids can be effective, but may not provide a long term solution (1). Another management strategy includes plant breeding for resistance to aphids. Aphid resistant plants can be effective to varying degrees. In soybean plants, _Rag_ (resistance to _Aphis glycines_) genes have been identified in certain soybean varieties that display some effectiveness in suppressing soybean aphid populations (126). However, biotypes with resistance to Rag genes have already been identified (127, 128). Aphid resistant maize and wheat lines have also been developed and have had some success in limiting aphid damage (129-132).

**Managing aphid transmitted viruses.** Managing the aphid vector can be an effective method to delay the spread of persistently transmitted viruses. However, insecticides are less effective in controlling non-persistent viruses due to the short time needed for aphids to inoculate the plant (133). Also, insecticide use may increase aphid dispersal and subsequently increase virus transmission (134). Unfortunately, nonpersistent viruses comprise 200 of the 275 viruses transmitted by aphids (1).

For virus resistance, a number of resistance genes in plants have been described (135), but only a few plant resistance genes that protect against luteovirus infection are available. Resistance to BYDV in wheat is not found naturally but resistance genes from relatives of wheat have been used to engineer BYDV resistant wheat cultivars (136). Some resistance to PLRV in potato has been observed and continues to be developed (137).

Using a unique approach, a plant virus transmission blocking peptide was identified that interferes with the aphid transmission of PEMV by the pea aphid (138). The pea aphid gut binding GBP3.1 was identified by screening a phage display library to identify peptides
that bound to the pea aphid gut. Feeding of aphids on this peptide prior to feeding on PEMV-infected plants reduced the amount of virus in the aphid hemocoel. Hence the peptide interfered with binding of PEMV to the midgut receptor and/or transcytosis of PEMV across the gut epithelium. Plant expression of such a plant virus transmission blocking peptide is expected to reduce the likelihood of virus transmission to other plants by aphids, but would not prevent inoculation of virus into the plant. Such a transmission blocking strategy may have application for management of non-persistently transmitted plant viruses.

All of the methods described above for management of aphids and aphid transmitted viruses have limitations. There is a need to increase our understanding of the molecular mechanisms involved in aphid transmission of plant viruses which may lead to novel strategies for management of aphid transmitted viruses. Knowledge gained from aphid-virus interactions may also be applicable to other agriculturally important plant virus-vector systems.

**Dissertation Organization**

Chapter 2 is focused on the identification of APN as the pea aphid gut receptor for PEMV and the roles of CP and RTD in this interaction. In addition, it was demonstrated that APN may not serve as the receptor for PEMV in the green peach aphid, a second vector species. The third chapter describes the effect of BSA on PEMV uptake into the pea aphid in relation to the role of plant proteins in aphid transmission. In particular, the role of RTD in virus binding and association with proteins that facilitate virus uptake into the aphid hemocoel is examined. Chapter 4 focuses on the potential role of glycans in PEMV-aphid interactions, including both the role of glycans in receptor recognition, and the presence of
glycans on the virion. In the last chapter general conclusions are outlined and potential
directions for future research are discussed.

References


CHAPTER 2

*Pea enation mosaic virus* uses aminopeptidase N as a gut receptor in the pea aphid but may use a different receptor in the green peach aphid vector

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Abstract

Aphids and the plant viruses transmitted by aphids cause tremendous economic loss worldwide. Viruses in the family Luteoviridae are strictly transmitted by aphids in a persistent- circulative manner. For this type of transmission, ingested virions cross the aphid gut and salivary gland epithelial barriers, but the aphid receptors involved in Luteovirus recognition and uptake have not been identified. We used two-dimensional far-western blot analysis to screen brush border membrane vesicle (BBMV) proteins of the pea aphid, *Acyrthosiphon pisum* for binding to a model luteovirid, *Pea enation mosaic virus* (PEMV).

Pea aphid membrane alanyl aminopeptidase N (APN) was identified by mass spectrometry following specific binding to PEMV virions and to a PEMV coat protein -eGFP fusion peptide (CP-P-eGFP). The coat protein readthrough domain fused to eGFP (eGFP-RTD) also bound to APN along with many other aphid proteins. The binding of PEMV to pea aphid APN was confirmed by pull-down assay, and by increased binding of CP-P-eGFP to baculovirus expressed pea aphid APN in Sf9 cells. A pea aphid gut binding peptide (GBP3.1; amino acid sequence TCSKKYPSPCM) previously shown to impede uptake of PEMV into the pea aphid (1), was also shown to bind APN. Unexpectedly, PEMV apparently bound to a different receptor protein in a second vector species, *Myzus persicae*, suggesting that different gut receptors are used by luteoviruses in different vector species.
These data strongly suggest APN is the gut receptor for PEMV in the pea aphid, which would be the first receptor to be identified in the vector for a plant virus.

Introduction

Aphids are efficient vectors of plant viruses, responsible for nearly half of insect-borne plant virus transmission (2). Viruses in the family Luteoviridae are phloem-limited RNA viruses transmitted exclusively by aphids and cause disease in multiple food crops (3-5). Luteoviruses are transmitted in a circulative and persistent manner which involves specific molecular interactions between the virus and the aphid (6). The first barrier in the circulative pathway of a luteovirus particle is the aphid digestive tract. The virus binds to a receptor in either the midgut, hindgut, or both for transcytosis across the gut epithelium and release into the hemocoel (7). Virions diffuse through the hemolymph prior to a second receptor-mediated transcytosis event at the accessory salivary glands (ASG) from which virus particles are secreted with saliva to inoculate the plant phloem during subsequent feedings (8).

Luteovirus-aphid interactions are mediated by the viral capsid proteins consisting of a major coat protein (CP, 22 kDa) and one minor coat protein read-through domain (CP-RTD, 35–55 kDa) (9, 10). The RTD is not required for virus particle assembly or for uptake of virus from the gut into the aphid hemocoel, but both CP and RTD are essential for aphid transmission (11-13) and are the sole determinants of vector specificity (14). Although many studies have investigated the specific molecular interactions that facilitate virus movement in the aphid (15-18), the receptors involved in virus recognition are unknown.

Identification of virus receptors in their insect vectors has been unsuccessful for circulative viruses as a whole. However, many insect proteins that bind virus particles in
vitro have been identified primarily using far-western blotting, also known as a virus overlay assay (15, 16, 19, 20). Two proteins isolated from the head of the aphid *Sitobion avenae*, SaM35 and SaM50, bound to Barley yellow dwarf virus-MAV (BYDV-MAV) particles and were thought to be potential receptors in the ASG (15). In the aphid *Myzus persicae*, Rack-1, GAPDH3, and actin were shown to bind to Beet western yellows virus (16). A similar study of the small brown planthopper vector (*Laodelphax striatellus*) also identified Rack-1 and GAPDH3 along with three ribosomal proteins capable of binding Rice stripe virus (19). The authors suggest that Rack-1, GAPDH3, and actin function in endocytosis and intracellular transport, but are not actual virus receptors. Yang et al. (17) used a different approach based on Two-Dimensional DIGE comparisons of proteins from the greenbug, *Schizaphis graminum*, differing in the ability to transmit Cereal yellow dwarf virus-RPV. Two proteins that were differentially expressed and also confirmed to bind virions were luciferase and cyclophilin (17). However, as in the previous studies, the authors could only link these proteins to the endocytosis pathway. Identification of the virus receptors utilized in their insect vectors is crucial for the development of novel strategies to block virus transmission.

To further investigate luteovirus-aphid interactions, we used Pea enation mosaic virus (PEMV) and two aphid vector species, the pea aphid, *Acyrthosiphon pisum* and the green peach aphid, *Myzus persicae*. PEMV provides an ideal model virus as it is the only luteovirid that is not phloem-limited and is thus mechanically transmissible to plants (21). PEMV consists of two taxonomically distinct positive-sense RNAs. PEMV-1 is the sole member of the genus *Enamovirus* (Luteoviridae) with genome organization similar to that of the Poleroviruses (22), while PEMV-2 belongs to the genus *Umbravirus* (21). In addition, the genome of the pea aphid has been sequenced (23), facilitating identification of putative
receptor proteins. While PEMV is transmitted by at least 10 aphid species, the pea aphid and the green peach aphid, *Myzus persicae*, are the most important vectors of this virus (24).

In this study we demonstrated that PEMV binds to membrane alanyl aminopeptidase N (APN) in the pea aphid using a far-western blot method. Pull-down and immunofluorescence binding assays, and surface plasmon resonance (SPR) were used to confirm the interaction of PEMV and APN. Previously, we identified a peptide (GBP3.1) that binds the pea aphid gut and impedes uptake of PEMV into the aphid hemocoel (1). We demonstrated that GBP3.1 also binds to APN, supporting the hypothesis that GBP3.1 competes with PEMV for receptor binding. These findings indicate that APN is the putative gut receptor for PEMV. In contrast, APN does not appear to function as a PEMV receptor in a second vector species, the green peach aphid, *M. persicae*.

**Materials and Methods**

*Insects*

Pea aphids, *Acyrthosiphon pisum* Harris (Aphidinae: Macrosiphini) were obtained from Berkshire Biological Supply Company (Westhampton, MA) and reared on broad bean, *Vicia faba*. Aphid colonies were maintained in growth chambers at 24°C with a 12h light: 12h dark cycle. Green peach aphids, *Myzus persicae*, were maintained on Michihli Chinese cabbage plants at room temperature.

*Preparation of aphid brush border membrane vesicles*

Brush border membrane vesicles (BBMV) of adult *A. pisum* and *M. persicae* were prepared from whole aphids as described in (25). The final BBMV pellets was resuspended in ice cold buffer (0.3 M Mannitol, 5 mM EGTA, 17 mM Tris–HCl pH 7.5) diluted 1:2 in
Protease inhibitor cocktail (Sigma-P8340) was added to a 1:100 dilution and samples were snap frozen in liquid nitrogen and stored at −80 °C until further use. The protein concentration was determined by Bradford assay. Aminopeptidase activity was measured as described in (26) and was typically enriched 10–15-fold in the final BBMV suspensions relative to the initial homogenates.

**Purification of Pea enation mosaic virus**

Seven day old pea plants (*Pisum sativum*) were mechanically infected with wild type PEMV or PEMV RNA1Δ as described in (27) and harvested at 10-14 days post infection. PEMV RNA1Δ is a naturally selected mutant that lacks the majority of the coding sequence for the readthrough domain (RTD) and does not produce the RTD polypeptide (Figure 1) (28). The method used to purify PEMV from plants was modified from Liu et al., 2009 (27). PEMV infected plant tissue was frozen in liquid nitrogen and homogenized in a blender with 0.2 M sodium acetate pH 6 (1mL/gram of tissue) and an equal volume of chloroform. The homogenized tissue suspension was centrifuged at 3000g for 10 min. The supernatant was transferred to clean tubes and centrifuged 17,200g for 2 h. Pellets were saved and supernatant was again centrifuged at 141,000g for 2.5 h. All pellets were soaked in 0.2 M sodium acetate pH 7 overnight at 4°C and then resuspended. The soluble fraction was centrifuged at 147,000g through a 30% sucrose cushion made with 0.2 M sodium acetate, pH 7. The final pellet was washed three times in 0.2 M sodium acetate buffer to remove excess sucrose and resuspended in the same buffer. Sample purity was assessed by SDS PAGE analysis. The protein concentration of PEMV was determined by densitometric analysis with Image J software (29) of the Coomassie stained bands with reference to known BSA concentrations resolved by SDS–PAGE.
Production of eGFP and eGFP-RTD

The pBAD/His B (Invitrogen) vector was used for expression of eGFP and the PEMV read through domain (RTD) fused to enhanced green fluorescent protein (eGFP) (Figure 1). The RTD was fused to the C-terminus of eGFP and inserted into pBAD/His B. Three oligonucleotides were used for integrating the RTD and eGFP DNA sequences: a forward primer containing a PstI site and the 5’ end of the eGFP sequence, an oligonucleotide connecting the 3’ end of the eGFP and the 5’ end of the RTD sequence, and a reverse primer complementary to the 3’ end of the RTD sequence. Primers used for construction of the eGFP-RTD fusion sequence were GFPPstI (5’-GCGCTGCTAGGCTgtgcaagggcaggagctg-3’, with the PstI site underlined and lower case text indicating the coding part of the fusion protein), GFPRTD (5’-GGACGAGCTGTACAAGGGGGACGACGCTCCCCCG-3’). The reverse primer was RTDHindIII (5’-GGAAGCTTTTAAatctagggacttgtg-3’, HindIII site underlined).

The eGFP-PCR products were excised from a 1% agarose gel and purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The DNA fragments were digested with Pst I and Hind III, cleaned by using a QIAquick Nucleotide Removal Kit (Qiagen), and ligated into pBAD/His B (previously linearized with PstI and Hind III). For expression of eGFP-RTD and eGFP, competent Top10 cells were transformed with the plasmids peGFP-RTD or peGFP. Cells were cultured in low salt LB medium containing ampicillin (50µg/ml) in an orbital shaker at 250 rpm (37 °C) until the OD600 reached 0.5-0.6. L-(+)-Arabinose (Sigma) was then added to the culture to a final concentration of 0.02% to induce protein expression. The culture was maintained overnight at ambient temperature with shaking at
220 rpm. The overnight cultures were centrifuged to pellet the cells and pellets were frozen at -80°C prior to protein purification.

The His-tagged fusion proteins were purified using Ni-NTA agarose resin (Qiagen) according to the manufacturer’s directions. Purification was conducted under native conditions using a batch purification method. All purification steps were performed either on ice or at 4°C. Purified proteins were concentrated as needed using an Amicon YM-3 Centricon Centrifugal Filter Device (Millipore) and dialyzed in Side-A-Lyzer Dialysis Cassettes (Pierce) with phosphate buffered saline (PBS). The fusion proteins were stored at -80°C.

**Baculovirus expression of CP-P-eGFP**

The CP-P-eGFP construct (Figure 1) was designed with the proline rich region of the RTD sequence (P) included between the CP and eGFP sequences. The first fragment (CP-P) was obtained by using a CP-RTD construct without the stop codon as template (30). To obtain the CP-P with a downstream 5’ EGFP flanking sequence, primers PEMV 9 F (5’-GCCTCCCTCATGGTGAGCAAGGGCGAG - 3’) and PEMV 18 R (5’-GCCCTTGCTCACCATTCCCT ACGGGAGTGGGACT - 3’) were used. To obtain the EGFP sequence with the upstream proline rich domain, primers PEMV 17F (5’-CCCACTCCCGTAGGAATGGTGAGCAAGGGCGA G- 3’) and PEMV 5R (5’-CCAAAAGCTTTGTTACTTGTACAGCTCGTCCATG - 3’, HindIII site underlined) were used. The full length CP-P-EGFP was PCR amplified by combining these two fragments using the PEMV 19 F (5’-ACGGGATCCACCatgggcacatcaccatcatgcatgcactagataaatca aa) and PEMV 5R primers. The BamHI site is underlined and lower case text indicates the coding part of the fusion protein.
The PCR-amplified CP-P-eGFP fragment was digested with BamHI and HindIII and cloned into the BamHI – HindIII sites of pFastBac1 (Invitrogen). Constructs were made with N-terminal His tags and Kozak sequences, as described previously (30). The recombinant bacmid was transfected into Sf9 cells to produce recombinant baculovirus vCP-P-eGFP. The recombinant baculovirus was plaque purified and recombinant CP-P-eGFP was expressed in baculovirus-infected cells using standard procedures (31). Expressed CP-P-eGFP protein was purified using the Ni-NTA agarose resin (Qiagen) as described above.

*Two-dimensional gel electrophoresis and far-western blotting*

To remove contaminants such as salts, lipids, or nucleic acids that interfere with isoelectric focusing, whole aphid BBMV protein derived from *A. pisum* or *M. persicae* was subjected to a 2D-Clean-Up kit (GE Health Sciences). The final pellets from 300 µg of initial protein were resuspended in rehydration buffer containing 2M thiourea, 7M urea, 2% ASB-14, 2% C7BzO, 0.5% IPG buffer (GE Healthcare), and a trace amount of bromophenol blue. A sonicating water bath was used to increase protein solubilization. Reconstituted proteins were centrifuged in a microcentrifuge at 15,200g for 5 min to remove insoluble material. The protein was then applied to 7 cm Immobiline DryStrip gels (GE Healthcare) for overnight rehydration. Both pH 3-10 and 4-7 strips were used. Focusing was performed using the IPGphor (Biorad) as follows: 50V for 10 h, 300V for 1 h, 1000V 1 h (gradient), 5000V 1.5 h (gradient), 5000V 1 h.

After isoelectric focusing, IPG strips were equilibrated for 15 min in Equilibration Buffer I (EB I) (6 M urea, 2% SDS, 0.125 M Tris–HCl (pH 8.8), 30% glycerol and 2% (w/v) DTT) followed by 15 min in EB II (same as EB I but containing 2.5% iodoacetamide instead of DTT). For the second dimension IPG strips were loaded and run on 10% SDS-PAGE gels.
Electrophoresis was run under constant voltage at 100V. Gels were either stained with Coomassie blue or equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 0.025% SDS, pH 8.3) prior to overnight transfer to a PVDF membrane at 4°C at 30V.

Membranes were blocked in 5% non-fat dry milk in phosphate buffered saline with 0.1% Tween-20 (PBS-T) overnight at 4°C. Membranes were then incubated with PEMV or PEMV RNA1Δ (20 µg/mL) or eGFP-fusion proteins (10 µg/mL) in 1% non-fat dry milk in PBS-T overnight at 4°C. Bound ligand was detected using affinity purified (32) PEMV coat protein antiserum (1:100) or GFP antiserum (Sigma; 1:5000) followed by an HRP-conjugated anti-rabbit IgG (1:5000). The PVDF membranes were incubated in HyGlo Chemiluminescent HRP detection reagent for 1 min, with luminescence detected on X-ray film using standard procedures. Antibody only controls were run in parallel by eliminating incubation with the ligand (virus or eGFP-fusion) to identify non-specific binding of the antibodies to aphid BBMV. The experiments were replicated at least four times with each ligand.

MALDI-TOF MS/MS protein identification

Protein spots bound by PEMV, CP-P-eGFP, or eGFP-RTD were picked from Coomassie stained gels with reference to probed membranes and submitted to the Iowa State University Protein Facility for identification using MALDI-TOF MS/MS mass spectrometry. Briefly, trypsin digested peptides were analyzed using the QSTAR XL quadrupole TOF mass spectrometer (AB/MDS Sciex, Toronto, Canada) equipped with an oMALDI ion source. After every regular MS acquisition, MS/MS acquisition was performed against most intensive ions. All spectra were processed by using a MASCOT (MatrixScience, London, UK) database search. Peak lists were generated by Analyst QS (AB/MDS Sciex, Toronto,
Canada) and were used for MS/MS ion searches. Protein identification was based on the probability based Mowse Score. The significance threshold $p$ was set to less than 0.05.

Expression of pea aphid APN in E. coli for production of polyclonal antibodies

Both a 28 kDa (amino acids 385-633) and 67 kDa (amino acids 216-794) fragment of full length pea aphid APN (gi|187179337) were generated by PCR and cloned into pBAD/His B. Proteins were expressed as described above. Due to low binding of His-tagged APN fragments to the nickel resin under native conditions, the proteins were purified under denaturing conditions using 6M guanidine hydrochloride. For production of antiserum, a mixture of the refolded soluble 28 kDa fragment and PAGE gel slices containing the 67 kDa fragment were used for inoculation of rabbits at the Iowa State University Hybridoma Facility.

Baculovirus expression of pea aphid APN in Sf9 cells

The Bac-to-Bac® Baculovirus Expression System (Invitrogen) was used to generate recombinant baculoviruses. To clone pea aphid APN (gi|187179337) into the baculovirus transfer vector pFastBac1 (Invitrogen), the cDNA was first amplified using gene specific primers containing NotI and HindIII restriction sites and ligated into pGEM T-easy vector (Promega). A version of APN lacking the glycophasphatidylinositol (GPI) anchor and containing a C-terminal histidine tag was also constructed by adding the 6X His-tag and a stop codon prior to the GPI signal sequence. The sequences were excised from the vector and ligated into NotI/HindIII digested pFastBac1. To generate the recombinant bacmids, chemically competent DH10Bac™ cells were transformed with pFastBac1 containing the A. pisum APN cDNA and the transformants were selected by plating on LB plates containing 10 μg/ml tetracycline, 50 μg/ml kanamycin, and 7 μg/ml gentamycin as well as 100 μg/ml Bluo-
gal and 40 μg/ml IPTG for blue/white screening. The recombinant bacmids (vAPN-GPI(-)
and vAPN-GPI(+)) were isolated and PCR was used to confirm the presence of inserts. Sf9
cells (33) grown in a monolayer and maintained in Sf900 medium at 28°C, were transfected
with recombinant bacmids using Cellfectin II reagent (Invitrogen). Supernatant was
harvested from transfected cells 4 days post infection. Recombinant baculoviruses were
plaque purified and recombinant APN was expressed in baculovirus-infected cells using
standard procedures (31). Untransformed bacmid retaining the lacZ insert (vLacZα) was
used in experiments as a negative control virus.

Purification of His-tagged APN

Sf9 cells were infected with recombinant baculovirus expressing APN and harvested
at 72 hours post infection. APN-GPI(-) was purified from both the Sf900 medium and cell
lysates. To purify from the medium, sodium phosphate and imidazole were added to a final
volume of 50 mM and 10 mM, respectively. The pH was not adjusted to avoid precipitation
of some components of the medium. The medium was incubated with the Ni-NTA agarose
resin (Qiagen) overnight at 4°C. The resin was washed and the protein eluted using standard
buffers described by the manufacturer. APN purification from the cells was conducted under
native conditions using a batch purification method with the Ni-NTA resin as described by
the manufacturer.

Immunofluorescence detection of pea aphid APN expressed in Sf9 cells

Two methods were used for the immunofluorescence assays. For the first method,
Sf9 cells were infected with the recombinant baculoviruses expressing APN. At 48 hrs post-
infection, cells were seeded on microscope slides, washed once in PBS, and fixed with 3.7%
formaldehyde in PBS for 30 min. All washes and incubations were done using a Coplin jar.
Following fixation, the cells were permeabilized with PBS containing 0.3% Triton X-100 (PT buffer) for 1 hr at 37°C followed by blocking for 1 hr in PT buffer containing 3% bovine serum albumin (PTB buffer). Cells were then incubated with a 1:250 dilution of anti-APN antiserum in PTB containing 0.5% BSA overnight at 4°C. The following day cells were washed 3 times in PT buffer and then incubated in 1:500 Alexa 488-conjugated goat anti-rabbit secondary antibody (Invitrogen) in PTB containing 0.5% BSA for 1 hr at 37°C. Cells were then washed 3X in PT buffer, incubated in 1:1000 DAPI (Kirkegaard & Perry Laboratories, Inc) in PT to stain the nuclei, then washed three times in PT buffer. Slides were mounted in 50% glycerol in PBS. Alexa 488 fluorescence was detected using standard epi-fluorescence microscopy employing a FITC HYQ filter (Nikon) with 460-500 nm excitation and 510-560 emission. DAPI fluorescence was visualized with a UV-2E/C filter (Nikon) with a 340-380 nm excitation and 435-485 emission. Images were also taken under bright field. Uninfected cells and cells infected with vLacZα served as negative controls.

For the second method cells were seeded and infected on Poly-L-Lysine coated 12mm glass coverslips (BD-Biosciences) in 24 well plates. After 48 hrs post infection, cells were washed three times in insect PBS on a shaker. Cells were fixed in 2% formaldehyde in PBS for 30 min at room temp. After a brief rinse in PBS, cells were permeabilized in PT buffer for 1 hr at 28°C followed by blocking in 3% PTB for 1 hr. Antibody and DAPI incubations were conducted as described above, but on a shaker. To investigate the binding of CP-P-eGFP to APN expressed in Sf9 cells, the same procedure was followed with the exception that the antibody steps were replaced with incubation with 10 µg/ml CP-P-eGFP. The Sf9 cells were incubated with the CP-P-eGFP for either 1 hr at 28°C or overnight at 4°C.
Uninfected cells and cells infected with vLacZα served as controls. Experiments for antibody detection of APN were repeated four times between the two methods. Experiments for CP-P-eGFP binding were repeated six times. Results for CP-P-eGFP binding were observed in six experiments.

*Pull-down assays with PEMV and GBP3.1*

Purified PEMV was labeled with a Sulfo-SBED Biotin label transfer reagent (Thermo Scientific). All steps were performed in the dark to avoid activation of the aryl azide group prior to crosslinking. The Sulfo-SBED reagent was dissolved in dimethylformamide (DMF) to increase solubility and then added to 250 µg of PEMV in 5 ml of 0.2M sodium phosphate at pH 7. A final concentration of 8% DMF was maintained in the solution to avoid precipitation of the labeled virus. The final concentration of the Sulfo-SBED in the reaction was 110 µM. The reaction was incubated at room temperature for 30 min mixing occasionally. To remove the unbound Sulfo-SBED reagent, virus solution was passed through a Sephadex G-25 column (GE Healthcare). The labeled virus was stored at 4°C protected from light. A double-derivatized GBP3.1 peptide with biotin at the N-terminus and a UV-crosslinker residue (Bpa; pbenzoyl-L- phenylalanine) in place of the tyrosine (Y) within the 8-amino acid loop was synthesized by Genemed Synthesis (San Antonio, TX) (Supplementary Figure 1).

Whole guts from pea aphids were dissected in PBS containing protease inhibitor cocktail at a dilution of 1:100 (Sigma). Protease inhibitors were present throughout the following procedure. Guts were washed three times in PBS and then incubated with 10 µg of labeled PEMV or GBP3.1 for 1 hr at 4°C in the dark. The guts were gently mixed every 5-10 min. The guts were then washed 7 times in PBS to remove unbound PEMV or GBP3.1. The
guts were exposed to UV for 15 min (PEMV) or 30 min (GBP3.1) to crosslink proteins interacting with the virus or GBP3.1. This was done on ice with occasional mixing. The guts were then briefly centrifuged and resuspended in lysis buffer (250 mM KAc, 10 mM MgAc, 50 mM HEPES, pH 7.4, 0.1% NP-40) and thoroughly homogenized. For GBP3.1, the suspension was then centrifuged at 25,000g to remove any insoluble material. For PEMV, the suspension was centrifuged at a slower speed (13,300g) to pellet the insoluble material without virus. The supernatants were added to 100 µl streptavidin agarose beads (Invitrogen) pre-equilibrated in lysis buffer without NP40. The beads were incubated end over end at room temperature for 1 hr. The beads were then washed seven times in lysis buffer without NP40, boiled in SDS loading buffer for 5min, and the supernatants loaded for SDS-PAGE and western blot detection with purified APN antiserum. APN pulled down with PEMV in test and control treatments and visualized by western blot was quantified using ImageJ analysis (29). Statistical differences were determined using the Student’s t-test. Controls for PEMV pull-down assays included streptavidin beads incubated with PEMV alone, beads incubated with aphid guts alone, and the beads incubated with buffer only. Controls for GBP3.1 pull-down assays included streptavidin beads incubated with GBP3.1 and aphid guts without UV crosslinking, and beads incubated with buffer only.

**Identification of GBP3.1-binding partners by LC MS/MS**

The GBP3.1-interacting protein was analyzed by LC-MS/MS on Dionex 3000 nanoRSLC series HPLC system (Thermo-Electron, Waltham, MA). LC effluent was directed to the electrospray source of a linear ion-trap mass spectrometer (LTQ/XL, Thermo-Electron, USA). MS/MS spectra were acquired in a data-dependent acquisition mode that automatically selected and fragmented the five most abundant peaks from each MS spectrum.
All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.4.0), Spectrum Mill (Agilent, Santa Clara, CA, USA; version Unknown) and X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1) with the PeaAphid_20130306 database (unknown version, 33591 entries) assuming digestion with the enzyme trypsin. Scaffold (version Scaffold_4.0.0, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Protein probabilities were assigned by the Protein Prophet algorithm.

**Surface plasmon resonance binding assays**

Surface plasmon resonance (SPR) assays using a BIAcore T100 (BIAcore, Uppsala, Sweden) (34) were used for quantification of the relative binding of CP-P-eGFP, eGFP and RTD-eGFP to APN-GPI(-). The buffer HBS-N (BIAcore) was used for all experiments. Preparations of CP-P-eGFP, eGFP and RTD-eGFP were dialyzed in HBS-N to a final concentration of 6 µM. APN-GPI(-) was immobilized on to the carboxymethylated dextran (CM5) sensor chip surface (BIAcore) through standard amine coupling method. Carboxyl groups along the CM-dextran chains of the sensor chip surface were activated by injecting a mixture of NHS (0.1 M N-hydroxysuccinimide) and EDC (0.1M 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride) (1:1, vol/vol). APN-NGP in coupling buffer (10 mM sodium acetate, pH 4.5) was injected over the chip surface at 0.1 µg/µl to obtain an immobilization target level of 2000 resonance units (RU). After coupling, unreacted surface ester groups were blocked by injecting 1 M ethanolamine (pH 8.5) on to the chip surface. Analysis of the interaction of APN-GPI(-) with CP-P-eGFP, eGFP and RTD-eGFP was performed by injecting proteins (6 µM in HBS-N) at 30 µl/min for 60 s, dissociation for 60 s, regeneration with 50 mM NaOH for 60 s and stabilization for 120 s.
Reference flow cells without immobilization of APN-NGP were included in the experiments. SPR assays were conducted at least twice and data analyzed by one-way ANOVA.

Results

*PEMV binds to pea aphid APN*

A far-western blot method was used to identify pea aphid membrane proteins that bind PEMV. To target membrane-associated proteins likely to interact with PEMV in the aphid gut, brush border membrane vesicles (BBMV) rather than proteins from whole aphids were used. BBMV were separated by two-dimensional gel electrophoresis, blotted to PVDF membranes, and overlaid with purified PEMV, coat protein-eGFP fusion (CP-P-eGFP), or the readthrough domain-eGFP fusion (eGFP-RTD; Figure 1). Bound PEMV or eGFP fusion protein was detected with antiserum specific to the PEMV coat protein or GFP, respectively. Wild type PEMV, PEMV lacking the RTD (PEMV RNA1Δ), and CP-P-eGFP all bound specifically to an aphid protein migrating above 150 kDa (Figures 2-4). This protein was isolated from Coomassie stained gels (Figure 4) with reference to probed membranes and identified as membrane alanyl aminopeptidase N (APN, gi|187179337) by MALD-TOF MS/MS (Table 1). Mascot search results also hit a partial sequence of an APN (gi|193713823) indicating that the proteins sequenced could have been a mixture of APN isoforms. There are six full length isoforms of APN (Supplementary Table 1). The eGFP-RTD fusion also bound to APN, along with many other aphid proteins (Figure 3). The additional binding observed in both the CP-P-eGFP and eGFP blots was attributed to binding of the GFP antibody to aphid BBMV proteins.
**PEMV binds to different proteins in the green peach aphid**

The same far-western blot method was used to assess if PEMV binds to APN in a second vector, the green peach aphid. PEMV and CP-P-eGFP did not appear to bind to APN from whole aphid BBMV, but consistently bound to a series of seven proteins migrating between 75 and 100 kDa (Figure 5). A protein spot on a Coomassie-stained gel migrating at a similar position as APN from the pea aphid was confirmed to be APN by MALDI MS/MS, however there was no binding of PEMV or CP-P-eGFP to this *M. persicae* APN (Figure 6). Protein spots from a Coomassie stained gel corresponding to PEMV-bound proteins on the probed membranes have been submitted for protein identification (Figure 6).

**Expression and purification of pea aphid APN in Sf9 cells**

To confirm the binding of PEMV to APN, we used recombinant baculoviruses to express a recombinant pea aphid full length APN and a truncated version lacking the GPI anchor in Sf9 cells. Both APNs were detected in the membrane and soluble fractions of cell lysates, but a larger proportion of the APN-GPI(-) was detected in the soluble fraction. APN has a secretory signal so in the absence of the GPI anchor it is released into the medium. This allowed for successful purification of APN-GPI(-) by nickel affinity from the medium (Figure 7). The theoretical molecular mass of pea aphid APN is 111 kDa. Anti-APN antiserum detects a band slightly larger than 150 kDa from pea aphid BBMV suggestive of extensive glycosylation of the native protein (Figure 7). APN lacking the GPI anchor is truncated by 29 amino acids resulting in a 3.2 kDa reduction in size consistent with the baculovirus expressed APNs appearing slightly smaller in size than the native protein.
Interaction of baculovirus-expressed pea aphid APN with CP-P-eGFP

Immunofluorescence assays were used to confirm the expression of recombinant pea aphid APN-GPI(+) and APN-GPI(-) in Sf9 cells (Figure 8A). The binding of the PEMV coat protein to cells with and without expressed recombinant pea aphid APN was visualized by incubation with CP-P-eGFP. An increase in fluorescence was detected in Sf9 cells expressing APN compared to the controls, indicating increased binding of CP-P-eGFP (Figure 8B). The low levels of background fluorescence in the LacZα and uninfected cells may result from weak CP binding to Spodoptera frugiperda APN in the Sf9 cells. Fluorescence was not observed in Sf9 cells incubated with eGFP alone supporting low level CP binding to Sf APN and indicating that the observed fluorescence from CP-P-eGFP was not due to eGFP binding. These fluorescence assays were conducted in six independent replicates.

Pull-down of APN with PEMV

A UV cross-linking pull-down assay was used to confirm the binding of PEMV to APN. PEMV was labeled with a biotin label transfer reagent as described in the methods. This molecule is designed to crosslink interacting proteins when activated by ultraviolet light. The biotin label was used to pull-down the protein complex with streptavidin-linked agarose beads. Purified anti-APN antiserum was used to detect the co-precipitated APN by western blot (Figure 9). PEMV consistently pulled down more APN than in the control; some APN was pulled down by streptavidin beads in the absence of PEMV (Figure 9). The band intensities of APN were measured by densitometric analysis using the Image J program. The amount of APN pulled down by PEMV averaged 4.5-fold more than the control across four replicates and was statistically significant as determined by Student’s t-test (p = 0.025;
Native pea aphid APN may associate with biotin, resulting in the background binding observed in these experiments.

**SPR analysis of CP-P-eGFP and eGFP-RTD interaction with APN**

Surface plasmon resonance (SPR) was used to quantify the real-time binding of the baculovirus-expressed APN-GPI(-) to CP-P-eGFP, eGFP-RTD, and eGFP alone as a control (Figure 10). The relative binding units ± SEM to APN were: eGFP, 0.00 ± 0.00; eGFP-RTD, 3.079 ± 0.37 (p = 0.021399); CP-P-EGFP, 100.38 ± 0.51 (p = 3.91E-05). The binding of CP-P-eGFP to APN was significantly stronger than that of eGFP-RTD.

The gut binding peptide GPB3.1 that impedes PEMV uptake also binds to APN

We previously identified the peptide GBP3.1 that binds the pea aphid gut and impedes uptake of PEMV into the aphid hemocoel (1). Pull-down assays were used to determine whether GBP3.1 also binds APN. GBP3.1 was synthesized with the addition of a photoreactive benzoyl phenylalanine for crosslinking and biotin for pull-down with the streptavidin-linked agarose beads. A protein of the correct size for APN that was detected by the purified APN antiserum was pulled down by GBP3.1 (Figure 11). The identity of this protein was confirmed as APN by LC MS/MS (Table 1). A protein (~140 kDa) detected in both the cross-linked and uncross-linked samples (Figure 11), was also pulled-down by streptavidin beads alone (data not shown). This experiment was repeated three times with western blot analysis conducted for one of the three experiments (Figure 11).

**Discussion**

Although luteovirus-aphid interactions have long been the focus of investigation, the specific aphid receptors involved in luteovirus transmission have not been previously
identified. In this study we demonstrated that PEMV binds to membrane alanyl aminopeptidase N (APN) using a 2-D far-western blot method with BBMV from whole pea aphids. In contrast to previous studies, we used BBMV to increase the probability of identifying a receptor by enriching for membrane proteins. Although we used whole aphid BBMV rather than pea aphid gut-derived BBMV, the majority of the protein from whole aphid BBMV preparations would be derived from the gut and APN is localized to the gut (35).

We were unable to detect PEMV-APN binding using a co-immunoprecipitation method with APN antiserum or by pull-down assay with biotin labeled PEMV without the cross-linking step, suggesting that PEMV-receptor binding is transient. Transient binding of luteoviruses to aphid receptors could explain why previous attempts to identify plant virus receptors were unsuccessful (15-17), and why UV crosslinking was necessary. In addition, GBP3.1 binding under in vitro conditions was difficult to detect without cross-linking to the bound protein.

Aminopeptidase N. APN is bound to the gut epithelial membrane with a primary function in insects to cleave N-terminal amino acids and amino-acid absorption (35). APN is a major component of some insect midguts, and is estimated to comprise 15% of the total midgut protein in the pea aphid (35). This midgut localization is consistent with its role as a gut receptor, given the midgut is the known site for PEMV uptake (7). Aminopeptidase activity is relatively low in the hindgut (36), which functions in absorption of water and other beneficial molecules prior to excretion (37). Consistent with the high molecular mass relative to the predicted molecular mass, APN is heavily glycosylated and known to be a target for aphicidal lectins (38-41). The role glycans play, if any, in luteovirus-aphid
interactions is unclear (42, 43). APN is also one of the receptors for *Bacillus thuringiensis* δ-endotoxins in insects, although these toxins are relatively ineffective against aphids (25, 44, 45). There is a precedent for APN functioning as a virus receptor as some group I coronaviruses such as Human coronavirus 229E use APN for cell entry (46).

*APN is not the receptor for PEMV in *M. persicae*. Unexpectedly, PEMV does not appear to bind APN from a second vector aphid, *M. persicae*. This result implies that PEMV has evolved to use different gut receptors in different aphid vectors. Notably, the site of PEMV uptake in *M. persicae* (midgut and/or hindgut) has not been determined. There are many examples of luteoviruses crossing the gut of non-vector aphids (47, 48) illustrating that the aphid gut is not as stringent a barrier to transmission as the accessory salivary glands. Given the structural similarity of the luteovirus coat proteins, it is expected that these virions have evolved to interact with similar proteins in their respective vectors. However, different luteoviruses use either the midgut or hindgut, and in some cases both, for uptake into the hemocoel as shown by transmission electron microscopy (7). The use of different sites for luteovirus acquisition argues against the idea of a common gut receptor, particularly in the case of APN, as the abundance of APN in the hindgut is low (36). *M. persicae* for example acquires *Soybean dwarf virus* (SbDV) through the hindgut (49), but *Potato leaf roll virus* (PLRV) and BWYV through the midgut (50, 51). *M. persicae* therefore has receptors in the mid- and hindgut bound by different luteoviruses. Given that the aphid gut is not a stringent barrier to luteoviruses; the inability of some viruses to be transmitted by certain aphids must come from incompatible interactions in either the hemocoel or at the accessory salivary glands.
In vivo confirmation of APN as the receptor for PEMV. GBP3.1 impedes the uptake of PEMV into the aphid hemocoel (1) and we have now shown that GBP3.1 also binds to APN. Hence in vitro binding of PEMV to pea aphid APN shown in this study has been confirmed under in vivo conditions (1).

Peptides such as GBP3.1 could be developed to bind very specifically to virus binding sites and be expressed in planta. Upon aphid feeding, these peptides would interfere with uptake of virions into the aphid hemocoel thereby reducing virus transmission in the field. In an agricultural setting this method would not protect the first plant fed on by an incoming viruliferous aphid, but would block plant to plant spread of the virus in the field. A single competing molecule could be effective in blocking transmission of related viruses, if common insect gut receptors are used. Identification of APN as the putative receptor for PEMV has significant value for increased understanding of plant virus-vector molecular interactions and for the development of novel strategies to control the spread of plant viruses.

Author Contributions

LBL performed all of the experiments described except for the pull-down assays with GBP3.1 and SPR analyses, which were performed by NPC. SL and BCB contributed to the research concept and experimental design.

Acknowledgements

We thank S. Sivakumar for construction of the recombinant baculovirus expressing CP-P-eGFP. This work was funded by the USDA AFRI 2008-03996, by the Iowa State University Plant Sciences Institute Virus-Insect Interactions Initiative, and by Hatch Act and State of Iowa funds.
References


Tables and Figures

**Figure 1.** Schematic diagrams of the viruses wild type PEMV and PEMV RNA1Δ, and fusion proteins CP-P-eGFP, and eGFP-RTD. The length of the genomic RNA is indicated on the right. The short remaining 5’ sequence of the RTD in PEMV RNA1Δ is in a different reading frame and does not produce a translated protein product (27). This figure is modified from (27).
Figure 2. Binding of WT PEMV and PEMV RNA1Δ to pea aphid BBMV. Far western blotting conducted using pH 3 to 10 and 4 to 7 was replicated at least 4 times with each virus. No binding was detected for the antibody (no ligand) control. The positions of molecular mass standards are indicated at left. Arrows indicate consistent binding observed for both viruses.
Figure 3. Binding of CP-P-eGFP and eGFP-RTD to pea aphid BBMV. Far western blotting conducted using pH 3 to 10 and 4 to 7 was replicated at least 4 times with each ligand. Arrows in the CP-P-eGFP blot indicate binding consistent with the viruses in Figure 1. The additional binding observed in both the CP-P-eGFP and eGFP control blots was attributed to binding of the GFP antibody to aphid BBMV proteins. The positions of molecular mass standards are indicated at left.
Figure 4. Identification of proteins bound by WT PEMV and CP-P-eGFP. Two-dimensional gel electrophoresis of pea aphid BBMV stained with Coomassie blue (left) and compared to the far-western blots with WT PEMV (A) or CP-P-eGFP (B) on the right. Proteins identified by MALDI-TOF MS/MS are indicated by arrows and are listed in Table 1.
Table 1. MALDI-TOF MS/MS identification of pea aphid proteins bound by WT PEMV and PEMV structural proteins (Figure 3) and LC MS/MS identification of APN (listed as Spot No. 6) from the GBP3.1 pull-down assay (Figure 11).

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Figure 5. Binding of PEMV and CP-P-eGFP to green peach aphid BBMV using far-western blotting. Aphid proteins consistently detected are circled. The control blots with eGFP or the PEMV antibody alone are shown. Images are representative of two replicates with each ligand.
Figure 6. Binding of CP-P-eGFP to green peach aphid BBMV compared to a Coomassie blue stained gel. A. Far-western blot with green peach aphid BBMV and CP-P-eGFP. Proteins bound by CP-P-eGFP are circled. The additional bands were also seen in the eGFP control. B. Green peach aphid proteins indicated by arrows were selected for MALDI-TOF MS/MS identification from the Coomassie stained gel with reference to probed membranes. The protein identified as *M. persicae* APN is boxed. There was no binding of CP-P-eGFP or WT PEMV to *M. persicae* APN in the far-western blots.
Figure 7. Purification and western blot detection of baculovirus-expressed APN-GPI(-). Purified APN-GPI(-) was stained with Coomassie blue (A) or probed with APN antiserum (B). Full length APN (APN-GPI(+)) detected from pea aphid BBMV (C) with APN antiserum is larger in size than the baculovirus-expressed APN-GPI(-) (B). The GPI anchor is expected to account for a 3.2 kDa difference in molecular mass.
Figure 8A. Baculovirus expression of recombinant APN in Sf9 cells. Sf9 cells were infected with the recombinant baculoviruses vAPN-GPI(+), vAPN-GPI(-), or vLacZα as a control and expression of APN at 48 hrs was detected using an anti-APN primary antibody and an Alexa 488-conjugated secondary antibody. Nuclei are stained with DAPI. Uninfected cells served as a control. Alexa 488 (top row) and DAPI fluorescence (middle row) were visualized by epi-fluorescence microscopy. Brightfield images are also shown. Images are representative of four experiments.
Figure 8B. Binding of CP-P-eGFP to Sf9 cells expressing recombinant APN. Sf9 cells were infected with vAPN-GPI(+), vAPN-GPI(-), or vLacZα and at 48 hrs post infection the cells were incubated with CP-P-eGFP or eGFP followed by DAPI to stain the nuclei. Uninfected Sf9 cells served as an additional control. GFP and DAPI fluorescence were detected using an epi-fluorescence microscope. Increased fluorescence was observed in Sf9 cells expressing recombinant APN. Brightfield images are also shown. No fluorescence was observed when cells were incubated with eGFP. Images are representative of six experiments.
Figure 9. PEMV binding of APN using a pull-down assay. PEMV was labeled with a biotin transfer reagent to UVcross-link proteins bound to PEMV. The PEMV-APN complex was pulled down using streptavidin-agarose beads and APN was detected by western blot using APN antiserum (A). Controls consisted of aphid guts + beads, PEMV + beads, and a beads + buffer control. Aphid gut protein was run as a positive control for the APN antiserum. PEMV consistently pulled down more APN than in the control although some APN was pulled down by streptavidin beads in the absence of PEMV (A). The band intensities of APN were measured by densitometric analysis using the Image J program (B). The amount of APN pulled down by PEMV averaged 4.5-fold more than the control lacking PEMV across four replicates and was statistically significant as determined by Student’s t-test (p = 0.025).
Figure 10. BIAcore surface plasmon resonance analysis of CP-P-eGFP, eGFP-RTD, eGFP interaction with APN. Sensorogram showing the real-time interaction between 6 µM of the eGFP- fusion proteins and immobilized APN-GPI(-). The eGFP protein was used as a negative control. L1 chip surfaces were prepared with 2000 RU of APN-GPI(-). The data shown are representative of two independent experiments.
Figure 11. GBP3.1 binds to APN using a pull-down assay. GBP3.1 was incubated with pea aphid guts and proteins bound to GBP3.1 were UV cross-linked and pulled down with streptavidin-agarose beads. A. A protein cross-linked to GBP3.1 (indicated by the arrow) was excised from a Coomassie stained gel and identified as APN by LC MS/MS (See Table 1). The ~140 kDa protein that appeared in the cross-linked and uncross-linked samples bound non-specifically to the beads and was seen in a control sample with streptavidin beads and guts only. B. Western blot analysis with APN antiserum detected APN in the cross-linked GBP3.1 sample (arrow). Controls consisted of GBP3.1 and guts without the crosslinking step and streptavidin beads and buffer only. Gut protein served as a positive control for the APN antiserum. The UV-crosslinking experiment was repeated three times with western blot performed for one of the three experiments.
**Supplementary Table 1.** Pea aphid APN isoforms and percent amino acid identity. Only full length sequences were analyzed. Data presented are based on sequences in the NCBI database only.


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Supplementary Figure 1. The Synthetic GBP3.1 used for receptor identification. A double derivatized synthetic GBP3.1, with a biotin residue at the N-terminus and a UV-crosslinking residue (pbenzoyl-L-phenylalanine, BPa) replacing the tyrosine residue in the loop was used in pull-down assays to identify the GBP3.1 receptor in the pea aphid gut.
CHAPTER 3

The Pea enation mosaic virus readthrough domain is critical for BSA-mediated enhanced transcytosis into the pea aphid hemocoel

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Abstract

Circulative transmission of luteoviruses by aphids relies on virus acquisition from the phloem of an infected plant. The association of phloem sap proteins with virus particles facilitates aphid transmission of the virus by an unknown mechanism. In this study we show that the readthrough domain (RTD) of Pea enation mosaic virus (PEMV) which protrudes from the surface of the virion is sticky, and binds multiple aphid proteins. We also demonstrate increased virus in the hemocoel of aphids fed on artificial diet containing purified PEMV with bovine serum albumin (BSA) compared to aphids fed on virus in the absence of BSA. In contrast, the presence of BSA reduced the amount of a mutant virus lacking RTD (PEMV RNA1Δ), in the aphid hemocoel. Models are presented to account for the possible role of RTD and mechanism by which BSA facilitates virus entry into the aphid hemocoel.

Introduction

Viruses in the family Luteoviridae replicate in parenchyma and companion cells of the host plant prior to movement via plasmodesmata to adjacent sieve elements (SE) (1, 2). Mature SE lack a nucleus and translational machinery and connect to form sieve tubes which are used for long distance movement of macromolecules in plants (3-5). Luteoviruses use
the sieve tubes for movement in the plant. Luteoviruses are transmitted between plants by aphids in a circulative, non-propagative manner (6). Aphids are phloem feeders and use specialized mouthparts called styles to navigate through the layers of plant tissue and probe into SE to ingest phloem sap (6). Plant sap is known to contain a variety of molecules including sugars, amino acids, RNA, and proteins (7-9), and recent studies indicate that sap proteins interact with luteoviruses and facilitate transmission by the aphid vector.

Luteoviruses have positive-sense single stranded RNA genomes that are encapsidated by two structural proteins: a major coat protein (CP, 22 kDa) and a minor coat protein readthrough domain (CP-RTD, 33-55 kDa) produced by readthrough of a leaky stop codon (10, 11). Ingested virions transcytose across the aphid gut epithelium, circulate in the hemocoel, and enter the accessory salivary gland before being released into a healthy plant with saliva during a subsequent aphid feeding (6, 12). It is well established that both the CP and CP-RTD are required for the efficient transport of virions through the aphid (13-19) and it is generally accepted that virus aphid-interactions are mediated by direct interactions between capsid proteins and surface receptors in the aphid. The CP alone is sufficient for transcytosis of the aphid gut epithelium, although the RTD may enhance this process and also determine tropism for the mid- or hindgut (20, 21). The RTD is thought to be involved in recognition and uptake of virions at the ASG (21) as virions lacking the RTD are not transmitted to plants. In the plant, the RTD has been linked to the restriction of virions to the phloem tissue (22).

Plant proteins that associate with luteovirions play a role in aphid transmission (23, 24). *Cucurbit aphid borne yellows virus* (CABYV, genus *Polerovirus*) binds plant phloem proteins and the addition of these proteins to virus solutions artificially fed to aphids
increased virus transmission to host plants (23). Interestingly, the addition of some (but not all) non-phloem proteins including bovine serum albumin (BSA) to artificial diet also increased aphid transmission of CAYBV, suggesting the observed effect was not specific to phloem proteins (23). The number of viral genomes detected in whole insects increased when these phloem proteins were added to the diet, but this was not tested following addition of non-phloem proteins. In a more recent study, the removal of *Cereal yellow dwarf virus* (CYDV, genus *Polerovirus*) associated plant proteins by treatment with sodium sulfite prevented uptake of virions into the hemocoel of aphids fed on artificial diet (24). Also, treated virions were not transmitted by aphids when injected directly into the hemocoel suggesting the inability of virions to cross the ASG (24). These results contradict previous reports suggesting the luteovirus structural proteins alone are sufficient for mediating interactions in the aphid however (13-19, 25, 26).

In contrast to other luteoviruses *Pea enation mosaic virus* (PEMV) is not restricted to the phloem of the plant. PEMV is unusual in that it has two genomic RNAs belonging to two taxonomically distinct groups. PEMV-1 is the sole member of the genus *Enamovirus* (*Luteoviridae*) with genome organization similar to that of the Poleroviruses (27), while PEMV-2 belongs to the genus *Umbravirus* (28). Both genomes are encapsidated separately from structural proteins encoded by PEMV-1. PEMV-2 encodes a putative movement protein necessary for virus movement and systemic infection of the host plant (29), which is lacking in PEMV-1. PEMV provides a useful model virus as it is not restricted to the phloem and can be mechanically inoculated into the plant.

In this study we examined the binding properties of CP and RTD, and tested the impact of BSA on movement of wild type PEMV and a mutant virus that lacks RTD (PEMV
RNA1Δ; Figure 1) into the hemocoel of the pea aphid, *Acyrthosiphon pisum*. Models for the role of RTD in virus uptake into the aphid vector, and for the mechanism of BSA-mediated enhanced transcytosis are presented.

**Materials and Methods**

*Production of eGFP and eGFP-RTD*

The pBAD/His B (Invitrogen) vector was used for expression of eGFP and the PEMV read through domain (RTD) fused to enhanced green fluorescent protein (eGFP). The RTD was fused to the C-terminus of eGFP and inserted into pBAD/His B. Three oligonucleotides were used for integrating the RTD and eGFP DNA sequences: a forward primer containing a PstI site and the 5’ end of the eGFP sequence, an oligonucleotide connecting the 3’ end of the eGFP and the 5’ end of the RTD sequence, and a reverse primer complementary to the 3’ end of the RTD sequence. Primers used for construction of the eGFP-RTD fusion sequence were GFPPstI (5’-GCGCTGCACTgtgcaaggcaggagctg-3’, with the PstI site underlined and lower case text indicating the coding part of the fusion protein), GFPRTD (5’-GGACGAGCTGTACAAGGGGGACGACGCTCCCCCG-3’). The reverse primer was RTDHindIII (5’-GGAAGCTTTAatcaaggactttg-3’, HindIII site underlined).

The eGFP-PCR products were excised from a 1% agarose gel and purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The DNA fragments were digested with Pst I and Hind III, cleaned by using a QIAquick Nucleotide Removal Kit (Qiagen), and ligated into pBAD/His B (previously linearized with PstI and Hind III). For expression of eGFP-RTD and eGFP, competent Top10 cells were transformed with the plasmids peGFP-RTD or peGFP. Cells were cultured in low salt LB medium containing ampicillin (50µg/ml) in an orbital shaker at 250 rpm (37 °C) until the OD600 reached 0.5-0.6. L-(+)-Arabinose
(Sigma) was then added to the culture to a final concentration of 0.02% to induce protein expression. The culture was maintained overnight at ambient temperature with shaking at 220 rpm. The overnight cultures were centrifuged to pellet the cells and pellets were frozen at -80°C prior to protein purification.

The His-tagged fusion proteins were purified using Ni-NTA agarose resin (Qiagen) according to the manufacturer’s directions. Purification was conducted under native conditions using a batch purification method. All purification steps were performed either on ice or at 4°C. Purified proteins were concentrated as needed using an Amicon YM-3 Centricon Centrifugal Filter Device (Millipore) and dialyzed in Side-A-Lyzer Dialysis Cassettes (Pierce) with phosphate buffered saline (PBS). The fusion proteins were stored at -80°C.

*Baculovirus expression of CP-P-eGFP*

The CP-P-eGFP construct (Figure 1) was designed with the proline rich region of the RTD sequence (P) included between the CP and eGFP sequences. The first fragment (CP-P) was obtained by using a CP-RTD construct without the stop codon as template (26). To obtain the CP-P with a downstream 5’ EGFP flanking sequence, primers PEMV 9 F (5’-GCCTCCC TCATGGTGAGCAAGGGCGAG - 3’) and PEMV 18 R (5’-GCCCTTGCTCACCATTCCCT ACGGGAGTGGAACT - 3’) were used. To obtain the EGFP sequence with the upstream proline rich domain, primers PEMV 17F (5’-CCCACTCCCGTAGGAATGGTGAGCAAGGGCGAG - 3’) and PEMV 5R (5’-CGTTACTTGTACAGCTCGTCCATG CCAGCTCGTCCATG - 3’, HindIII site underlined) were used. The full length CP-P-EGFP was PCR amplified by combining these two fragments using the PEMV 19 F (5’-ACGGGATCCACAtgacactcactacca ctatcgagcaaa
tcaaa) and PEMV 5R primers. The BamHI site is underlined and lower case text indicates the coding part of the fusion protein.

The PCR-amplified CP-P-eGFP fragment was digested with BamHI and HindIII and cloned into the BamHI – HindIII sites of pFastBac1 (Invitrogen). Constructs were made with N-terminal His tags and Kozak sequences, as described previously (26). The recombinant bacmid was transfected into Sf9 cells to produce recombinant baculovirus vCP-P-eGFP. The recombinant baculovirus was plaque purified and recombinant CP-P-eGFP was expressed in baculovirus-infected cells using standard procedures (30). Expressed CP-P-eGFP protein was purified using the Ni-NTA agarose resin (Qiagen) as described above.

*Far-western blotting with CP-P-eGFP and eGFP-RTD*

Brush border membrane vesicles (BBMV) of *A. pisum* were prepared from whole aphids as described in (31). To remove contaminants such as salts, lipids, or nucleic acids that interfere with isoelectric focusing, the BBMV was subjected to a 2D-Clean-Up kit (GE Health Sciences). The final pellets from 300 µg of initial protein were resuspended in rehydration buffer containing 2M thiourea, 7M urea, 2% ASB-14, 2% C7BzO, 0.5% IPG buffer (GE Healthcare), and a trace amount of bromophenol blue. A sonicating water bath was used to increase protein solubilization. Reconstituted proteins were centrifuged in a microcentrifuge at 15,200g for 5 min to remove insoluble material. The protein was then applied to 7 cm Immobiline DryStrip gels (GE Healthcare) for overnight rehydration. Both pH 3-10 and 4-7 strips were used. Focusing was performed using the IPGphor (Biorad) as follows: 50V for 10 h, 300V for 1 h, 1000V 1 h (gradient), 5000V 1.5 h (gradient), 5000V 1 h.
After isoelectric focusing, IPG strips were equilibrated for 15 min in Equilibration Buffer I (EB I) (6 M urea, 2% SDS, 0.125 M Tris–HCl (pH 8.8), 30% glycerol and 2% (w/v) DTT) followed by 15 min in EB II (same as EB I but containing 2.5% iodoacetamide instead of DTT). For the second dimension IPG strips were loaded and run on 10% SDS-PAGE gels. Electrophoresis was run under constant voltage at 100V. Gels were either stained with Coomassie blue or equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 0.025% SDS, pH 8.3) prior to overnight transfer to a PVDF membrane at 4°C at 30V.

Membranes were blocked in 5% non-fat dry milk in phosphate buffered saline with 0.1% Tween-20 (PBS-T) overnight at 4°C. Membranes were then incubated the eGFP-fusion proteins (10 µg/mL) in 1% non-fat dry milk in PBS-T overnight at 4°C. Bound ligand was detected using GFP antiserum (Sigma) (1:5000) followed by an HRP-conjugated anti-rabbit IgG (1:5000). The PVDF membranes were incubated in HyGlo Chemiluminescent HRP detection reagent for 1 min, with luminescence detected on X-ray film using standard procedures. Antibody only controls were run in parallel by eliminating incubation with the ligand (eGFP-fusion) to identify non-specific binding of the antibodies to aphid BBMV. The experiments were replicated at least four times with each ligand.

**MALDI-TOF MS/MS protein identification**

Protein spots bound by eGFP-RTD were picked from Coomassie stained gels with reference to probed membranes and submitted to the Iowa State University Protein Facility for identification using MALDI-TOF MS/MS mass spectrometry. Proteins from the stained gels that readily aligned with the probed membrane were selected. Briefly, trypsin digested peptides were analyzed using the QSTAR XL quadrupole TOF mass spectrometer (AB/MDS Sciex, Toronto, Canada) equipped with an oMALDI ion source. After every regular MS
acquisition, MS/MS acquisition was performed against most intensive ions. All spectra were processed by using a MASCOT (MatrixScience, London, UK) database search. Peak lists were generated by Analyst QS (AB/MDS Scix, Toronto, Canada) and were used for MS/MS ion searches. Protein identification was based on the probability based Mowse Score. The significance threshold $p$ was set to less than 0.05.

**Purification of Pea enation mosaic virus**

Seven day old pea plants (*Pisum sativum*) were mechanically infected with wild type PEMV or PEMV RNA1Δ as described in (32) and harvested at 10-14 days post infection. PEMV RNA1Δ is a naturally selected mutant that lacks the majority of the coding sequence for the readthrough domain (RTD) and does not produce the RTD polypeptide (Figure 1) (33). The method used to purify PEMV from plants was modified from Liu et al., 2009 (32). PEMV infected plant tissue was frozen in liquid nitrogen and homogenized in a blender with 0.2 M sodium acetate pH 6 (1mL/gram of tissue) and an equal volume of chloroform. The homogenized tissue suspension was centrifuged at 3000g for 10 min. The supernatant was transferred to clean tubes and centrifuged 17,200g for 2 h. Pellets were saved and supernatant was again centrifuged at 141,000g for 2.5 h. All pellets were soaked in 0.2 M sodium acetate pH 7 overnight at 4°C and then resuspended. The soluble fraction was centrifuged at 147,000g through a 30% sucrose cushion made 0.2 M sodium acetate, pH 7. The final pellet was washed three times in 0.2 M sodium acetate buffer to remove excess sucrose and resuspended in the same buffer. Sample purity was assessed by SDS-PAGE analysis. The protein concentration of PEMV was determined by densitometric analysis with Image J software (34) of the Coomassie Brilliant Blue R-250 stained bands with reference to known BSA concentrations resolved by SDS–PAGE.
Aphid feeding assays and hemolymph collection

Prior to feeding, pea aphids were starved for 3 hr at room temperature. Aphids were fed for approximately 16 hr by Parafilm® membrane feeding (35) on 20% sucrose in phosphate buffered saline (PBS). The sucrose diet was supplemented with 200 ng/µl of wild type PEMV or PEMV RNA1Δ (Figure 1) with or without the addition of 600 ng/µl of bovine serum albumin (BSA, Promega). Aphids were fed on the 20% sucrose with BSA alone as a negative control. Hemolymph was collected from 30 aphids per treatment using a wax embedding method (36) and stored in Trizol reagent (Invitrogen) prior to RNA purification using the manufacturer’s protocol. Total RNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific).

Semi-quantitative RT-PCR detection of PEMV RNA

Superscript III reverse transcriptase (Invitrogen) was used according to the manufacturer’s protocol to generate cDNA from both PEMV RNA and pea aphid actin using gene specific primers (Table 1). Equal amounts of cDNA from each treatment were amplified by PCR using the primer sets in Table 1. PCR conditions were as follows: 94°C for 2 min, 28-32 cycles of 94°C for 30 sec, 52°C for 30 sec, 72°C for 30 sec, followed by a single cycle of 72°C for 3 min. PCR products were run on 1.5 % agarose gels, stained with ethidium bromide, and imaged using a gel imager (Fotodyne). Band intensities of both PEMV and actin products were quantified by densitometric analysis using Image J software (34). Image J values for wild type PEMV were normalized to the corresponding values obtained for actin. Three independent experiments were performed for both wild type and PEMV RNA1Δ. Statistical differences between the treatments for wild type PEMV were determined by a Students t-test.
Quantitative RT-PCR detection of RNA1Δ

For quantitative RT-PCR (qRT-PCR) analysis, 25 ng of total RNA and the gene-specific primers (Table 1) at a concentration of 5 pmol were used. The qRT-PCR was performed using a qScript One-Step SYBR Green qRT-PCR kit (Quanta Biosciences) according to the manufacturer's recommendations in the iCycler IQ system (Bio-Rad). PCR cycles were as follows: 1 cycle of 10 min at 50°C and 5 min at 95°C, followed by 45 cycles each of 10 sec at 95°C and 30 s at 60°C. A dissociation curve was produced at the end of the cycling phase to ensure that a single PCR product was produced with no primer dimers. Relative quantifications of PEMV RNA accumulation in the hemolymph were calculated using the comparative C_T method (ΔΔC_T method) (37). Three technical replicates of each reaction were used. Only one biological replicate yielded quality qRT-PCR data (with Ct values < 30) for PEMV RNA1Δ.

Surface plasmon resonance binding assays

Surface plasmon resonance (SPR) assays using a BIAcore T100 (BIAcore, Uppsala, Sweden) (38) were used for quantification of relative binding of CP-P-eGFP, eGFP and RTD-eGFP to BSA. The buffer HBS-N (BIAcore) was used for all experiments. Preparations of CP-P-eGFP, eGFP and RTD-eGFP were dialysed in HBS-N to a final concentration of 6 µM. BSA (Promega) was immobilized on to the carboxymethylated dextran (CM5) sensor chip surface (BIAcore) through standard amine coupling method. Carboxyl groups along the CM-dextran chains of the sensor chip surface were activated by injecting a mixture of NHS ((0.1M 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride) (1:1, vol/vol). BSA in coupling buffer (10 mM sodium acetate, pH 4.5) was injected over the chip surface at 0.1 µg/µl to obtain an immobilization target level of 4000
resonance units (RU). After coupling, unreacted surface ester groups were blocked by injecting 1 M ethanolamine (pH 8.5) on the chip surface. Analysis of the interaction between BSA and CP-P-eGFP, eGFP and RTD-eGFP was performed by injecting proteins (6 µM in HBS-N) at 30 µl/min for 60 s, dissociation for 60 s, regeneration with 50 mM NaOH for 60 s and stabilization for 120 s. Reference flow cells without immobilization of BSA were included in the experiments. SPR assays were conducted twice and data analyzed by one-way ANOVA.

Results

RTD binds multiple aphid proteins

A far-western blot method was used to identify pea aphid membrane proteins that bind CP-P-eGFP, eGFP-RTD and eGFP only (Figure 2). While CP-P-eGFP bound specifically to the APN (identified in Chapter 2), eGFP-RTD bound to APN and many other aphid proteins (Figure 2). The identities of some of the proteins bound by RTD are listed in Table 2. The additional binding observed in both the CP-P-eGFP and eGFP blots was attributed to binding of the GFP antibody to aphid BBMV proteins.

BSA increases the uptake of wild type PEMV but not PEMV RNA1Δ into the aphid hemocoel

Purified PEMV virions were fed to pea aphids in artificial diet with or without the addition of BSA to investigate whether the presence of BSA affects virus uptake. Increased WT PEMV RNA was detected by semi-quantitative RT-PCR in the hemolymph of aphids fed with BSA compared to aphids fed on virus alone (Figure 3). There was a 2.8-fold average increase in WT PEMV in the hemolymph when co-fed with BSA across three independent experiments. The data were statistically significant (p < 0.05) as determined by
a Student’s t-test with a p-value = 0.017. In contrast, the presence of BSA did not increase movement of PEMV RNA1Δ into the aphid hemocoel, but rather impeded uptake of the virus (Figure 4). Relative quantification from qRT-PCR data indicated 2.3-fold less PEMV RNA1Δ when aphids were fed on virus with BSA. Semi-quantitative data supported the qRT-PCR data for PEMV RNA1Δ showing less virus detected when co-fed with BSA in three independent experiments (Figure 4). RT-PCR products for pea aphid actin were unattainable for the PEMV RNA1Δ experiments preventing normalization of virus amounts. QRT-PCR experiments indicated that more WT PEMV was present in the aphid hemocoel compared to PEMV RNA1Δ, but this needs to be repeated.

**BSA binds more strongly to CP than to RTD**

Surface plasmon resonance (SPR) was used to quantify the real-time binding of CP-P-eGFP and eGFP-RTD to BSA with eGFP used as a negative control protein (Figure 5). The binding of CP-P-eGFP to BSA was significantly stronger than that of eGFP-RTD. The relative binding units ± SEM to BSA were: eGFP, 13.60 ± 0.25; eGFP-RTD, 48.73 ± 0.00 (p = 8.06E-05); CP-P-EGFP, 344.52 ± 2.88 (p = 0.0001). In contrast to CP-P-eGFP, eGFP-RTD dissociated rapidly from BSA (Figure 5).

**Discussion**

**Function of RTD.** We previously identified APN as the gut receptor for PEMV and the CP alone is sufficient for binding to APN (Figure 2). This result agrees with the observation that luteovirions lacking the RTD can cross the gut barrier and enter the aphid hemocoel (12, 17, 20, 39). However, the RTD may enhance virion transcytosis of the aphid gut epithelium (20). The RTD is required for aphid transmission, although its role is not fully understood. The RTD may determine tropism for the mid- or hindgut and determine aphid transmission
specificity, possibly through interactions with the accessory salivary glands (20, 21). In this study we demonstrated that PEMV RTD bound to APN, along with many other aphid proteins indicating that RTD is “sticky”. The hydrophilicity profile for RTD confirms that this protein would be accessible for binding (Supplementary Figure 1). However, using SPR analysis the binding of the RTD to APN was weak compared to CP binding to APN (Chapter 2). One aphid protein of note that was bound by the RTD was glyceraldehyde 3-phosphate dehydrogenase (GAPDH3). GAPDH3 was also bound by Beet western yellows virus (BWYV) and Rice stripe virus, in their respective vectors, Myzus persicae (green peach aphid) and Laodelphax striatellus (small brown planthopper) (40, 41). It is unknown whether RTD accounts for virus binding to these proteins in these cases.

*Multiple proteins can increase luteovirus uptake into aphids.* The restriction of luteoviruses to the plant phloem is ideal for acquisition and transmission of these viruses by aphids. The implication of plant proteins in virus-aphid interaction (23, 24) questions the hypothesis that the viral structural proteins (CP and RTD) are the sole determinants of virus transport through the aphid. In this study we investigated whether co-feeding aphids on PEMV with BSA alters virus uptake into the aphid hemocoel. We demonstrated that BSA increased uptake of WT PEMV into aphids, consistent with increased transmission when CABYV virions were co-fed with several non-phloem proteins, including BSA (23). Bencharki et al. (23) showed that the addition of BSA to aphid artificial diet increased the efficiency of CABYV transmission from 2.7% (2/72 plants) to 66.7% (28/42 plants). Other non-phloem proteins that also increased CABYV transmission were casein, lysozyme, cytochrome C, and carbonic anhydrase (23). In that same study, two phloem lectins from cucumber (*Cucumis sativus*), CsLec26 and CsLec17, that bound to CABYV also increased virus transmission.
These proteins belong to the PP2 lectin family, which bind poly-GlcNAc and high mannose N-glycans (42, 43). The authors used recombinant orthologs of these PP2 lectins from *Arabidopsis thaliana* for their experiments. Addition of these lectins to the artificial diet increased transmission efficiency to 80%. Interestingly, two other lectins, LeH from *Lens culinaris* (specific for α-D-mannose and α-D-glucose) and MPA from *Maculura pomifera* specific for Gal(β1-3)GalNAc did not enhance CABYV transmission (0/9 plants for each) (23). In our experiments, the presence of BSA increased uptake of WT PEMV into the hemocoel 2.8-fold compared to aphids fed on virus alone, which is comparable to the 2.5-fold increase in the amount of CABYV genomes detected in whole insects when co-fed with the PP2 lectin (23).

**BSA impedes uptake of PEMV RNA1Δ.** When BSA was fed with the RTD deletion mutant, PEMV RNA1Δ, there was a decrease in the amount of viral RNA in the hemolymph compared to aphids fed on virus alone (Figure 4). Although PEMV RNA1Δ virions do not produce any readthrough protein and are not aphid transmissible, the virus is still capable of crossing the aphid gut (32). Our lab has previously shown that PEMV RNA1Δ accumulates in the aphid hemolymph to levels comparable to WT PEMV when feeding on infected plants (32). However, it was noted that levels of PEMV RNA1Δ in plants were greater than levels of WT PEMV, which complicates comparison of the efficiency of gut transcytosis between the two viruses based on data provided for that study (32). Our current results for aphids fed on equal amounts of the two viruses, WT PEMV and PEMV RNA1Δ, suggest the RTD is critical for both virus interaction with the gut epithelium and for the enhanced virus uptake conferred by BSA. SPR analysis showed that binding of CP to BSA was much stronger than the binding of the RTD to BSA, which implies that BSA should interact with both WT
PEMV and PEMV RNA1Δ. While qRT-PCR experiments indicated that more WT PEMV was present in the aphid hemocoel compared to PEMV RNA1Δ, additional replication is required to confirm these results.

Two models for the impact of BSA on PEMV uptake. BSA has non-specific binding properties and is often used as a blocking agent. Albumin also binds to some glycoproteins in a saturable and specific manner however (44, 45). Both BSA and plant lectins cross the gut epithelium into the hemocoel in several insect species (46-51). Hence, these proteins may have specific receptors in the insect gut epithelium which promote transcytosis into the hemocoel. We propose two possible models to account for role of RTD and the impact of BSA on the movement of PEMV and PEMV RNA1Δ into the aphid hemocoel. For the first model (Figure 6), in the absence of BSA, the RTD provides an anchor to the gut epithelium allowing PEMV to efficiently bind APN. When BSA is present, BSA binds to the CP (and the RTD to a lesser extent) and the entry of the PEMV-BSA complex is mediated by both BSA receptors, and PEMV receptors on the aphid gut epithelium. The use of two separate receptors results in more efficient delivery of the virus into the hemocoel. In the absence of RTD, PEMV RNA1Δ is not anchored to the membrane thus reducing virus binding and uptake. Although BSA could still bind the virus, excess BSA may prevent uptake by blocking the BSA receptor. A critical component for this model is that the RTD in WT PEMV provides an anchor that increases the efficiency of uptake relative to PEMV RNA1Δ. Therefore, uptake of PEMV RNA1Δ would be less efficient than uptake of WT PEMV in the absence of BSA. Liu et al. (32) concluded that WT PEMV and PEMV RNA1Δ accumulate to comparable levels in the aphid hemolymph. However, this was determined by feeding aphids on infected plants where the virus titers in the plant could differ between the two
viruses. Membrane feeding assays with equal concentration of virus would be more appropriate to compare transcytosis efficiencies. In support of the RTD anchor model, Reinbold et al. (20) demonstrated that a RTD deficient mutant of Beet western yellow virus (genus Polerovirus) was less efficiently transcytosed across the aphid gut epithelium. These authors also described an RTD anchor model to explain the reduced uptake of the RTD-deficient BWYV (20, 52).

For the second model (Figure 7), the RTD functions to bind proteins such as BSA that might compete with PEMV for binding to APN. The binding of CP and RTD to BSA still facilitates uptake of the PEMV-BSA complex by using both the BSA and virus receptors. Without the RTD, free BSA competes with PEMV RNA1Δ for binding to the receptors thereby reducing virus entry. This model assumes that the efficiency of virus uptake into the aphid is similar between WT PEMV and PEMV RNA1Δ in the absence of BSA. It is unknown whether BSA binds to APN, but it is known that some plant lectins bind APN (53). Such lectins might compete with plant viruses for receptor binding. The validity of the second model may depend on the relative concentration of BSA (or plant protein) and virus, as to whether excess BSA (or plant protein) would be available to impede virus uptake. Additional work is needed to identify the receptors for BSA in the aphid gut and to address whether BSA binds to APN.

Critical components to both of these models are: i) the ability of BSA to independently transcytose across the gut epithelium and ii) an interaction between BSA and the virus. Other proteins that are also transcytosed across the gut epithelium may facilitate virus movement, while proteins that do not move across the gut epithelium may not facilitate virus entry into the hemocoel. Casein is known to cross the insect gut (54) and accordingly
this protein also increased transmission efficiency of CABYV (23). The snowdrop lectin, *Galanthus nivalis* agglutinin (GNA), is known to cross the gut of some insects (48-51). While it is unknown whether the PP2 lectins in the Bencharki et al. study (23) cross the aphid gut, the mannose binding properties of these lectins (42, 43) similar to GNA suggest they may also move across the gut epithelium. Some lectins, such as Concanavalin A (ConA) and GNA are known to bind to APN (53), which functions as the receptor for PEMV (Chapter 2). The fact that some plant lectins are known to bind to the same receptor proteins could provide evolutionary selection for viruses that enter via the hindgut of the vector. By the time gut contents reach the hindgut, most of the plant lectins (which are designed for anti-herbivore defense) may already be bound to midgut epithelial proteins such that competition for binding is reduced in the hindgut.

**BSA protection of virions.** In alternative scenarios, the association of BSA with PEMV may protect the virus in the aphid gut from degradation by proteolytic enzymes. However, if true, increased uptake of PEMV RNA1Δ would be expected in the presence of BSA, which would provide an alternative substrate for proteolytic enzymes for both viruses.

If BSA remains associated with PEMV in the hemocoel, BSA may protect the virus from the insect immune response, or from uptake from the hemolymph by the pericardial cells for subsequent degradation (55). Transcriptomic analysis of intestinal gene expression in the pea aphid following PEMV acquisition revealed that only 1.9% of genes were differentially expressed in the presence of the virus (56). The maximum levels of up or down regulation were 1.37-fold and 3.45-fold, respectively, suggesting the virus does not trigger much of a response from the aphid (56). However, this transcriptome was limited to the gut and may not reflect the complete insect response to virions. Knowledge of the fate of
luteoviruses within the aphid hemocoel is based largely on TEM studies. There have been no reports of virions in the pericardial cells.

We hypothesize that luteovirus binding of plant phloem proteins increases the efficiency of virus uptake by the aphid vector and subsequent virus transmission. Although this was shown using in vitro feeding assays with purified virus, it is possible that plant proteins copurified with WT virions and that these proteins were absent from the purified PEMV RNA1Δ mutant virus. Plant proteins that co-purified with WT virus may then have facilitated virus uptake by the vector. Interestingly, a plant glycoprotein from Montia perfoliata co-purified with CABYV and Turnip yellows virus (TuYV) virions (57). We have not analyzed our virus samples for co-purifying plant proteins, but there is no apparent difference in the protein profiles of our purified virus preparations using Coomassie Blue – stained SDS-polyacrylamide gels (data not shown). The RTD is also hypothesized to determine tropism for the mid- or hindgut (21). Swapping the RTD sequences between luteoviruses altered the intestinal sites of virus endocytosis (21). This result could be linked to a difference in plant protein association between the RTD sequences and associated receptors used for uptake by the virus – plant protein complex.

Role of plant proteins in virus uptake. Cilia et al. (24) reported that treating virus preparations of CYDV with sodium sulfite to remove associated host proteins abolishes the ability of virions to cross the aphid gut or salivary gland barriers. Sodium sulfite reduces disulfide bonds which could disrupt bonds in a single CP subunit, between CP subunits of a virus particle, or between the virus and associated plant proteins. Although virion morphology did not appear altered by the sodium sulfite treatment when observed by TEM, it is difficult to rule out the possibility that the virus capsid was not disrupted in any way. The
requirement for plant proteins to associate with virions in order to cross the aphid epithelial barriers seems unlikely given baculovirus expressed luteovirus coat proteins that form virus particles are capable of crossing the aphid gut (25, 26). Additionally, we have shown that both PEMV virions and a recombinant PEMV CP-eGFP fusion bind to the pea aphid gut receptor, aminopeptidase N (Chapter 2). Taken together, this suggests the virus structural proteins alone are sufficient for virus binding and uptake from the aphid gut into the hemocoel. Although plant proteins may enhance uptake of luteovirions, removal of virus associated plant proteins should not completely eliminate the ability of virions to the cross gut and salivary gland barriers.

The presence of plant proteins may allow sufficient titers of virus to accumulate in the hemolymph and subsequently move into the ASG for transmission. Luteovirus transmission requires a latent period, or time between virus acquisition and when the aphid can transmit the virus. This implies that the virus must accumulate to a certain threshold before virions are able to enter the ASG and be transmitted with the aphid saliva. The association of plant proteins with virions may allow for higher viral titers in the hemolymph, thereby indirectly enhancing movement across the ASG. Virions lacking the RTD can cross the gut but are not transmitted (6).

Our models for the uptake of PEMV may help explain why RTD deficient viruses are not efficiently transmitted. Additional work is needed to determine how these interactions between virus, aphid, and plant proteins facilitate virus movement through the aphid. Our results with PEMV and BSA provide insight into the possible roles of phloem proteins in virus-aphid interactions.
Author Contributions

LBL set up feeding assays, performed semi-quantitative RT-PCR, analyzed the data, and wrote the manuscript. BRD assisted with feeding assays, hemolymph isolations, and RT-PCR analyses. SL assisted with hemolymph isolation. VP conducted quantitative RT-PCR experiments. NPC performed the SPR analyses. BCB contributed to the experimental design and writing of the manuscript.

Acknowledgements

This work was funded by the USDA AFRI 2008-03996, by the Iowa State University Plant Sciences Institute Virus-Insect Interactions Initiative, and by Hatch Act and State of Iowa funds.

References


Tables and Figures

**Figure 1.** Schematic diagrams of wild type PEMV, PEMV RNA1Δ, CP-P-eGFP, and eGFP-RTD constructs. The length of the genomic RNA is indicated on the right. The short ORFs remaining of the RTD in PEMV RNA1Δ are in a different reading frame and do not produce a translated protein product (32). This figure is modified from (32).
Table 1. Primers used for semi-quantitative and quantitative RT-PCR detection of PEMV and actin in pea aphid hemolymph.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<th>Product size (bp)</th>
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<tr>
<td>PEMV3621-F</td>
<td>5’-TCCGTGCCCCTGATGTCGTT-3’</td>
<td>PEMV-1</td>
<td>196</td>
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<tr>
<td>PEMV3779-R</td>
<td>5’-ATCCCCAACCCCGAGATGCT-3’</td>
<td>PEMV-1</td>
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<tr>
<td>q-Actin-F</td>
<td>5’-ATTGAACCCCAAGCTAATC-3’</td>
<td><em>A. pisum</em> actin</td>
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</tr>
<tr>
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<td><em>A. pisum</em> actin</td>
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</table>
Figure 2. Binding of CP-P-eGFP and eGFP-RTD to pea aphid BBMV. Far western blotting conducted using pH 3 to 10 and 4 to 7 was replicated at least 4 times with each ligand. Arrows in the CP-P-eGFP blot indicate binding to pea aphid APN. The additional binding observed in both the CP-P-eGFP and eGFP control blots was attributed to binding of the GFP antibody to aphid BBMV proteins. The bottom panels indicate the proteins bound by eGFP-RTD that were identified by MALDI-TOF MS/MS from a Coomassie stained gel (left) with reference to the probed membrane (right). The selected proteins are indicated by arrows and are listed in Table 2.
Table 2. MALDI-TOF MS/MS identification of pea aphid proteins bound by eGFP-RTD in Figure 2. Proteins that could be aligned with confidence to the far-western blot were selected for analysis.

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Protein Name</th>
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<th>Mowse Score</th>
<th>Coverage (%)</th>
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<td>gi</td>
<td>31214985</td>
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**Figure 3.** BSA increases the uptake of WT PEMV into the pea aphid hemocoel. Band intensities of semi-quantitative RT-PCR products for both PEMV and actin (at left) were quantified using Image J software and normalized to the amount of actin (at right). There was a 2.8 -fold average increase in the amount of PEMV in the hemocoel when co-fed with BSA across three replicates. The presence of BSA resulted in significantly more virus in the aphid hemolymph (Student’s T-test, \( p = 0.017 \)).
**Figure 4.** BSA decreases the amount of PEMV RNA1Δ in the aphid hemocoel. Less viral RNA was detected in the hemolymph using semi-quantitative RT-PCR when co-fed with BSA (left) in three independent experiments. A 2.3-fold reduction in viral RNA in aphid hemolymph was detected by qRT-PCR when BSA was present (right). For qRT-PCR, relative quantifications were determined by the comparative ΔΔCt method (37) with actin as the reference gene. Only one biological replicate yielded quality qRT-PCR data (with Ct values < 30) for experiments with PEMV RNA1Δ.
**Figure 5.** Analysis of CP-P-eGFP, eGFP-RTD, eGFP interaction with BSA. Sensorgram from BIAcore surface plasmon resonance analysis showing the real-time interaction between 6 µM of the eGFP-fusion proteins and immobilized BSA. The eGFP protein was used as a negative control. L1 chip surfaces were prepared with 4000RU of BSA. The data shown are representative of two experiments.
Figure 6. Model 1: RTD functions to anchor the virion to the aphid gut epithelium. a) In the absence of BSA, the RTD provides an anchor to the gut epithelium allowing PEMV to bind APN. b) When BSA is present, BSA binds to the CP (and the RTD to a lesser extent) and the entry of the PEMV-BSA complex is mediated by both BSA receptors and PEMV receptors on the aphid gut epithelium. The use of two separate receptors results in more efficient delivery of the virus into the hemocoel. c) In the absence of RTD, PEMV RNA1Δ is not anchored to the membrane thus reducing virus binding and uptake. The presence of BSA further reduces binding and uptake by competing for binding sites. For this model, the uptake of PEMV RNA1Δ would be less efficient than that of WT PEMV in the absence of BSA.
Figure 7. Model 2: RTD functions to bind proteins that would otherwise compete with virus for receptor binding. a) Virus binding to the aphid gut epithelium is largely mediated by CP. b) Binding of BSA to CP and RTD facilitates uptake of the PEMV-BSA complex by using both the BSA and virus receptors. c) Without the RTD, free BSA competes with PEMV RNA1Δ for binding to the receptors. For this model, the efficiency of uptake of PEMV RNA1Δ would be comparable to that of WT PEMV in the absence of BSA.
Supplementary Figure 1. Predicted properties of PEMV readthrough domain using Protean prediction software (DNAsar Inc. v. 5.0). The antigenicity profiles were predicted using the Jameson–Wolf index and DNAsar 5.0. The y axes represent probability. The hydrophilicity plot indicates that the RTD would be available for binding to other proteins in the plant and the aphid gut.
CHAPTER 4

The role of carbohydrates in PEMV-pea aphid interactions

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Abstract

Glycans are known to function in the binding of animal viruses to host cells, but little has been done on the potential role of glycans mediating plant virus-aphid vector interactions. The aphid gut is heavily glycosylated with mannose residues. We previously identified the glycoprotein aminopeptidase N (APN) as the gut receptor for the luteovirus, \textit{Pea enation mosaic virus} (PEMV). APN is glycosylated with mannose residues and is a receptor for mannose binding plant lectins. By using lectin blot analysis of brush border membrane vesicles (BBMV) with the lectins Concanavalin A (ConA) and \textit{Galanthus nivalis} agglutinin (GNA), we confirmed that pea aphid gut proteins are glycosylated with mannose and possibly glucose residues. We tested for PEMV binding to a synthesized tri-mannose glycan that is common in insects. There was no binding of PEMV to the mannose sugars using isothermal titration calorimetry (ITC) or a carbohydrate microarray. These results suggest mannose is not involved in PEMV-APN binding. ITC showed binding of ConA to PEMV indicating the potential glycosylation of virus structural proteins. The potential role of PEMV virion glycosylation in interaction with plant lectins that enhance aphid-mediated virus transmission, and with aphid gut and salivary gland receptors are discussed.
Introduction

*Pea enation mosaic virus* (PEMV) is vectored by aphids in a circulative-nonpropagative manner. PEMV consists of two taxonomically distinct positive-sense RNAs. PEMV-1 is the sole member of the genus *Enamovirus* (*Luteoviridae*) with genome organization similar to that of the Poleroviruses (1), while PEMV-2 belongs to the genus *Umbravirus* (2). Successful virus transmission involves specific interactions between the virus and the aphid vector. The virus binds a receptor for transcytosis across the aphid gut epithelium and is released into the hemocoel (3). Receptor(s) are also involved in movement of the virus from the hemolymph into the accessory salivary glands (ASG) from which virus particles are secreted with saliva into the phloem of the plant (4). The viral coat proteins consist of a major coat protein (CP) and a minor coat protein readthrough domain (CP-RTD) which are the sole determinants of vector specificity (5). Both the CP and RTD are essential for aphid transmission, although the CP alone is sufficient for virus particle assembly and transcytosis across the aphid gut epithelium (6-8). The RTD is hypothesized to function in uptake of virions at the ASG as virions lacking the RTD are not transmitted to plants by the aphid vector (3, 9).

Protein glycosylation is important for many biological processes such as cell-cell interactions, signal transduction, and intracellular protein trafficking (10, 11). Protein glycosylation is a co- or post-translational modification that is either N-linked, i.e. sugars are attached by a glycosidic bond to asparagine residues, or O-linked in which sugars are connected to serine or threonine (11). N-linked glycans are found on glycoproteins from bacteria to mammals (12, 13). Although the enzymes required to generate complex glycans have been found in insects, the glycan structures typically consist of high mannose or
paucimannose structures and are less complex than in mammals (10). The most common insect N-glycan structures share a common terminal tri-mannoside structure containing a β-mannoside with two α-mannosides attached at the O-3 and O-6 positions (Figure 1).

More complex insect glycans have recently been discovered and the greater diversity of glycans across insect species is being realized (11). It is still evident that mannose residues are the most abundant glycans in insects. Mannose binding lectins exhibit toxicity to various insects including aphids (14-20). Insect midgut proteins including ferritin, sucrase, or aminopeptidase N are known targets for these mannose-binding plant lectins (14, 16, 19, 21). Aminopeptidase N (APN) is of interest because we have determined that APN is the gut receptor for Pea enation mosaic virus (PEMV, Chapter 2). An estimated 16% of the molecular mass of pea aphid APN is comprised of carbohydrates (20) and glycans may therefore be involved in PEMV-APN interaction.

While several examples of glycan involvement in host cell recognition of animal viruses exist (22-24), little is known about the role of glycans in plant virus-insect interactions. Sialic acid residues are sufficient for attachment of influenza virus (25). In some cases viruses interact with glycoproteins for initial attachment which stimulates a conformational change in the host cell allowing virus binding to a second receptor (24). Increasing evidence suggests that glycans are critical for uptake of insect vectored pathogens (26-29). Glycans on the surface of mosquito-borne Dengue virus and West Nile virus may be important for receptor attachment (29, 30). A non-viral pathogen, the malaria parasite, Plasmodium yoelii also uses glycans in vector interactions. An antibody that binds α-mannose on mosquito midgut microvilli blocked development of the parasite (27) indicating that mannose may be required for binding.
There is relatively little information on the glycosylation of plant viruses. The *Potato virus X* and the aphid transmitted *Plum pox virus* have been shown to be O-glycosylated (31-33). The role of these modifications has not been determined. Another aphid transmitted virus, *Lettuce necrotic yellow virus*, is reported to have N-linked complex oligosaccharides (34). *Tomato spotted wilt virus* (TSWV) is an enveloped virus in the family *Bunyaviridae* that is transmitted by Western flower thrips, *Frankliniella occidentalis* (35). TSWV encodes two glycoproteins G\textsubscript{N} and G\textsubscript{C}. G\textsubscript{N} is critical to infection of thrips and is required for binding to the thrips midgut (35). Only two studies have investigated the glycosylation of luteovirus structural proteins (36, 37). The earlier of the two studies suggested the capsid proteins of *Turnip yellows virus* (TuYV, formerly named *Beet western yellows virus*) contain α-D-galactose residues that when altered by N-glycosidase or α-D-galactosidase disrupted aphid transmission (36). In contrast, there was no evidence that the structural proteins of TuYV and a related polerovirus, *Cucurbit aphid-borne yellows virus* (CABYV) are glycosylated and consequently argued against the role of virus glycosylation in aphid transmission (37). The number and location of predicted glycosylation sites is variable among luteoviruses (37) (Table 1). The structural proteins of PEMV contain four predicted sites for N-glycosylation based on sequence analysis. The four sites are found in the coat protein (CP) sequence. There are also five predicted O-glycosylation sites, two sites in the CP and three in the RTD.

In this study we used a carbohydrate microarray and isothermal titration calorimetry (ITC) to investigate the binding of PEMV to a synthesized insect tri-mannose designed based on common glycan structures in insects. We also used ITC to show that the lectin ConA binds to PEMV confirming glycosylation of the virus capsid proteins.
Materials and Methods

Prediction of PEMV glycosylation sites

O-linked N-acetylgalactosamine glycosylation sites in the PEMV coat protein sequence were predicted using NetOGlyc 3.1 (http://www.cbs.dtu.dk/services/NetOGlyc/). N-linked glycosylation sites for PEMV were predicted using NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/). Putative glycosylation sites for the other luteoviruses were taken from Revollon et al. (37) where the authors used the same glycosylation prediction programs. The parameters for the glycosylation prediction programs were used as described in (37).

Lectin blotting of pea aphid BBMV and gut protein

BBMV were prepared from whole pea aphids using the method described in (38). To extract total gut protein, pea aphid guts were dissected in PBS in the presence of protease inhibitors at a 1:100 dilution (Sigma) and homogenized with a pestle. The homogenate was clarified by centrifugation at 14,000g to remove cell debris and the supernatant was collected. All protein concentrations were determined by Bradford assay. For lectin blotting, 10 µg of BBMV or gut extract was separated by SDS-PAGE, transferred to PVDF membrane, and probed with two different lectins, ConA and GNA, at a concentration of 5 µg/ml. Two µg of bovine serum albumin (BSA, Promega) was loaded as a negative control. A commercially available Con A conjugated to horse radish peroxidase (AMS Biotechnology) was used for detection of ConA binding. Bound GNA was detected with GNA antiserum (39) (1:5000) and an anti-rabbit HRP-conjugated secondary antibody (1:5000). Purified GNA (500 ng) was used as a positive control for the GNA antibody.
HyGlo Chemiluminescent HRP detection reagent (Denville Scientific) was used and luminescence detected on autoradiography film using standard procedures.

*PEMV purification and FITC labeling*

PEMV was purified using a method modified from (40). PEMV infected plant tissue was frozen in liquid nitrogen and homogenized in a blender with 0.2 M sodium acetate pH 6 (1mL/gram of tissue) and an equal volume of chloroform. The homogenized tissue suspension was centrifuged at 3000g for 10 min. The supernatant was transferred to clean tubes and centrifuged 17,200g for 2 h. Pellets were saved and supernatant was again centrifuged at 141,000g for 2.5 h. All pellets were soaked in 0.2 M sodium acetate pH 7 overnight at 4°C and then resuspended. The soluble fraction was centrifuged at 147,000g through a 30% sucrose cushion made 0.2 M sodium acetate, pH 7. The final pellet was washed three times in 0.2 M sodium acetate buffer to remove excess sucrose and resuspended in the same buffer. Sample purity was assessed by SDS PAGE analysis. The protein concentration of PEMV was determined by densitometric analysis with Image J software (41) of the Coomassie stained bands with reference to known BSA concentrations resolved by SDS–PAGE.

The purified virus was labeled with fluorescein isothiocyanate (FITC) using an FITC Antibody Labeling Kit (Pierce) following the manufacturer’s protocol. The labeling reaction was completed in the virus purification buffer (0.2M sodium acetate, pH 7), instead of the recommended 50mM sodium borate at pH 8.5. This was done to avoid precipitation of the PEMV virions at a more basic pH. To confirm FITC labeling of PEMV, the viral protein were separated by SDS-PAGE and the gel scanned using a Typhoon 9410 Variable Mode Imager (Amersham Pharmacia Biotech) in the green-excitation mode (532nm).
Carbohydrate microarray and isothermal titration calorimetry

The detailed protocols for synthesis of insect glycans, the carbohydrate microarray, and ITC experiments can be found in (42). Briefly, for the carbohydrate microarray the N-glycan trimannosides (Figure 1) were synthesized with fluorous tags (F-tag) and attached to a fluorous coated glass slide. The control sugars in the experiment were F-tag modified α-mannoside and β-galactoside. The slides were incubated for 1hr with the FITC-labeled PEMV or FITC-labeled ConA (positive control), washed twice in PBS and once in deionized water, and then scanned at the Iowa State University DNA Facility to visualize fluorescence.

For ITC, the fluorous tags were removed from the sugars. The ITC experiments involved titration of the mannoses into a mixing cell containing PEMV. ConA was used as a positive control. The temperature of the mixing cell was compared with a reference cell and the difference in heat was measured over the course of the titration. At the end of each experiment, $K_d$ (dissociation constant), $\Delta H$ (enthalpy), $\Delta S$ (entropy), and $N$ (reaction stoichiometry) were extracted from the resulting data.

Results

The pea aphid gut is glycosylated with mannose groups

To determine the extent of glycosylation in the pea aphid gut, BBMV and gut protein extracts were probed with the lectins ConA and GNA. ConA binds to α-mannose and α-glucose sugar groups and GNA recognizes high α-mannose structures. Based on comparison of the lectin blots to the Coomassie stained gels a large percentage of the proteins were bound by the lectins (Figure 2). This indicates an abundance of mannose and possible glucose residues present in the gut. There was a difference in the profiles of the two lectins.
The lectins did not bind to BSA which was used as a negative control. The molecular mass of GNA is 50 kDa and exists as four identical subunits of approximately 13Kda. The GNA antibody recognized proteins in the positive control migrating at about 20-25 kDa and 200 kDa (Figure 2), which are likely to be multimers of GNA. A protein migrating slightly higher than 150 kDa that corresponds to the size of APN was recognized by ConA in both BBMV and gut protein samples (Figure 2).

*PEMV does not bind to mannose*

A carbohydrate microarray and ITC were used to test the binding of PEMV to α-mannose and the synthesized insect tri-mannose. In the microarray, synthesized sugars were attached to a fluorous chip and incubated with FITC-labeled PEMV. There was no binding to either the α-mannoside or tri-mannose at the concentrations used (Figure 3). FITC-labeled ConA was used as a positive control for binding to the sugars. β-galactose is not recognized by ConA and was used as a negative control.

Iosthermal titration calorimetry was used as a second method to investigate PEMV binding to the sugars. The tri-mannoside was used for the ITC experiment with a commercially available methyl α-mannoside as a control sugar. ConA was the positive control for comparison with PEMV. Molar concentrations of PEMV were calculated based on the size of a single coat protein subunit with each subunit considered one potential glycan binding site. The ITC experiments involved titration of the solution with the sugar into a cell with the PEMV solution. At the end of each titration experiment, $K_d$, $\Delta H$, $\Delta S$, and $N$ were determined from the resulting data. There was obvious binding between the two saccharides and ConA with a $K_d = 4.65 \mu M$ for methyl α-mannoside, and a $K_d = 3.27 \mu M$ for the tri-mannoside (Figures 4 and 5). The $K_d$ values are calculated by the formula $K_b = 1/K_d$ where...
Kₐ is the K value found on the ITC graphs. The smaller the Kₐ, the stronger the binding.

There was no binding of PEMV to the α-mannoside and tri-mannoside (Figure 4 and 5).

*ConA binds to PEMV*

To determine if the PEMV structural proteins are glycosylated, ITC was used to test for the binding of ConA to PEMV. There was weak binding of ConA to PEMV (Figure 6). The Kₐ of the reaction was 50.5 indicating a low level of binding compared to the Con A-mannose controls (4.65 and 3.27). This result suggests a low level of glycosylation of PEMV virions.

**Discussion**

*Glycosylation of aphid gut proteins.* Aphid gut proteins are known to be glycosylated with mannose residues which correlates with the toxicity of ConA, GNA, and other mannose binding lectins in these insects (14-20). In our study, the mannose-binding lectins ConA and GNA bound to pea aphid BBMV and gut extracts confirming the abundance of mannose in the pea aphid (14, 20, 43). Sauvion et al. (43) immunolocalized ConA to the gut epithelial cells of pea aphids fed on the lectin. A mannose-specific garlic lectin bound to many pea aphid proteins of which APN and sucrase were identified as receptors for the lectin (14). Cristofoletti et al. (44) showed GNA bound a single band from midgut homogenates which was identified as APN. In our lectin blots ConA appeared to bind APN, but it was unclear whether GNA bound to APN. Our lectin blots with BBMV and gut protein showed binding of ConA and GNA to many pea aphid proteins. This result is in contrast to the Cristofoletti et al. study (44) where a single band (identified as APN) was detected from midgut homogenates using GNA. The total gut protein in our study was prepared using the same method as in (44), so the reason for the different results is unclear. BBMV preparations are
enriched for membrane associated gut proteins, such that a difference in the lectin blot profiles between BBMV and total gut homogenates is expected. Our results suggest that the pea aphid gut is heavily glycosylated with mannose and possible glucose residues.

**PEMV does not bind mannose and tri-mannose.** The carbohydrate microarray did not show binding between PEMV and the α-mannose or insect tri-mannose sugars. In the microarray there was evident binding of ConA to these sugars, but not to the negative control sugar, β-galactose as expected. The results from the carbohydrate microarray were confirmed by ITC. There was obvious binding between ConA and the mannose moieties, but no binding between PEMV and the mannose sugars. The $K_d$ for the binding of ConA to the tri-mannose (3.27) was smaller than the $K_d$ for ConA to α-mannose (4.65) indicating a stronger binding to the tri-mannose. This is not surprising given the structure of the tri-mannose. The lack of binding observed between the mannose sugars and PEMV suggests the virus does not use these glycans on the receptor for receptor binding or interact with mannose residues on other proteins during transport through the aphid. However, the methods used may not represent *in vivo* conditions. For example, the synthesized insect tri-mannose only consist the terminal three mannose groups. In the actual insect glycan structure the mannose residues are attached to two N-Acetylglucosamine (GlcNAc) residues that contain a fucose group (10)(Figures 1 and 2). These additional carbohydrate groups could be required for binding. Also, the glycan component alone may not be sufficient and both protein and glycan components may be required for virus binding. Aphid BBMV, or APN, could be tested with enzymes to remove the carbohydrates to look for any deleterious effects to PEMV binding.

**PEMV virions are glycosylated.** The binding of ConA to PEMV in the ITC experiments suggests that sugars are present on the viral structural proteins. Although few studies have
investigated luteovirus glycosylation, a recent study based on lectin binding assays and mass spectrometry analysis of the structural proteins suggested the structural proteins of two poleroviruses CABYV and TuYV are not glycosylated (37). Also, mutations to disrupt potential N-glycosylation sites of CAYBV structural proteins did not affect aphid transmission (37). However, the CAYBV mutants had mutations to a single glycosylation site, which leaves the possibility that more than one site could be glycosylated. These results for CAYBV and TuYV may not apply to all luteoviruses.

Interestingly it was recently determined that phloem lectins bind to CAYBV in vitro and can increase aphid transmission in in vitro feeding assays (45). The two phloem lectins from cucumber (Cucumis sativus) that bound to CAYBV were CsLec26 and CsLec17 which belong to the PP2 lectin family (45). PP2 lectins are known to bind poly-GlcNAc and high mannose N-glycans (46, 47). This supports the hypothesis that glycosylation of virions is important for binding to plant lectins which facilitates plant virus transmission. However, some lectins may inhibit aphid transmission such as α-D-galactose specific lectin inhibition of BWYV (36) so there could be multiple factors involved including lectin specificity, ability of the lectin to cross the insect gut, and whether the lectin blocks virus binding to the receptor.

The ability of proteins to increase virus transmission in vitro is not specific to lectins. In the same study with CAYBV, other randomly selected proteins including BSA, (but not all of the proteins tested) increased transmission of virus when co-fed to aphids (45). This result agrees with our results demonstrating that BSA can increase uptake of PEMV into the pea aphid hemocoel (Chapter 3). Notably, GNA, casein and BSA which facilitated virus uptake are all known to transcytose across the insect gut epithelium and hence these proteins may
provide additional receptor sites thereby increasing virus movement from the gut into the hemocoel (17, 48-53). We have shown that BSA binds to the recombinant PEMV CP (Chapter 3), but although BSA is known to bind glycoproteins (54, 55) it is unknown whether BSA binds to sugars. Sugars on the surface of the virus might be important for both lectin and BSA binding to provide additional movement of the virus across the gut epithelium. Alternatively, glycosylation of viral proteins may function in the virus lifecycle in the plant. More work will be needed to characterize the glycosylation of luteovirions and to assess the potential role of glycans and associated lectins in aphid transmission.

**Author Contributions**

LBL performed the lectin blots and completed the purification and FITC labeling of PEMV. ST synthesized the sugars and performed the carbohydrate microarray and ITC experiments. NP was involved in experimental design. BCB contributed to the experimental design and editing of the manuscript.

**Acknowledgements**

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## Tables and Figures

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### Key to symbols
- N-Acetylglucosamine
- Mannose
- Fucose

**Figure 1.** A comparison of the N-glycan structures in insects (left) with the synthesized mannose moieties used in this study (right). The N-glycan structures are modified from (10). The synthesized mannose moieties have a fluorous tag added for attachment to the glass slides used in the carbohydrate microarrays.
Table 1. Location of putative N- and O-linked glycosylation sites among luteovirus structural protein sequences. The table is modified from (37) with the addition of PEMV. The parameters for the glycosylation prediction programs were used as described in (37). The number of predicted glycosylation sites is followed by the position on the amino acid sequence in parentheses.

| Accession numbers ID: Pea enation mosaic virus (PEMV/NP_840025.2), Cucurbit aphid-borne yellows virus (CABYV/NC_003688.1), Cereal yellow dwarf virus (CYDV/NP_840025.2), Potato leafroll virus (PLRV/NC_001747.1), Turnip yellows virus (TuYV/NP_620487.1) and Melon aphid-borne yellows virus (MABYV/NC_010809.1) |}

| Accession numbers ID: Pea enation mosaic virus (PEMV/NP_840025.2), Cucurbit aphid-borne yellows virus (CABYV/NC_003688.1), Cereal yellow dwarf virus (CYDV/NP_840025.2), Potato leafroll virus (PLRV/NC_001747.1), Turnip yellows virus (TuYV/NP_620487.1) and Melon aphid-borne yellows virus (MABYV/NC_010809.1) |}

<table>
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<th>Predicted N-glycosylation sites</th>
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<tr>
<td>CP</td>
<td>RTD</td>
</tr>
<tr>
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<td>4 (47, 64, 137, 186)</td>
</tr>
<tr>
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<td>TuYV</td>
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<td>MABYV</td>
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<td>CYDV</td>
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Figure 2. Pea aphid proteins are glycosylated with mannose and glucose moieties. Pea aphid BBMV and gut protein extracts were separated by SDS-PAGE and stained with Coomassie blue or transferred to PVDF membranes and probed with the lectins ConA or GNA. Arrows, position of APN based on molecular mass.
Figure 3. PEMV did not bind to the insect N-glycan trimannoside or α-mannoside using a carbohydrate microarray. The fluorous tagged carbohydrates were attached to commercially available slides in the pattern shown above. The control sugars in the experiment were β-galactoside (−) and α-mannoside (+). The attached sugars were incubated with various concentrations of FITC-labeled PEMV (A – F) or FITC-labeled ConA as a positive control (G).
Figure 4. PEMV does not bind to α-mannoside using ITC. ConA was used as a positive control for binding. In the top panel the amount of heat released is measured after each addition of α-mannoside and the signal should diminish over time as the binding sites are saturated. The bottom panel shows the amount of heat plotted against the molar ratio of the ligands in the reaction which is fit to a binding curve.
Figure 5. PEMV did not bind to the synthesized insect tri-mannoside in the ITC experiment. ConA was used as a positive control. In the top panel the amount of heat released is measured after each addition of tri-mannoside. In the bottom panel the amount of heat is plotted against the molar ratio of the ligands in the reaction. The data from PEMV could not be fit to a binding curve as seen for ConA.
Figure 6. ConA bound weakly to PEMV in the ITC experiment indicating the presence of carbohydrates associated with the PEMV structural proteins. In this experiment ConA was titrated into the cell containing PEMV. The $K_d$ for ConA-PEMV is 50.5 compared to a $K_d$ of 3.27 for ConA binding to the insect tri-mannoside. The lower the $K_d$, the stronger binding.
CHAPTER 5

General Conclusions

The focus of the research described in this dissertation was to increase our understanding of the molecular interactions involved in aphid transmission of luteoviruses. Our knowledge of the insect receptors for plant viruses is limited, with no protein receptors identified. For circulative transmission of luteoviruses, recognition by specific receptors is critical for transcytosis of virus across gut epithelial cells. Virions disseminate through the hemolymph before being taken up into the accessory salivary glands (ASG) by a second receptor mediated endocytosis event. To complete the transmission cycle, virions are secreted with the aphid saliva to inoculate a healthy plant. We used *Pea enation mosaic virus* (PEMV) and the pea aphid (*Acyrthosiphon pisum*) as a model system for the study of luteovirus-aphid interactions. There are many advantages to using this model system: PEMV is the only luteovirus that can be mechanically transmitted, which facilitated large scale infection of pea plants for purification of the virus used in our experiments. The relatively large size of the pea aphid simplifies gut isolation and extraction of protein. In addition, the complete genome of the pea aphid is available (1) which was instrumental in identifying proteins of interest. The primary goal of this project was to identify gut receptor molecules for PEMV in the pea aphid and to study the impact of glycosylation and other proteins on virus-receptor interaction.

Chapter Two of this dissertation describes the identification of membrane alanyl aminopeptidase N (APN) as a gut receptor for PEMV in the pea aphid. A far-western blot method with pea aphid brush border membrane vesicles and purified PEMV was used. The PEMV CP-eGFP fusion (CP-P-eGFP) showed similar binding and provided confirmation of
the results with PEMV. APN was identified by MALDI-TOF MS/MS of the ligand binding protein extracted from stained gels with reference to the probed membranes. Preparing BBMV enriches for membrane associated proteins that would be exposed on the surface of the gut and therefore potentially interact with ingested virus. The use of BBMV likely increased our chances of identifying the gut receptor and may have been the reason for the specific binding to APN in the far-western blots with low background binding to other aphid proteins. Previous attempts to identify plant virus receptors using total insect protein extracts in similar far-western blot methods have failed (2-4). This failure may have resulted from a low concentration of the receptor in total aphid protein extracts. We confirmed the binding of PEMV to APN by three additional methods. The first was an immunofluorescence binding assay in which there was more binding of CP-P-eGFP to Sf9 cells infected with a baculovirus expressing pea aphid APN compared to the controls. The second method demonstrated PEMV-APN binding using a pull-down assay. In this experiment PEMV was labeled with a biotin crosslinking reagent that would allow UV crosslinking of PEMV to APN from aphid guts followed by pull-down with streptavidin-agarose beads. Western blot analysis was used to detect pulled-down APN. This cross-linking method was used because of problems with high background and the inability to detect PEMV-APN binding using a co-immunoprecipitation method with protein A-agarose beads. The high background resulted from non-specific pull-down of multiple proteins by both the PEMV and GFP antibodies. PEMV binding to APN may be weak or transient and could be why cross-linking was necessary for pull-down assays. The third method used to confirm binding was surface plasmon resonance (SPR). In this experiment the CP-P-eGFP bound a baculovirus expressed APN. The binding of eGFP-RTD to APN was weak relative to CP-P-eGFP, but was still
significant compared to the eGFP control. CP-P-eGFP binding correlates with the fact that CP alone is sufficient for transcytosis of PEMV across the gut epithelium.

A common problem with the far-western blot method is false positives. The ability of an insect protein to bind a virus may not directly correlate with a receptor function. Many of the luteovirus binding proteins identified in other studies including rack-1, actin, luciferase, and cyclophilin, would not serve as receptors based on their location in the cell or on the inner membrane. Given the mechanism by which luteoviruses are internalized into coated vesicles and transported directly across the cell, it seems unlikely that these proteins would interact directly with the virus. APN is a highly abundant membrane associated gut protein in the pea aphid (5) which supports its role as a virus receptor. A peptide (GBP3.1) that binds to the pea aphid gut and impedes uptake of PEMV into the aphid hemocoel was previously identified in our lab (6). In this study, we showed that APN is also the receptor for GBP3.1 using a pull down assay. Taken together, these results provide additional evidence that PEMV uses APN as a receptor in the pea aphid gut.

Interestingly, PEMV does not appear to bind to APN in a second vector, the green peach aphid, *Myzus persicae*. This result suggests that PEMV uses a different receptor in different aphid vectors. Different luteoviruses can be acquired through the midgut, hindgut, and in some cases both (7). PEMV is acquired through the midgut in the pea aphid (8) but it is unknown what route PEMV takes in *M. persicae*. Transmission electron microscopy of viruliferous gut tissues has been the primary method used to assess the site of intestinal uptake (9). It is important in these studies to understand the anatomy of the aphid digestive tract when discussing luteovirus acquisition sites in the gut, and there is certainly scope for error. The alimentary canal begins with a narrow chitin lined foregut which leads to an
enlarged anterior midgut. The anterior midgut is not chitin lined and contains many microvilli protruding into the gut lumen. Luteoviruses are not known to associate with this region of the gut (10). The posterior midgut is long and narrow and the amount of microvilli decreases with proximity to the hindgut. The hindgut lacks well-defined microvilli and is not chitin lined as seen in some insects. There is not a sharp boundary separating the mid and hindgut as the two regions blend together (10). From our observations of dissected pea aphid guts, the hindgut is very short and appears more transparent than the midgut. This change in appearance may be due to the change in gut structure, i.e. the lack of complex microvilli. The hindgut opens to a short chitin-lined rectum.

Species of *Barley yellow dwarf virus* (BYDV-PAV and BYDV-MAV) are in the genus *Luteovirus* and are taken up in the hindgut (11). Poleroviruses PLRV and *Beet western yellow virus* (BWYV) are acquired through the midgut, as is PEMV (*Enamovirus*) (7, 8, 12, 13). However, another polerovirus *Cucurbit aphid-borne yellows virus* (CABYV) can be acquired from the mid- or hindgut (7). Regardless of the site of uptake, the process of endocytosis and transport in tubular vesicles across the cells appears to remains the same (7). The protein composition of the mid- and hindgut is likely different based on the known structure and function of these regions. The insect midgut functions in digestion and absorption, whereas the hindgut functions in excretion of waste and retention of water. CABYV entry through both sites suggests either a common protein in both tissues or the virus has adapted to use one receptor in the midgut and a different receptor in the hindgut. The acquisition site in the aphid vector seems to be segregated based on virus genus suggesting there may be differences in structural surface features between the coat proteins of different genera. While comparison of the CP and RTD sequences did not reveal any
obvious differences that would explain this tissue specificity (7, 14, 15), the N-terminal half of the RTD is more conserved across luteoviruses than the C-terminal half, which is highly variable. The potential role of the C terminal region of RTD in binding the midgut versus the hindgut warrants further investigation.

APN is present throughout the midgut but aminopeptidase activity decreases towards the posterior end of the gut (16). In the case of PEMV therefore, uptake of this virus through the midgut and the primary localization of APN to the midgut fits with use of APN as a receptor. Identifying receptors for viruses acquired through the hindgut will help understanding of why luteoviruses use different routes for uptake into the aphid vector.

Based on our results, APN may represent the first receptor identified for a plant virus in the insect vector. Modeling of the interaction of pea aphid APN and PEMV to determine the specific coat protein domains involved in binding would be informative. One-dimensional ligand blots suggest that the BC (amino acids 65-78) and GH (amino acids 147-159) loops of PEMV CP are not involved in receptor interaction (Appendix 2). Understanding the specific receptor-virus interactions is crucial for developing strategies to disrupt aphid transmission of plant viruses. To date, there is no crystal structure for a luteovirus, only a model for *Potato leaf roll virus* (PLRV) based on homology to *Rice yellow mottle virus* (genus *Sobemovirus*) (17). Crystal structures of virus-receptor interactions have been resolved for some viruses (18, 19). The protrusion of the RTD from the surface of luteovirions may hinder the ability to produce crystal structures, so the use of both recombinantly expressed CP (which self assembles into virus-like particles) and receptor (APN) may suffice. Constructing a short peptide library from the PEMV CP to test for binding to APN would also narrow down specific regions of the CP involved in binding.
This method was used to test the binding of *Barley yellow dwarf virus* (BYDV) structural proteins to GroEL from the aphid endosymbiont, *Buchnera* (20). A method involving chemical cross-linking followed by mass spectrometry has recently been used to study functional domains in the CP and RTD of *PLRV* (21). Interacting proteins are cross-linked and subsequently identified by mass spectrometry to determine sites between CP molecules, between CP and RTD, and within RTD that interact. This method may have the potential to determine the interactions of luteoviruses with non-viral proteins in the aphid and plant.

GBP3.1 was shown to impede uptake of PEMV into the aphid hemocoel (6) and we demonstrated that GBP3.1 also binds to APN. Peptides similar to GBP3.1 developed to bind to virus receptors could be expressed in planta. Upon aphid feeding, these peptides could interfere with uptake of virions into the aphid hemocoel thereby reducing virus transmission in the field. In an agricultural setting this method would not protect the first plant fed upon by an incoming viruliferous aphid, but would block plant to plant spread of the virus in the field. A single competing molecule could be effective in blocking transmission of related viruses if common insect receptors are used, but this remains to be seen. This strategy could also be applied to block binding of stylet-borne, non-persistent viruses if peptides that bind the same site are identified. Such a virus transmission blocking strategy would need to be used in conjunction with a method to control aphid populations such as aphid resistant plants. This approach may be a way to limit the use of environmentally damaging insecticides. The methods used successfully for identification of APN as a receptor for PEMV can now be applied for discovery of virus receptors in other virus-vector systems.

In Chapter Three, the impact of bovine serum albumin (BSA) on uptake of PEMV into the pea aphid was investigated. This study was designed based on the findings of
Bencharki et al. (22) showing that CABYV virions bound to phloem lectins and the addition of these lectins (and other non-plant proteins) to artificial diet increased the efficiency of virus transmission by the aphid vector. However, not all proteins fed with virus had this effect. In our study, we showed increased virus in the hemocoel of aphids fed on artificial diet containing purified PEMV with bovine serum albumin (BSA) compared to aphids fed on virus in the absence of BSA. SPR analysis indicated that CP binds BSA strongly, while only weak binding was detected between RTD and BSA. We propose that BSA binds CP and the entry of the PEMV-BSA complex is mediated by both BSA receptors and PEMV receptors on the aphid gut epithelium; thus providing more efficient delivery of the virus into the hemocoel. In contrast, BSA did not increase uptake of virus lacking the coat protein readthrough domain (CP-RTD, PEMV RNA1Δ). A proposed model to explain these observations is that the RTD serves as an anchor to enhance virus association with the gut epithelium. While BSA can further enhance uptake in the presence of the RTD anchor, the absence of the RTD removes any benefit of virus association with BSA. This model implies that under BSA free conditions WT PEMV crosses the gut more efficiently than PEMV RNA1Δ. Decreased uptake of RTD-deficient BWYV has been observed (12). WT PEMV and PEMV RNA1Δ accumulate to comparable levels in the aphid when acquired from the plant (23), but our preliminary data suggest WT PEMV is acquired more efficiently than PEMV RNA1Δ by membrane feeding. This result needs to be confirmed. In an alternative model, the RTD functions to bind proteins such as BSA that might compete with PEMV for binding to APN. The binding of CP and RTD to BSA still facilitates uptake of the PEMV-BSA complex by using both the BSA and virus receptors. Without the RTD, free BSA competes with PEMV RNA1Δ for binding to the receptors thereby reducing virus entry.
Important to both of these models is the ability of BSA to independently transcytose across the gut epithelium and for BSA to interact with the virus. Not all proteins are able to cross the gut and/or interact with the virus and these proteins may not enhance uptake of the virus. Importantly, BSA has been reported to cross the gut of some insects (24, 25), along with several additional proteins shown to improve plant virus transmission (22).

The function of the RTD is not fully understood. Both the CP and RTD are required for aphid transmission. The CP alone is sufficient to cross the gut (12, 26, 27), however virions lacking the RTD do not move as efficiently (12). Virions without RTD have not been observed to associate with the ASG, with the exception of baculovirus-expressed virus-like particles of PLRV lacking the RTD that were detected in the ASG (27). RTD binding of aphid gut epithelial proteins may serve to anchor the virus to specific regions of the gut epithelium, enhancing uptake and the accumulation of virus in the hemolymph. Virus association with plant proteins may further enhance uptake and these increased virus titers in the hemocoel may be sufficient for successful entry of virions into the ASG. Examination of the interaction between CP-P-eGFP and RTD-eGFP with plant lectins would indicate whether BSA behaves in a similar manner to facilitate virus uptake.

The presence of BSA or plant proteins is expected to reduce the latent period, or time between virus ingestion and when the aphid can transmit the virus. Luteoviruses can typically be detected in the hemolymph within 30 min of feeding, but can require up to 24 hrs before the aphid will efficiently transmit the virus (28). This suggests the virus must accumulate to a certain threshold to allow enough virus to enter the aphid saliva and be transmitted. Plant proteins associated with virus particles that enhance virus accumulation in the hemolymph to the level required for efficient transmission would reduce the latent period.
In Chapter Four, the role of glycans in PEMV-pea aphid interactions was assessed. While glycans are known to mediate animal virus-vector interactions (29-31), little is known about glycan involvement in insect transmission of plant viruses. We have identified APN as the putative receptor for PEMV. APN is a glycosylated protein and a known receptor for mannose binding lectins (5, 32). We have not determined whether glycans are required for PEMV binding to APN. An insect tri-mannose or “paucimannose” structure commonly found in insects (33) was synthesized by our collaborators and tested for binding to PEMV using a carbohydrate microarray and isothermal titration calorimetry (ITC). Binding between the insect tri-mannose and PEMV was not detected by either method. The results suggest the virus does not use this insect glycan for receptor binding or interact with mannose residues on other proteins during transport through the aphid. However, virus binding may require both a protein and a glycan component or the addition of N-Acetylglucosamine (GlcNAc) groups normally found in insect glycans. Determining whether the glycosylation of APN is critical for PEMV binding may be a direction for future work. This could be addressed by enzymatically removing carbohydrate groups from APN and monitoring the impact on PEMV binding.

Using ITC, we observed binding of the lectin Concanavalin A (ConA) to PEMV indicating glycosylation of the PEMV structural proteins. ConA binds both mannose and glucose. Only two studies have investigated the glycosylation of luteoviruses (34, 35). The first study suggested glycosylation of Turnip yellows virus (TuYV) structural proteins was important for aphid transmission (34). The second study disputed this claim and provided additional evidence that the structural proteins of TuYV and a related polerovirus, Cucurbit aphid-borne yellows virus (CABYV) were not glycosylated or that virus glycosylation was
not involved in aphid transmission (35). However, the results from these studies may not apply to all luteoviruses, and presumably the role of virus glycosylation, if any, on aphid transmission could differ depending on the system (plant, virus, and aphid vector) being studied. Glycosylation of luteoviruses may relate to the function of phloem proteins in aphid transmission as discussed above. The plant proteins previously shown to bind CABYV and increase aphid transmission were plant lectins (22). Lectins are defined as sugar-binding proteins, so it is plausible that these lectins bind sugars on the surface of the virions and enhance virus uptake as described above. The carbohydrate specificities of these lectins and the ability of the lectins to cross the aphid gut may be important. *Galanthus nivalis* agglutinin (GNA) is known to cross the gut of some insects (36-39). Interestingly, not all of the lectins used in the Bencharki et al. study increased transmission (22). These lectins, LeH from *Lens culinaris* (specific for α-D-mannose and α-D-glucose) and MPA from *Maculura pomifera* (specific for Gal(β1-3)GalNAc) did not enhance CABYV transmission. However the sample size used for these lectins was low (only 9 plants). ConA, GNA, and other mannose binding lectins (5, 32), bind to APN but it is unknown whether these lectins would compete with virus binding or enhance virus uptake. APN is not the only glycosylated protein in the aphid gut, so lectins could still enhance virus uptake by a route that uses a different receptor. More work is needed to determine if the enhanced uptake of virus from plant lectins (or other proteins) is sugar mediated.

As outlined in Chapter Three, plant viruses may bind and sequester plant lectins that would otherwise compete for binding to the aphid gut receptor. In addition, the fact that some plant lectins are known to bind to the same receptor proteins (e.g. APN), could provide evolutionary selection for viruses that enter via the hindgut of the vector. By the time gut
contents reach the hindgut, most of the plant lectins (which are designed for anti-herbivore defense) may already be bound to midgut epithelial proteins such that competition for binding is reduced in the hindgut.

It is apparent that aphid transmission of luteoviruses involves multiple complex interactions between the aphid, virus, and plant. To our knowledge, APN is the first putative gut receptor identified for a circulative virus, but the approaches used successfully for this study can now be expanded not only to other luteoviruses, but to other persistently transmitted viruses. The aphid gut is the first physical barrier encountered by the virus in the vector and therefore represents an appropriate target for developing strategies to block plant virus transmission. However, identifying receptors at ASG will also provide valuable information. Insect transmission of plant viruses will continue to pose a threat to agriculture, but what we have learned from the study of PEMV-pea aphid interactions may allow for advancement in the development of alternative strategies to manage plant virus epidemics.

References


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Finally, I wish to thank my family for their support and encouragement over the years. I especially thank my wife, Michelle, for her endless support, understanding, and patience throughout this process.
APPENDIX 1

Strand-specific detection of *Pea enation mosaic virus* in plants, aphids, and purified virions: Detection of negative strand RNA in virions

Introduction

In early studies with *Pea enation mosaic virus* (PEMV), virus particles were observed by transmission electron microscopy (TEM) not only in the aphid gut and salivary glands, but also in tissues such as the fat body, midgut muscle cells, and even nuclei of midgut cells suggesting the possibility of replication in the aphid (1-3). However, in more recent electron microscopy studies, detection of luteoviruses in tissues other than the gut, salivary glands, or hemocoel has not been reported, nor has luteovirus replication in an aphid vector been substantiated (4-6). Luteoviruses are classified as circulative, non-propagative and are thought to move through the aphid with minimal disturbance to the insect (7).

Luteoviruses have positive sense single strand RNA genomes. Synthesis of subgenomic RNAs and replication of the genome requires a negative strand intermediate (8). However, only the positive strand is thought to be incorporated into the virion (9). In this study we demonstrate the presence of negative strand RNA in purified virus preparations using a strand-specific detection method (10). We were unable to detect the negative strand in aphids fed on suspensions of purified virus, supporting the fact that luteoviruses are non-propagative in the aphid vector.
Materials and Methods

Aphid feeding assays and RNA purification

Aphids were fed for approximately 16 hr by Parafilm® membrane feeding (11) on 25% sucrose in phosphate buffered saline (PBS). The diet was supplemented with 500 ng/ul of wild type PEMV purified as described in Chapter 2. Three plates were set up with 15 aphids per plate. Aphids were fed on the sucrose diet alone as a control. The next day, aphids fed on virus or the control diet were pooled from the three plates (~45 aphids per group) and RNA was purified using Trizol reagent (Invitrogen) according to the manufacturer’s protocol.

Seven day old pea plants (*Pisum sativum*) were mechanically infected with wild type PEMV as described in (12). At 10-14 days post infection total RNA was purified from 100 mg of tissue using Trizol. RNA was purified from uninfected plant tissue as a control. RNA was also extracted from 250 µg of purified wild type PEMV.

Generating in vitro transcripts of positive and negative strand PEMV

The plasmid pPERI described in (13) is the pUC19 vector containing the PEMV RNA1 genome with a T7 promoter to drive productions of positive strand RNA. The plasmid pNS-RNA1 is the pUC18 vector containing the PEMV RNA1 genome with a T7 promoter for production of negative strand RNA. The mMessage mMACHINE T7 Kit (Ambion, AM1344) was used following the manufacturer’s protocol to generate *in vitro* transcripts from the plasmids that were previously linearized with PstI. The RNA transcripts were purified using Trizol reagent remove to the DNA template.
Strand-specific RT-PCR detection of PEMV RNA

Superscript III reverse transcriptase (Invitrogen) was used according to the manufacturer’s protocol to generate cDNA from both PEMV RNA and pea aphid actin using gene specific primers (Table 1). The volume of the reactions was reduced to 10 µl instead of 20 µl as listed in the manufacturer’s protocol. From aphid, plant, or purified virus samples, 2.5 µg of RNA was used for each cDNA reaction. Approximately 100 ng of the in vitro transcripts was used per reaction. The primer Tag-N2636 was used to generate cDNA from the negative strand RNA. The primer Tag-P4719 was used to generate cDNA from the positive strand RNA. The cDNA was amplified using primers TagOnly1 and RNA1-4719 (for negative strand, expected size: 2,117bp) or primers RNA1-4123 and TagOnly1 (for positive strand, expected size: 617 bp). PCR conditions were as follows: 94°C for 2 min, 30 cycles of 94°C for 30 sec, 53°C for 45 sec, 72°C for 1 min, followed by a single cycle of 72°C for 5 min. PCR products were run on 1.0 % agarose gels, stained with ethidium bromide, and imaged using a gel imager (Fotodyne). A diagram of the strand-specific RT-PCR detection method is shown in Figure 1.

Results

Strand-specific detection using in vitro transcripts

The use of the tagged primers was necessary to detect specific strands of the PEMV-1 genome. If the TagOnly1 primer was not used in the PCR step, a PCR product was detected regardless of the primers used for cDNA synthesis (Figure 2A). This means that there is mis-priming to the incorrect strand during cDNA synthesis and therefore template for the PCR reaction which results in false positives. If the TagOnly1 primer was used with the appropriate primer, the specific products (617bp for (+) strand and 2117bp for (-) strand)
were detected only when the correct primer was used for cDNA synthesis (Figures 2B and 2C). No products were detected when the RNA alone was used as a template for PCR indicating DNA template was not contaminating the \textit{in vitro} transcripts.

\textit{Strand-specific detection of PEMV RNA1 in aphid, plant, and purified virus}

The positive strand of PEMV RNA1 was detected in both the infected plant and aphids that were fed on the purified virus (Figure 3). There was no (+) strand product observed in the uninfected plant or aphids fed on diet only. The (-) strand was only detected in the infected plant and not in the aphids fed on virus. These experiments were replicated three times.

Positive strand was detected from RNA of three separate virus purifications. On two occasions, negative strand RNA was detected from purified virus (Figures 4 and 5). Three different virus purifications were tested. Purification #2 tested positive for negative strand (one replicate) (Figure 5), whereas purification #3 tested positive in one replicate (Figure 4), but not in the second replicate (Figure 5). The negative strand RNA was detected at lower levels than the positive strand.

\textbf{Discussion}

We were unable to detect the negative strand of PEMV RNA1 in aphids fed overnight on purified virus. It is well established that luteovirus particles are not observed outside of the vesicles in which they are transported across the gut and salivary glands (6). Except for the early studies with PEMV (1-3), luteovirus particles have not been found in tissues other than the gut and ASG and it is now generally accepted that luteoviruses do not replicate in the aphid (6).
Unexpectedly, negative strand RNA was detected from purified virus preparations. The genomic RNA of luteoviruses is plus sense and is considered the only RNA packaged by luteoviruses. In our study, the negative strand was detected at levels much lower than the positive strand. In the cell, many copies of the positive strand are generated from a few copies of the negative strand intermediate. Our results suggest that the negative strand is packaged at a low level. This work needs to be repeated to confirm these findings. RNA packaging is thought to involve interactions between the viral coat protein and a specific RNA signal (9). More work is needed to determine the mechanisms by which the virus preferentially packages the genomic RNA and whether packaging of negative strand RNA has any biological significance.

References


Table 1. Primers used for strand-specific detection of PEMV RNA1. The upper case letters indicate the Tag region. The strand specificity and purpose of each primer is indicated.

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<tr>
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<td>Tag-P4719</td>
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<td>(+)</td>
</tr>
<tr>
<td>PCR</td>
<td>RNA1-4123</td>
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**Figure 1.** Diagram of the process for two-step strand-specific RT-PCR detection of PEMV RNA1. The tagged-primers used to generate cDNA from each strand are shown with the primers used in the PCR step. The expected PCR product sizes from each RT-PCR reaction are listed.
Figure 2. Testing of tagged-primers for strand-specific detection of PEMV RNA1 using *in vitro* transcripts of the positive and negative strand. Without the use of the TagOnly1 primer in the PCR step, a PCR product is generated regardless of the primer used for cDNA synthesis (A). For (+) strand detection (B), a product of ~600 bp was produced only when cDNA is generated with the Tag-P4719 primer as expected. There was a faint band detected for (+) strand when cDNA was generated with the incorrect primer, Tag-N2636, but this may be overflow from the previous lane (B). For (-) strand detection (C), a band of the correct size of ~2000bp was observed with the correct cDNA primer, Tag-N2636. When the incorrect primer was used (Tag-P4719) for cDNA synthesis there were two bands of incorrect size observed (C). When the *in vitro* transcripts were directly used for PCR, there were no observed products indicating the RNA was free of DNA template contamination.
Figure 3. Strand-specific detection of PEMV RNA1 in infected plants and aphids fed on purified virus. The (+) strand was detected from both infected plants and aphids fed on virus (left). Negative strand was only detected in the infected plants and not from the aphids fed on the virus (right). No PCR products were observed in uninfected plants or aphids fed on diet only (left and right). Images are representative of three independent experiments.
Figure 4. Negative strand detection of PEMV RNA1 from purified virions. Negative strand was not detected in virus fed aphids but was detected at a low level in RNA extracted from purified virions. *In vitro* transcribed negative strand RNA served as a positive control for the reactions.
Figure 5. Positive and negative strand detection of PEMV RNA1 from multiple PEMV purifications. The RNA from three separate virus purifications from infected plants were tested for positive (left) or negative strand (right). All three samples had positive strand, but only virus purification #2 tested positive for the negative strand (arrow). The negative strand was not detected from purification #3 as it was in Figure 4. This was the second technical replicate for purification #3. *In vitro* transcripts served as positive controls for the respective reactions. For virus purifications #1 and #2 this was the only technical replicate.
APPENDIX 2

Binding of the βB-βC and βG-βH loops of the PEMV coat protein
to pea aphid brush border membrane vesicles

Introduction

The PEMV coat protein (CP) domains involved in binding to the aphid gut are unknown. A model for the coat protein of Potato leaf roll virus (PLRV, Luteoviridae) has been created by comparison to the crystal structure of Rice yellow mottle virus (RYMV, genus Sobemovirus) (1). A CP epitope on the surface of the PLRV CP (1, 2) is involved in virion assembly, systemic movement, and aphid transmission (3). This epitope, the βG-βH loop, has the sequence ‘HDSSEDQ’ and is predicted to be an acidic surface loop. There is a similar motif, ‘GPSSDCQ’, in the PEMV CP. The βB-βC loop of PLRV is conserved among luteoviruses (1, 2). Sequence conservation of these surface loops suggests that these regions could be important for virus interaction with aphid receptors. Here we analyze the binding of PEMV coat protein βB-βC (amino acids 65-78) and βG-βH (amino acids 147-159) loops to pea aphid BBMV. We also test the binding of the gut binding peptide (GBP3.1) -eGFP fusion.

Materials and Methods

Production of peptide-eGFP fusions

The method for the production of GPB3.1-eGFP is described in (4). Production of CP-P-eGFP is described in Chapter 2. The βB-βC and βG-βH peptides and a nonbinding, control peptide (C2) were fused to the N-terminus of eGFP and inserted into pBAD/His B (Invitrogen). The cDNA encoding the proteins was generated by PCR using the primers sequences listed in Table 1. To add the C2 peptide (AYCPSSAAVSGCSA) to the N-terminus of eGFP, the primers C2-EGFP and EGFP-HindIII were used with eGFP cDNA as a template. The SacI site was
added at the 5’ end by a second PCR reaction with primers C2-Sac-I and EGFP-HindIII. The BC peptide was added to the N-terminus of eGFP by a PCR reaction with primers BC-EGFP and EGFP-HindIII. The SacI site was added to the 5’ end with a second PCR reaction with primers BC-SacI2 and EGFP-HindIII. The GH peptide was added in the same way with a PCR reaction with GH-EGFP and EGFP-HindIII. A 5’ SacI site was added with a second reaction with primers GH-Sac-I2 and EGFP-HindIII.

The eGFP-PCR products were excised from a 1% agarose gel and purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The DNA fragments were digested with the SacI and HindIII restriction enzymes, cleaned by using a QIAquick Nucleotide Removal Kit (Qiagen), and ligated into pBAD/His B (previously linearized SacI and HindIII restriction enzymes). Expression and purification of the peptide-eGFP fusion proteins was performed as described in Chapter 2.

Far-western blotting

Brush border membrane vesicles (BBMV) were prepared as described in Chapter 2. Twenty µg of BBMV per lane was separated by 10% SDS-PAGE. The gel was equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 0.025% SDS, pH 8.3) prior to overnight transfer to a nitrocellulose membrane (Hybond-ECL, Amersham) at 4°C at 30V. Each lane on the membrane was separated and individually processed. Membranes were blocked in 5% non-fat dry milk in phosphate buffered saline with 0.1% Tween-20 (PBS-T) overnight at 4°C. Membranes were then incubated with the eGFP-fusion proteins (10 µg/mL) in 1% non-fat dry milk in PBS-T overnight at 4°C. Bound ligand was detected using GFP antiserum (Sigma; 1:5000) followed by an HRP-conjugated anti-rabbit IgG (1:5000). The PVDF membranes were incubated in HyGlo Chemiluminescent HRP detection reagent for 1 min, with luminescence
detected on X-ray film using standard procedures. An antibody only control was run in parallel by eliminating incubation with the ligand (eGFP-fusion) to identify non-specific binding of the antibodies to aphid BBMV. The experiments were replicated twice with each ligand.

Results

The βB-βC and βG-βH loops of the PEMV coat protein did not bind to pea aphid BBMV using a one-dimensional far-western blot. CP-P-eGFP bound a protein migrating above 150 kDa which corresponds to the size of APN as seen in the two-dimensional far-western blots. In the two-dimensional far-western blots with CP-P-eGFP and eGFP alone there are two proteins of about 65 kDa and 90 kDa in size that are detected. These proteins were determined to be non-specifically bound by the anti-GFP antiserum. In the one-dimensional far-western blots CP-P-eGFP, there is a band of about 65 kDa, but a band is not observed at the 90 kDa size. Also, this 65 kDa band does not appear in all the lanes which is expected if it is a result of non-specific antibody binding. CP-P-eGFP also binds to seven proteins between 15-37kDa, which is not seen in the two-dimensional gels. The GBP3.1-eGFP fusion did not show any binding. Binding that was seen in the blots for peptide-eGFP fusions (excluding CP-P-eGFP) after longer exposures was also either seen in the C2-eGFP or eGFP only controls.

Discussion

Although the βB-βC and βG-βH loops did not bind to aphid BBMV using the one-dimensional far-western blot method, it may be necessary to try other methods before concluding these loops are not involved in virus binding. GBP3.1 did not bind using this method, but we have shown that GBP3.1 binds to APN using a cross-linking pull-down assay. As described by Liu et al. (4), the structural features of GBP3.1 are more similar to those of the βG-βH loop than to the βB-βC loop. Future work could look at the binding of these loops using a similar pull-
down assay used for GBP3.1. Alternatively, the method used by Bouvaine et al. (5) to test the binding of Barley yellow dwarf virus (BYDV) structural proteins to GroEL from the aphid endosymbiont, Buchnera may be a better option. This approach would involve synthesizing a short peptide library based on the PEMV CP sequence to test the peptides for binding to APN. This would determine the specific regions of the CP capable of binding the virus receptor.

References


Table 1. Primers used to generate the peptide-eGFP fusions. The protein coding sequences are in lower case text. The restriction sites are underlined.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>C2-EGFP</td>
<td>5'-gcctattgtccgagttcgctctgtgctgtcgttgcttagctgcttgagcaagggcaggg-3'</td>
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<tr>
<td>GH-EGFP</td>
<td>5'-acgtgccttggtgtcaaccgggtgtaacgagtcctcaaatagggactagctgtcgttgagcaagggcagggag-3'</td>
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<tr>
<td>BC-EGFP</td>
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</tr>
<tr>
<td>GH-Sac-I2</td>
<td>5'-CGGGAGCTCGacgtgccttggtgtcaac-3'</td>
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<td>BC-Sac-I2</td>
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<tr>
<td>EGFP.HindIII</td>
<td>5'-CCAAAAGCTTGTaatgtgacagctgtccatg-3'</td>
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Figure 1. Binding of the peptide-eGFP fusion proteins to pea aphid BBMV. Far-western blot with 5 min exposure (left) or 30 min exposure (right). Each lane was individually incubated with the indicated ligands. The arrow in the CP-P-eGFP lane indicates binding to a protein corresponding to the size of APN. There was no binding observed for the BC- and GH-eGFP fusions proteins that was not also observed in the C2-eGFP and eGFP only controls. The positive control (+) for the western blot is purified eGFP protein. This experiment was repeated twice.