Virology 401 (2010) 107–116

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

A peptide that binds the pea aphid gut impedes entry of Pea enation mosaic virus into the aphid hemocoel

Sijun Liu [a], S. Sivakumar [a], Wendy O. Sparks [a,c], W. Allen Miller [b], Bryony C. Bonning [a,*]

[a] Department of Entomology, Iowa State University, Ames, IA 50011, USA
[b] Department of Plant Pathology, Iowa State University, Ames, IA 50011, USA
[c] Interdepartmental Graduate Genetics Program, Iowa State University, Ames, IA 50011, USA

ARTICLE INFO

Article history:
Received 26 June 2009
Returned to author for revision
4 November 2009
Accepted 5 February 2010
Available online 11 March 2010

Keywords:
Pea enation mosaic virus
Luteoviridae
Plant virus transmission
Aphid vector

ABSTRACT

Development of ways to block virus transmission by aphids could lead to novel and broad-spectrum means of controlling plant viruses. Viruses in the Luteoviridae enhanced are obligately transmitted by aphids in a persistent manner that requires virion accumulation in the aphid hemocoel. To enter the hemocoel, the virion must bind and traverse the aphid gut epithelium. By screening a phage display library, we identified a 12-residue gut binding peptide (GBP3.1) that binds to the midgut and hindgut of the pea aphid *Acyrthosiphon pisum*. Binding was confirmed by labeling the aphid gut with a GBP3.1–green fluorescent protein fusion. GBP3.1 reduced uptake of Pea enation mosaic virus (Luteoviridae) from the pea aphid gut into the hemocoel. GBP3.1 also bound to the gut epithelia of the green peach aphid and the soybean aphid. These results suggest a novel strategy for inhibiting plant virus transmission by at least three major aphid pest species.

Introduction

Insects with piercing–sucking mouthparts transmit many plant viruses that cause widespread crop losses (Brown and Bird, 1992; Tatchell, 1989). The major insect vectors are aphids (Aphididae) and whiteflies (Aleyrodidae), with some 275 plant viruses in 19 virus genera transmitted by aphids alone (Nault and Ammar, 1989).

The transmission of plant viruses involves specific molecular interactions between the virus and the aphid vector. For persistent transmission of luteoviruses by aphids, the virus must bind to receptor(s) for transcytosis across the midgut or hindgut epithelium and release into the hemocoel where the virus persistently circulates in a nonpropagative manner (Gray and Gildow, 2003). Receptor(s) are also involved in movement of virus from the hemocoel into the accessory salivary gland, from which the virus is delivered with the aphid saliva into the phloem of the plant. The viral coat proteins, consisting of one major coat protein (CP, 22 kDa) and one minor coat protein (read-through domain, CP-RTD, 35–55 kDa) (Mayo and Ziegler-Graff 1996; Miller et al., 2002), are the sole determinants of vector specificity (Rochow, 1970). Although the RTD is not essential for particle formation (Reutenauer et al., 1993), RTD plays an important role in the transmission process (Brault et al., 1995; Bruyere et al., 1997; Chay et al., 1996; Leiser et al., 1992; Mohan et al., 1995; Prüfer et al., 1995; Veidt et al., 1992) and has been implicated in vector specificity of luteoviruses (Brault et al., 2005). CP alone is sufficient for transport of some luteoviruses through the gut membrane (Chay et al., 1996; Gildow, 1999; Reinbold et al., 2001; Rouze-Jouan et al., 2001; van den Heuvel et al., 1994). Uptake of luteoviruses from the aphid gut into the hemocoel appears to be relatively non-specific; many luteoviruses are taken up into the hemocoel, but few are actually transmitted, presumably being blocked at the salivary gland barrier (Gildow et al., 2000; Gray and Gildow, 2003).

The genome of Pea enation mosaic virus (PEMV) consists of two positive-sense RNAs that belong to two taxonomically different groups. PEMV-1 is the type member of monotypic genus Enamovirus (Luteoviridae), while PEMV-2 belongs to genus Umbravirus. The sequence and genomic organization of PEMV-1 is Polerovirus-like (Demler and deZoeten, 1991). PEMV provides an ideal model virus for study of luteovirus–aphid interactions: PEMV is the only Luteovirid that is not phloem-limited and is thus mechanically transmissible to plants (Demler et al., 1996). The major vector of PEMV is the pea aphid, *Acyrthosiphon pisum* Harris, an emerging model aphid (Brisson and Stern, 2006) whose genome has been sequenced (The International Aphid Genomics Consortium, 2010). PEMV binds to the mid- and hindgut of *A. pisum* and is also vectored by at least six other aphid species (de Zoeten and Skaf, 2001; Demler et al., 1996).

* Corresponding author. Department of Entomology, Iowa State University, 418 Science II, Ames, IA 50011-3222, USA. Fax: +1 515 294 5957.
E-mail addresses: sliu@iastate.edu (S. Liu), sivaento@iastate.edu (S. Sivakumar), wosparks@iastate.edu (W.O. Sparks), wamiller@iastate.edu (W.A. Miller), bbonning@iastate.edu (B.C. Bonning).

0042-6822/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2010.02.009

Viruses in the Luteoviridae are managed primarily by spraying environmentally damaging pesticides to kill the aphid vectors, or by genes that confer tolerance or resistance to the virus. However, there are few plant resistance genes that protect against luteovirus infection, which greatly limits the germplasm available for crop breeding. Numerous transgenic plant lines have been produced that resist aphid-transmitted viruses, for example, by expression of viral coat protein or viral dsRNA (Baulcombe, 2002; Fuchs and Gonsalves, 2007; Goldbach et al., 2003; Prins et al., 2008). However, these strategies are effective only for plant viruses with high sequence similarity to the transgene. There is clearly a need for additional tools for management of aphids and the diseases that they transmit. Here, we report the isolation of an aphid gut binding peptide that appears to compete with PEMV for binding in the gut of the aphid vector, thereby reducing uptake of PEMV virions into the hemocoel. This may lead to a new approach for reducing transmission of aphid-transmitted viruses and may also provide a tool for increased understanding of the molecular interactions of the luteovirus virion with the aphid gut.

Results

Isolation of the pea aphid gut binding peptide GBP3.1

To screen for peptides that bind the pea aphid gut, we devised a biopanning protocol that involved feeding the f88.4-LX8 phage library to aphids and eluting bound phage from dissected gut epithelium. Briefly, aphids were allowed to feed for several hours through parafilm membranes on a sucrose solution containing the f88.4-LX8 phage library. The guts were then dissected, unbound phage washed away, and bound phage removed, amplified and used for another round of feeding (see Materials and methods for details). After each round of biopanning, between 100 and 400 phage plaques were recovered from the aphid gut epithelium. After the third round of biopanning, the phage DNA from 16 plaques was extracted, and the DNA sequences encoding the peptides displayed by each phage were determined. All 16 of the eluted phage isolated after the third round of selection encoded the same peptide sequence, named gut binding peptide 3.1 (GBP3.1): TCSKKYPRSPCM. Replication of the entire experiment gave the same result. To confirm that the phage display library encoded diverse peptide sequences, 10 phages from the library were sequenced, along with 10 phages from each of the first and second rounds of eluted phage. All 10 phage genomes sequenced from the original library had different sequences. Of phage eluted from the first and second rounds, zero and four had the same sequence as GBP3.1, respectively. Use of the same f88.4 phage display library to identify gut binding peptides in other insects in our lab (unpublished data) and elsewhere (Ghosh et al., 2001; Jacobs-Lorena, 2003; James, 2003), resulted in recovery of diverse peptides, indicating that isolation of a single pea aphid gut binding peptide did not result from limitations of the phage display library. A nonbinding peptide, C6 with the sequence FCRTADVIDACT, was randomly selected from the library for use as a negative control in subsequent experiments. GBP3.1 does not have significant amino acid similarity to PEMV CP, but it is predicted to have structural similarity to the predicted βG-βH loop of the PEMV CP, with high hydrophilicity and surface probability (Fig. 1).

GBP3.1 binds the aphid gut but does not enter the hemocoel

To visualize potential gut binding by GBP3.1, pea aphids were allowed to feed on purified protein consisting of the GBP3.1 or C6 sequence fused to enhanced green fluorescent protein (EGFP). After feeding, aphids were examined for fluorescence. Areas of fluorescence in the gut area could be seen in whole aphids fed on GBP3.1–EGFP, but not in aphids fed on C6–EGFP (Fig. 2). Fluorescence was observed in 30% of the aphids fed on the GBP3.1–EGFP ($n=60$). Dissection of aphids that did not appear to fluoresce revealed fluorescence in the gut in some but not all instances. Injection of aphids with GBP3.1–EGFP or C6–EGFP, to assess the distribution of fluorescence in the event that GBP3.1–EGFP entered the hemocoel following feeding, did not result in any areas of concentrated fluorescence, although fluorescence was clearly visible in the pericardial cells that line the dorsal aorta (Fig. 2). The distribution of fluorescence was distinct from that seen following ingestion of GBP3.1–EGFP. Examination of guts dissected from pea aphids fed on GBP3.1–EGFP confirmed extensive binding of GBP3.1–EGFP to both the mid- and the hindgut (Fig. 3). No fluorescent signal was detected in guts from aphids fed on the control fusion, C6–EGFP. These results show that GBP3.1 binds to the aphid gut.

Interference of PEMV entry into the hemocoel

We conducted experiments to determine whether GBP3.1 can block movement of PEMV virions into the hemocoel. First, we determined the minimum time required for detection of viral RNA by RT–PCR in the aphid hemocoel from onset of feeding on a virus-infected plant. For aphids fed on PEMV-infected plants, viral RNA could be detected in the pooled hemolymph of five aphids by RT–PCR after 25 min (Fig. 4). For initial experiments to address whether GBP3.1 competes with PEMV for binding, aphids were fed overnight by membrane feeding on PhD3.1 (the phage that expresses the peptide GBP3.1), negative control phage (PhDC6), or negative control diet (no phage) and then transferred to PEMV-infected plants for acquisition of PEMV. Aphids were then tested for the presence of viral RNA in the hemolymph by RT–PCR at various times after onset of feeding on the infected plants. PhD3.1 delayed penetration of detectable amounts of virus from the aphid gut into the hemocoel with no viral RNA detected up to 80 min after onset of feeding on virus-infected plants (Fig. 4 and Table 1). For aphids fed first on control phage, PEMV was detected in the hemolymph 30 min after virus acquisition, similar to the results for aphids fed on PEMV alone (Fig. 4). In PhD3.1, it is possible that the large size of the phage particle attached to the GBP3.1 peptide may confer steric hindrance that contributes to inhibition of virus uptake. To test this, we conducted the same experiment using the smaller fusion protein GBP3.1–EGFP in place of PhD3.1, and C6–EGFP as the negative control in place of PhDC6. In contrast to PhD3.1, PEMV RNA was detected in the hemolymph of aphids that had fed on GBP3.1–EGFP. However, this was not until 80 min after the start of feeding on the PEMV-infected plant in aphids fed previously with GBP3.1–EGFP (Fig. 4). In the negative control, PEMV RNA was detected by RT–PCR in the hemolymph of all samples derived from C6–EGFP-fed aphids 30 min after the start of feeding on the PEMV-infected plant (Fig. 4). This result supports the hypothesis that GBP3.1 competes with PEMV for binding to the aphid gut, thereby hindering uptake of PEMV into the aphid hemocoel. We speculate that either the large PhD3.1 phage fusion sterically hinders PEMV access to a receptor or it displays the GBP3.1 peptide in a slightly different or more accessible conformation that allows the peptide to bind the gut (receptor) more tightly than in the GBP3.1–EGFP context.

Impact of GBP3.1 on relative virus load in the aphid hemocoel

We used qRT–PCR to examine the relative virus loads within the hemocoel of aphids fed on GBP3.1–EGFP or C6–EGFP prior to PEMV acquisition from infected plants (Fig. 5). The relative virus titers were significantly lower for aphids fed on GBP3.1–EGFP following 30 min and 60 min of virus acquisition on infected plants when compared to aphids fed on C6–EGFP ($P<0.05$, one-way ANOVA). There was no significant difference between treatments in virus load by 90 min of virus acquisition. Variation in aphid feeding behavior

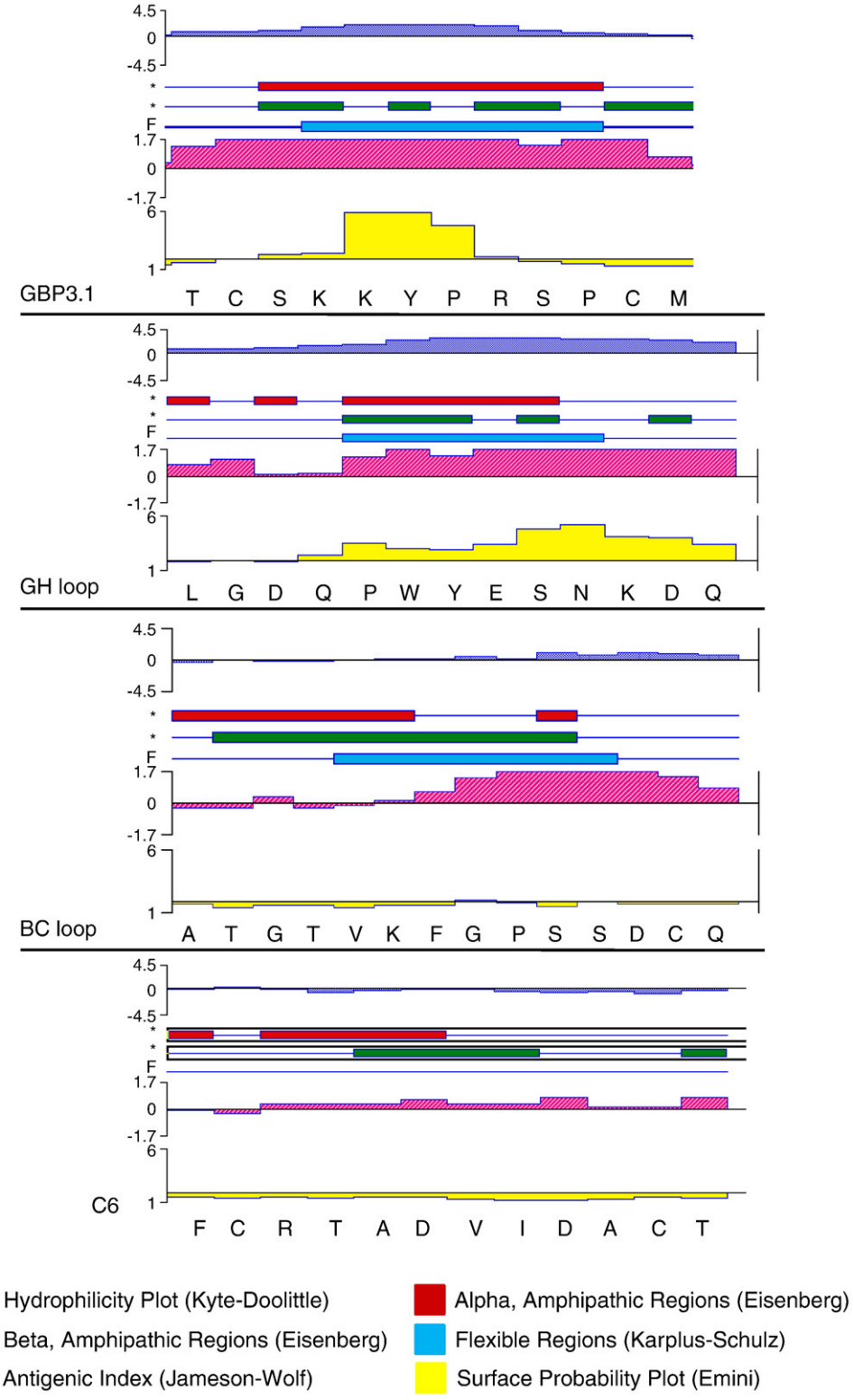

Fig. 1. Predicted properties of peptides and regions of PEMV coat protein. The hydrophilicity plots, surface probability and structural features of GBP3.1, were compared with those of the PEMV βB–βC (BC) and βG–βH (GH) putative surface loops using Protean prediction software (DNAstar Inc. v. 5.0). The properties of the nonbinding control peptide, C6, are also shown. The antigenicity profiles were predicted using the Jameson–Wolf index and DNAstar 5.0. The *y* axes represent probability. The structural features of GBP3.1 are similar to those of the PEMV βG–βH loop. The surface probability plot for the βB–βC loop sequences does not support a surface location for this sequence.

during the relatively short virus acquisition period could mask differences at these higher virus levels. These results further support the hypothesis that GBP3.1–EGFP impedes entry of PEMV into the aphid hemocoel, resulting in reduced virus loads relative to the control treatment.

GBP3.1 binds the gut of other aphid species

To determine whether GBP3.1 bound the midgut epithelia of other aphid species, we used fluorescent 5FAM tag-labeled synthesized GBP3.1 and C6 peptides. Binding of GBP3.1 to the pea aphid gut was

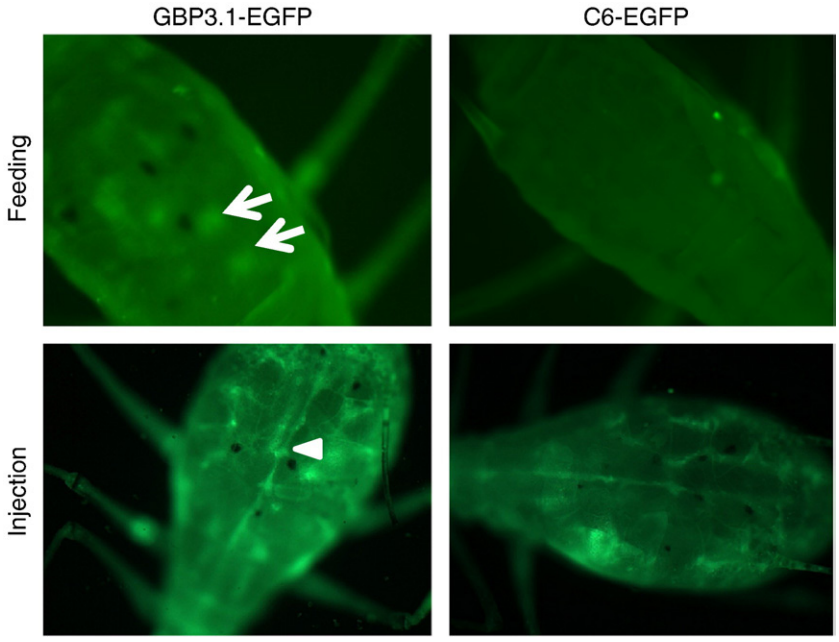

Fig. 2. GBP3.1–EGFP does not enter the pea aphid hemocoel. Fluorescence light microscopy images of aphids fed or injected with GBP3.1–EGFP or C6–EGFP. Aphids were fed on the test proteins in 25% sucrose solution by membrane feeding for 16 h before being observed under UV light. Regions of fluorescence were observed in the whole aphid for GBP3.1–EGFP in the general area of the gut (arrows). Arrowhead indicates pericardial cells lining the dorsal aorta that appear to take up injected GBP3.1–EGFP. For injection experiments, pea aphids were examined 1 to 2 h after injection.

confirmed, in contrast to the low level of gut association apparent for the C6 peptide (Fig. 6). Multiple sections examined on four slides of individual guts were all positive. Binding of GBP3.1 to the guts of the green peach aphid, *Myzus persicae* and the soybean aphid, *Aphis glycines* was also demonstrated (Fig. 6). Bound GBP3.1 was observed in all gut cross sections for the three or four guts examined for each

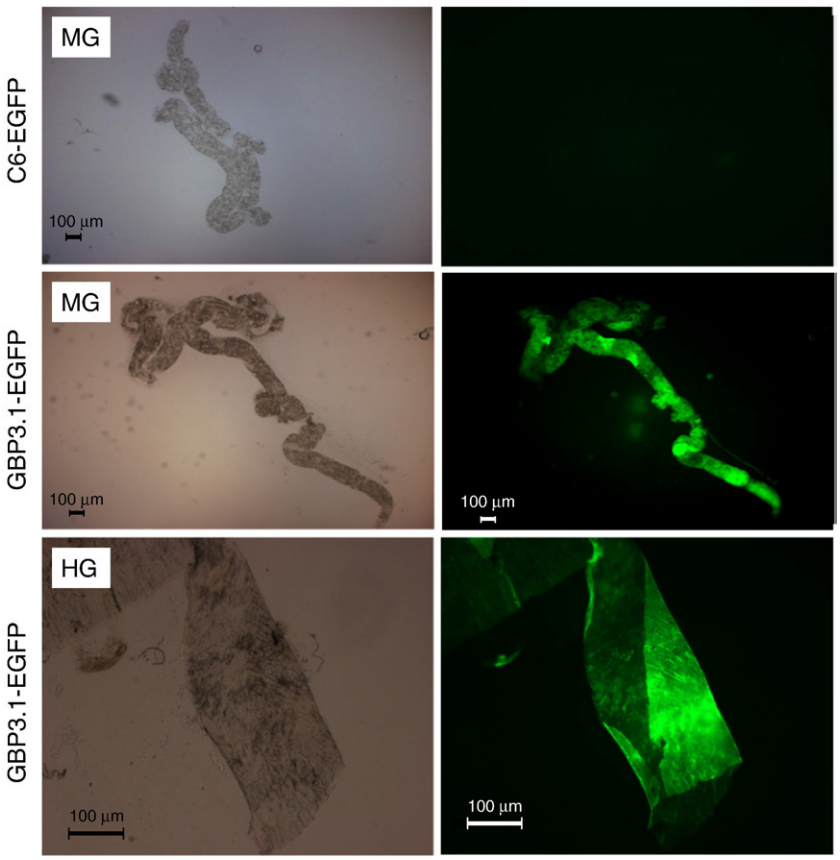

Fig. 3. Distribution of GBP3.1–EGFP in the midgut and hindgut of the pea aphid. Representative aphid guts (from 30 guts examined per treatment in 3 replicate experiments) observed under normal light (left column) and under UV light (right column). No fluorescence was detected in guts isolated from aphids fed with the C6–EGFP fusion protein. GBP3.1–EGFP bound to the surface of both the midgut (MG) and hindgut (HG) of aphids fed GBP3.1–EGFP. Scale bars, 100 µm.

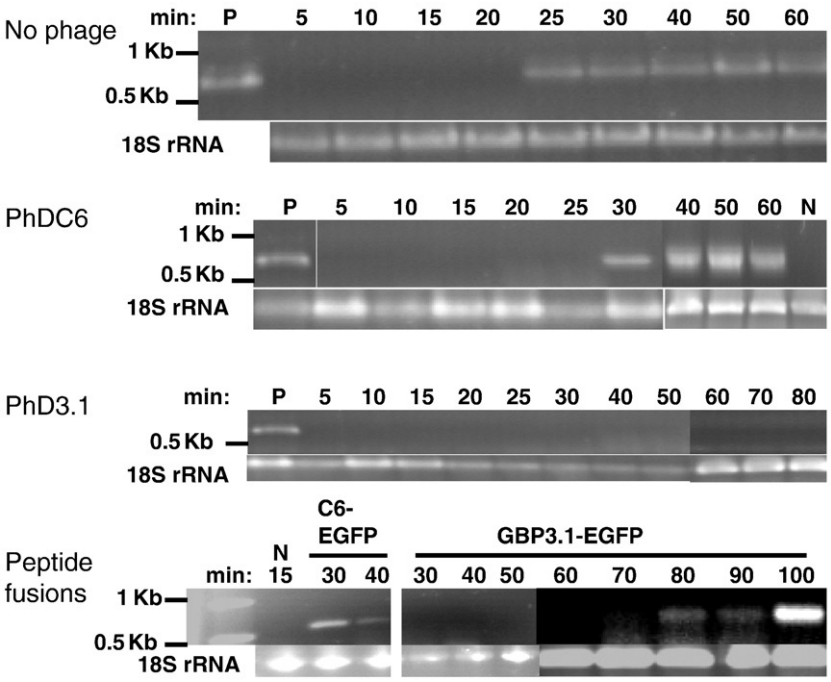

Fig. 4. Effect of aphid feeding on phage or peptides on uptake of PEMV into the hemolymph. Before being fed on PEMV-infected plants, aphids were fed overnight on buffer alone (no phage), control phage, (PhDC6), phage expressing GBP3.1 (PhD3.1), or the peptide fusion GBP3.1–EGFP, or C6–EGFP. PEMV RNA1 was detected in the aphid hemolymph by RT–PCR after time intervals on PEMV-infected plants, indicated by numbers (min) above each panel. Each sample contained total hemolymph RNA pooled from five aphids. P, positive control prepared by damaging the gut of aphids fed on infected plants for 15 min, during hemolymph collection such that PEMV present in the aphid gut would be detected. N, negative control aphids: RNA was extracted from the hemolymph of aphids fed on infected plants for 15 min only. Lower panels, 18S rRNA to confirm that negative results were not caused by loss of RNA during the RNA extraction process.

species. These results indicate that GBP3.1 binds to the guts of multiple aphid species, including one (*A. glycines*) not known to be a vector of PEMV.

Discussion

By screening a phage display library for peptides that bind the gut of the pea aphid, we identified a gut binding peptide, GBP3.1, that clearly hinders uptake of PEMV into the aphid hemocoel. Isolation of only one aphid gut binding peptide sequence was unexpected. Selection of a single peptide may have resulted from high abundance of the receptor in the aphid gut, or tight binding.

Blast analysis of the GBP3.1 peptide sequence did not reveal any proteins with obviously similar sequences. Although structural similarities have not been examined in detail (Reineke and Schneider-Mergener, 1998; Tsonis and Dwivedi, 2008), we assessed the properties of the GBP3.1 and C6 peptide sequences and compared them to the PEMV βB–βC and βG–βH loops. The sequence of amino acids 65-78 of PEMV CP is conserved among luteoviruses and this conserved region is located in a putative surface βB–βC loop of potato leafroll virus (PLRV) (Terradot et al., 2001; Torrance, 1992). Eight of 12 amino acids are identical among five luteoviruses and most of the conserved amino acids in this region are hydrophobic (Table 2). Conservation of the surface structure in this region suggests that this region may serve as a ligand for interaction with aphid gut receptors. However, the surface probability plot for the βB–βC loop is not strong (Fig. 1). The PEMV βB–βC loop has the motif 72-GPSSDCQ-78, similar to the PLRV "HDSSEDQ" epitope (Terradot et al., 2001; Torrance, 1992). This epitope was predicted to be on an acidic surface loop (βG–βH) of PLRV, which is involved in virion assembly, systemic movement, and aphid transmission (Lee et al., 2005). Based on peptide properties, GBP3.1 is similar to the βG–βH loop of PEMV (Fig. 1). We hypothesize that the PEMV βG–βH loop binds to the aphid gut epithelium and that GBP3.1 outcompetes the βG–βH loop for binding to the same site. This is consistent with the proposed mechanism of interference of aphid transmission by related isolates of Barley yellow dwarf luteovirus (BYDV) (Gildow and Rochow, 1980). These authors showed that feeding aphids with an isolate of BYDV reduced subsequent transmission efficiency of a closely related isolate. They proposed that the virions of the first virus occupied receptors in the aphid, thus reducing access to the receptors by the second isolate. GBP3.1 may be acting similarly by binding a receptor,

Table 1
Detection of PEMV RNA 1 in aphid hemolymph following ingestion of phage (PhD) or peptide fusion proteins prior to feeding on PEMV-infected plants. The RNA in each sample was extracted from the pooled hemolymph of five aphids for detection of PEMV RNA1 by RT–PCR. — , not tested.

Treatment	Time fed on PEMV-infected plants (min)									
	5–10	15–20	25–30	35–40	45–50	55–60	65–70	75–80	85–90	>90
Buffer	0/6[a]	0/8	9/15	6/6	6/6	2/2	2/2	2/2	3/3	–
PhDC6	0/4	0/4	2/4	2/2	2/2	2/2	2/2	2/2	2/2	–
PhD3.1	0/6	0/6	0/6	0/6	0/6	0/6	0/2	0/2	–	–
C6–EGFP	–	–	3/3	3/3	–	–	–	–	–	–
GBP3.1–EGFP	–	–	0/6	0/5	0/3	0/3	1/5	3/6	4/5	6/6

[a] Number of positive pools/number of pools tested.

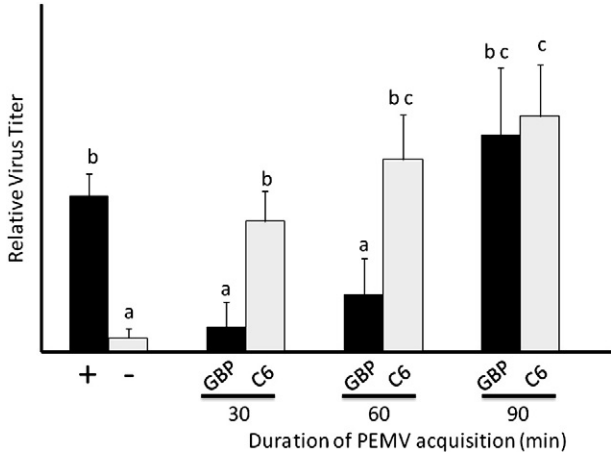

Fig. 5. Quantification of the relative virus load in hemolymph of aphids fed on GBP3.1–EGFP. Aphids fed on GBP3.1–EGFP (GBP) or C6–EGFP (C6) by membrane feeding were transferred to PEMV infected plants. After 30 to 90 min of virus acquisition, hemolymph was isolated from five aphids per treatment per time point. Relative virus titer was determined by qRT–PCR detection of viral RNA with reference to 18S RNA levels. +, positive control: five aphids were fed on infected plants for a period of 20 min and RNA isolated from whole aphids; −, negative control: five aphids were fed on infected plants for 15 min, and RNA isolated from hemolymph. Different letters indicate significant differences in relative virus levels ($P<0.05$, ANOVA).

but GBP3.1 binding may not trigger endocytosis with subsequent transport into the hemocoel. In addition, Whitfield et al. demonstrated the use of a virion glycoprotein to block transmission of Tomato spotted wilt virus (TSWV) by thrips (Whitfield et al., 2008).

Analysis of the fate of GBP3.1–EGFP following feeding and injection (Figs. 2 and 3) indicates that GBP3.1 binds to the aphid mid- and hindgut, but does not appear to enter the aphid hemocoel. If GBP3.1 entered the hemocoel, one would expect distribution of fluorescence following feeding to be similar to that observed following injection, which was not the case (Fig. 2). In particular, some concentration of fluorescence in the pericardial cells that regulate hemolymph composition, was observed following injection, but not following feeding on GBP3.1–EGFP. The molecular determinants required for binding to the gut epithelium and for triggering endocytosis to traverse the epithelium remain to be determined. GBP3.1 also binds the gut epithelium of the green peach aphid, *M. persicae*, an important vector of many viruses important to agriculture, including PEMV (Demler et al., 1996), and the soybean aphid, *A. glycines*, a major invasive pest in the mid-western United States (Fig. 6).

We have shown that feeding on GBP3.1 (fused to EGFP or expressed by a phage) impedes movement of PEMV into the pea aphid hemocoel. We hypothesize that with ongoing feeding on the PEMV-infected plant in our experiments, the fixed initial level of GBP3.1 fusion protein was eventually outcompeted for receptor binding by steadily increasing levels of PEMV accumulating in the gut during feeding (by 80 min; Fig. 4), eventually allowing movement of PEMV into the aphid hemocoel. Also, with no access to new GBP3.1 as the aphid gut sheds cells over time, the GBP3.1 fusion protein would be lost, and the replacement cells would provide free receptors available for PEMV binding. Further analysis to examine the relative virus load within the aphid hemocoel by qRT–PCR (Fig. 5) showed that low levels of virus were indeed present in the hemolymph even after a short time on the infected plant. These low levels of virus were too low to be detected by the conditions used for RT–PCR. The biological significance, if any, of these low levels of virus in terms of virus transmission remains to be established. Continuous exposure of aphids to GBP3.1 expressed by transgenic plants is expected to impede movement of PEMV into the aphid hemocoel and potentially reduce the incidence of virus transmission.

Despite the fact that insects transmit or are infected by a wide variety of viruses (Miller and Ball, 1998), in no case has the receptor(s) for uptake of virus into the insect been identified. Given that luteoviruses enter the hemocoel of both vector and non-vector aphid species (Gildow, 1993; Gray and Gildow, 2003), proteins and glycans that are common across aphid species are likely to be exploited by luteoviruses for uptake into the hemocoel. Aphid proteins that may bind to luteoviruses have been isolated (Li et al., 2001; Seddas et al., 2004; Yang et al., 2008), but a gut receptor has not been identified. GBP3.1 may provide a useful tool for identification of the pea aphid gut receptor for PEMV.

In summary, we have isolated an aphid gut binding peptide that impedes uptake of PEMV into the aphid vector. When expressed in transgenic plants, this peptide has potential to reduce transmission of circulative viruses in aphids and/or whiteflies. A similar approach could be used to interfere with cuticular attachment of plant viruses that are semipersistently or nonpersistently transmitted by their aphid vectors (Fereres and Moreno, 2009; Ng and Falk, 2006). Because of the economic damage caused directly by the aphid vectors of plant viruses, however, use of a plant virus "transmission blocking" technology alone may be insufficient for crop protection. Such a transmission blocking peptide could be useful to minimize plant virus transmission in conjunction with aphid resistant cultivars that inadvertently increase aphid sampling behavior and subsequent plant virus transmission (Fereres and Moreno, 2009; Tarn et al., 1992). Similar transmission blocking strategies with phage-display-identified peptides were used to identify a receptor for the *Plasmodium falciparum* agent of malaria (Ghosh et al., 2001) and are under consideration for management of mosquito-borne malaria (Targett and Greenwood, 2008).

Conclusions

1. An aphid gut binding peptide, GBP3.1, was isolated by screening a phage display library
2. GBP3.1 has structural similarity to the βG–βH loop of PEMV CP
3. GBP3.1 binds the midgut and hindgut epithelium of the pea aphid but does not appear to enter the hemocoel
4. GBP3.1 impedes the movement of PEMV into the hemocoel of the pea aphid
5. GBP3.1 also binds the guts of the green peach aphid and the soybean aphid

Materials and methods

Insects

Pea aphids, *A. pisum* Harris (Aphidinae: Macrosiphini), were obtained from Berkshire Biological Supply Company (Westhampton, MA) and reared on broad bean, *Vicia faba*. Green peach aphids, *M. persicae* (Sulzer) (Aphidinae: Macrosiphini) were reared on Chinese cabbage, *Brassica rapa*, and soybean aphids, *A. glycines* Matsumura (Aphidinae: Aphidini) were reared on soybean, *Glycine max*. All aphid colonies were maintained in growth chambers at 24 °C with a 12-h light/12-h dark cycle.

Phage selection

A phage display library consisting of random 12 amino acid peptides displayed on the filamentous phage vector f88.4 by fusion to the N-terminus of the phage coat protein VIII was used (Bonnycastle et al., 1996). Cysteine residues that produce a disulfide bond are included at each end of the random sequence in this library to maintain a constrained loop structure. The library was amplified by infecting K-91 cells and phage were precipitated using 20% PEG 8000 (Sigma, St. Louis, MO) and 2.5 M NaCl. Methods used for preparing

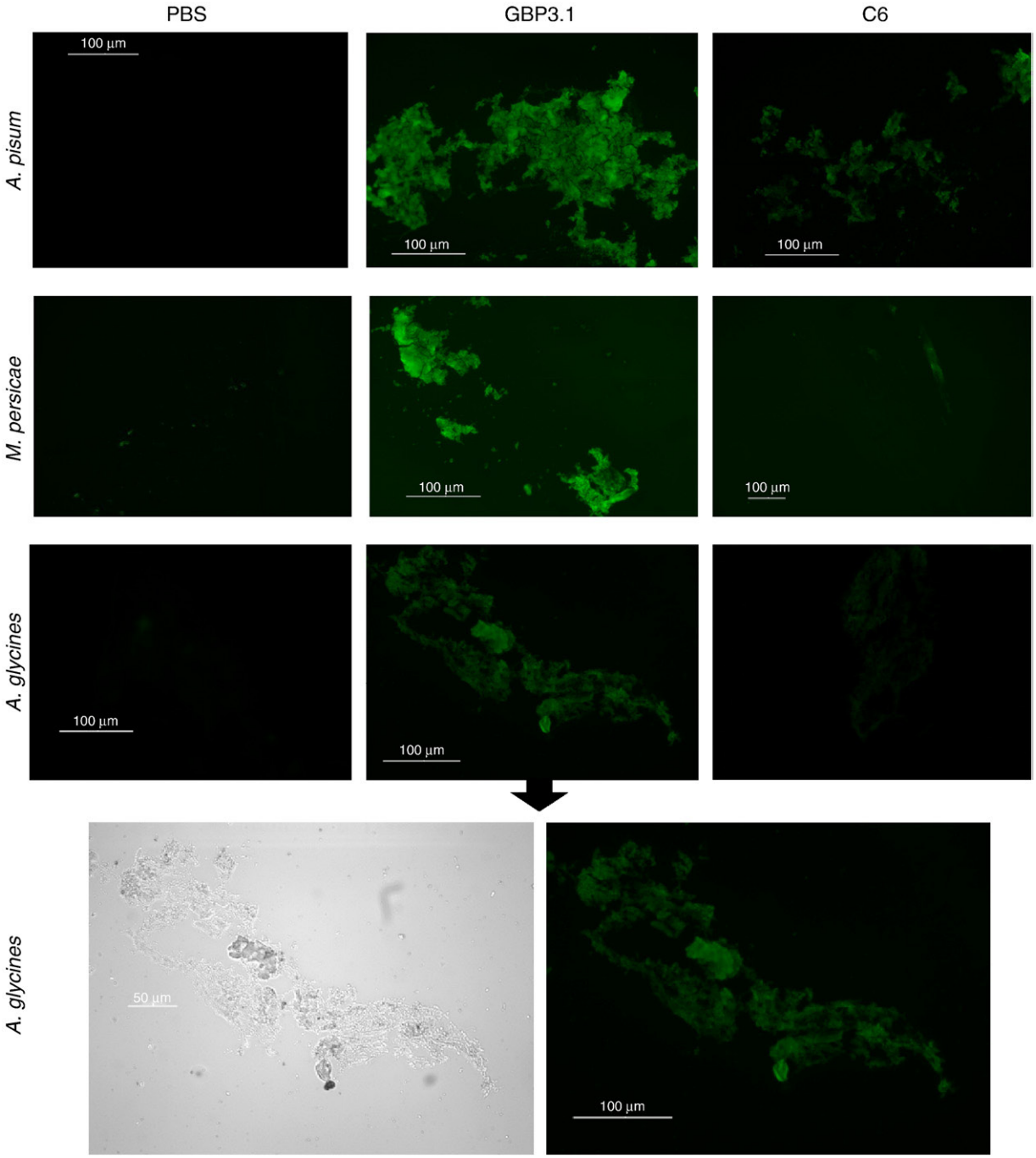

Fig. 6. Binding of GBP3.1 to the gut epithelia of three aphid species. GBP3.1 and C6 peptides were synthesized with N-terminal fluorescent 5FAM labels. These peptides were incubated with guts dissected from *M. persicae*, *A. pisum*, or *A. glycines*, and the guts were examined for fluorescence. With reference to the C6 nonbinding peptide and PBS control treatments, this qualitative approach confirmed binding of GBP3.1 to the gut of *A. pisum* and also showed binding of GBP3.1 to the gut of *M. persicae* and *A. glycines*. A bright field image of the GBP3.1 treatment of the *A. glycines* gut is also shown to facilitate interpretation. Images are representative of 2 to 4 cryosections examined per treatment. Bars, 100 μm; Bright field image, 50 μl.

Table 2
Alignments of conserved amino acids in selected domains of luteovirid CPs. PEMV CP fragment 65–78 is located in the βB–βC loop. A comparison of the PEMV and PLRV βG–βH loop sequences is also shown. Conserved amino acids are shown in bold. The sequences correlating to epitope 5 in PLRV are underlined (Terradot et al., 2001). SbDV, soybean dwarf virus; BMYV, beet mild yellowing virus; BWYV, beet western yellows virus.

Virus	βB–βC loop		βG–βH loop
PEMV	65 AT**G**TVK**FGPS.SDC**Q 78	PEMV	147 LGDQPWYE**S**NK**DQ** 159
SbDV	SK**G**YIT**FGPSLSEC**	PLRV	166 INGVEWHD**S**SE**DQ** 178
PLRV	SQ**G**SFT**FGPSLSDC**		
BMYV	SS**G**AIT**FGPSLSDC**		
BWYV	SS**G**AIT**FGPSLSDC**		

cells for infection, amplifying and preparing phage libraries were as previously described (Bonnycastle et al., 1996; Smith and Scott, 1993). The phage preparations precipitated from 250-ml cultures, were resuspended in 5 ml of PBS and 15% glycerol, aliquoted (200 μl), and stored at −80 °C. The phage concentration was determined by titration (Smith and Scott, 1993).

For selection of phage that bound to the aphid gut, fourth instar nymphs, were starved at 4 °C overnight (16 h), and then fed with 100 μl of PBS containing 25% sucrose and ~1×10^{14} pfu of f88.4 phage by membrane feeding (Chay et al., 1996) at room temperature for 4 to 16 h. The guts of 30–50 aphids were isolated after feeding (Liu et al., 2006) and suspended in 100 μl of PBS (100 mM, pH 7.0) containing 1%

BSA. The gut tissues were gently ground by using a Kontes pellet pestle (Fisher Scientific, Pittsburgh, PA) and the tissue suspensions spun at 1000×*g* in a bench top centrifuge for 1 min. The supernatant was removed and the pellets were resuspended in 500 µl of PBS, 1% BSA to wash out unbound phage. The resuspensions were centrifuged as previously, and the washing step was repeated once. The bound phage were eluted by adding 300 µl of elution buffer (50 mM glycine–HCl, pH 2.2, 1 mg/ml BSA) and rotated gently in a Labquake™ Shaker (Barnstead/Thermolyne, Dubuque, IA) for 5 to 8 min at room temperature, and then briefly centrifuged at 1000×*g*. The supernatants were transferred to a 1.5-ml tube and neutralized by adding 8 µl of 2 M Tris–base (pH 9.1). Twenty microliters of the eluted phage was used for titration to estimate the number of recovered phage from the biopanning process. The remainder of the eluted phage was amplified immediately in K-91 cells as described previously (Smith and Scott, 1993). The enriched phages were titrated and used for the next round of biopanning. The biopanning process was repeated three times, and the entire phage display library screen was repeated twice.

After the third round of selection, eluted phage were plated on to NZY + tetracycline (20 µg/ml) plates. Individual randomly selected bacterial colonies were cultured and used for isolation of phage DNA following standard phage DNA purification procedures (Sambrook and Russell, 2001). The sequencing primer (5′-CTGAAGAGAGT-CAAAAGC-3′) (Bonnycastle et al., 1996) was used for sequencing to determine the predicted amino acid sequence of inserts in the selected phage.

Production of peptide–EGFP fusions

The pBAD/His B expression vector was used for expression of peptide–EGFP fusions with His tags (Invitrogen, San Diego, CA). A gut binding peptide (GBP3.1) and a nonbinding, control peptide (C6) were fused at the N-terminus of EGFP and inserted into pBAD/His B. The DNA molecules encoding the fusion proteins were generated by PCR. Three oligonucleotides were used for integrating the peptide and EGFP DNA sequences: a forward primer containing a *Sac*I site and the peptide sequences, an oligonucleotide connecting the short peptide and the 5′end of the EGFP sequence, and a reverse primer complementary to the 3′ end of the EGFP sequence. The primers used for construction of the GBP3.1–EGFP fusion sequence were GBP3.1-*Sac*I (5′-CCGGAGCTCGgccacgtgtagtaagaagtatcc-3′, with the *Sac*I site underlined and lower case text indicating the coding part of the fusion protein) and FGBP3.1–EGFP (5′-gccacgtgtagtaagaag-tatccgcgttctccgtgtatggctgtgagcaagggcgagg-3′). The primers used for the C6–EGFP fusion were C6-*Sac*I (5′-GCGGAGCTCGgccttttgtcg-tacggctgatgtg -3′) and FC6–EGFP (5′-gccttttgtcgtacggctgatgtgatt-gatgcgtgtacggctgtgagcaagggcgagg-3′). The reverse primer used was EGFP-*Hind* III (5′-CCAAAAGCTTGGttacttgtacagctcgtccatg-3′). PCR was performed in a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA) with high fidelity pfu DNA polymerase (Stratagene, La Jolla, CA). The PCR was conducted in a volume of 50 µl containing 25 pm GBP3.1/C6-*Sac*I, 1 pm FGBP3.1/FC6–EGFP, 25 pm reverse primer, and 5 U of pfu DNA polymerase. Conditions for PCR were 1× 94 °C for 2 min; 5× of 94 °C for 45 s, 60 °C for 1.5 min, 72 °C for 4 min; 30× of 94 °C for 45 s, 60 °C for 35 s, 72 °C for 4 min, followed by 72 °C for 7 min.

The PCR products were run into a 1% agarose gel with ethidium bromide staining and the 0.78 kb fragments were isolated and purified by using a QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA). The DNA fragments were digested with *Sac* I and *Hind* III, cleaned by using a QIAquick Nucleotide Removal Kit (QIAGEN), and ligated into pBAD/His B (previously linearized with *Sac* I and *Hind* III). For expression of GBP3.1–EGFP and C6–EGFP, competent Top10 cells were transformed with the plasmid pGBP3.1EFGP or pC6EGFP. Cells were cultured in 50-ml low-salt LB medium containing ampicillin (100 µg/ml) in a 500-ml flask and shaken in a orbital shaker at 250 rpm (37 °C) until the OD450 reached 0.4 to 0.5. L-(+)-arabinose (Sigma, St. Louis, MO) was then added to the culture to a final concentration of 0.02% to induce protein expression. The culture was maintained overnight at 30 °C with shaking at 250 rpm. The overnight cultures were then chilled at 4 °C for 15 min, transferred to a 50-ml centrifuge tube, and the cells harvested by centrifugation in a swinging bucket rotor at 4500 rpm for 25 min. The resulting cell pellets were frozen at −80 °C for at least 30 min before being used for protein purification.

The His-tagged fusion proteins were purified using Ni-NTA agarose resin (QIAGEN, Germantown, MD) 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, Billerica, MA) and dialyzed in Side-A-Lyzer Dialysis Cassettes (0.1–0.5 ml capacity and 3500 MWCO) (PIERCE, Rockford, IL) with PBS buffer. The fusion proteins were stored at −80 °C.

The structural features of the test and control peptides isolated from the biopanning procedure were determined using the Protean prediction software (DNAstar Inc. v. 5.0) and compared to the putative βB–βC (BC) and βG–βH (GH) surface loop sequences of PEMV CP (Lee et al., 2005). The antigenicity profiles were predicted using the Jameson–Wolf index and DNAstar 5.0.

Confirmation of peptide binding to the aphid gut

To test whether the peptides selected from the biopanning procedure and the control peptide bound to the aphid gut and whether the fusions would enter the aphid hemocoel, the peptide–EGFP fusions were fed to prestarved aphids (30 aphids per membrane feeding sachet, three replicates per treatment) and the aphids subsequently examined for green fluorescence using a fluorescence microscope. Fusion proteins (1 µg) were resuspended in 100 µl of 25% sucrose in PBS, and fed to fourth instar *A. pisum* by membrane feeding for 16 h. Sixty aphids and thirty dissected guts per treatment were then observed under a compound microscope (Zeiss Axioplan II fluorescence microscope with an FITC filter). The gut contents were removed by breaking the gut tissues and washing with PBS. Images were recorded under natural and UV light using a Zeiss Axiocam digital camera.

Peptide binding to different aphid species

To address whether the pea aphid gut binding peptide GBP3.1 bound to the guts of other aphid species, GBP3.1 and the C6 control peptide were synthesized by Neo-Peptide (NeoBioPharma, Inc, Cambridge, MA) with N-terminal 5-FAM (5-carboxyfluoroscein) tags and a 6-aminohexanoic acid (ahx) spacer. The sequences of the peptides were GBP3.1FAM: TCSKKYPRSPCM-OH, and C6FAM: FCRTADVIDACT-OH. Aphid guts (3–4 per species) were dissected from the pea aphid *A. pisum*, the green peach aphid, *M. persicae*, and the soybean aphid, *A. glycines* in PBS buffer, pH 7.4, immediately placed in Tissue-Tek O.C.T. mounting medium (Ted Pella, Inc., Redding, CA) and frozen. Cryosections, approximately 20 µm in width, were made using an International Cryostat model CTI (International Equipment Company, Needham Heights, MA, USA) on to ProbeOn Plus slides (Fisher Scientific). The slides were allowed to air dry for 2 h. Slides were then washed twice with PBS for 5 min to remove the O.C.T., and sections were circled with a PAP pen to localize reagent on the sample. The sections were then incubated in PBS alone, or with 71 or 142 µl of peptide solution at a concentration of 14 ng/µl in PBS (1 µg of peptide) for 1 h at room temperature in the dark with a humidity source. Slides were then washed twice in PBS for 5 min, mounted using Fluoro-Gel (Electron Microscopy Services, Hatfield, PA), and coverslips sealed with clear fingernail polish. The slides were stored at 4 °C in the dark until viewed using a Zeiss Axioplan II fluorescesce

microscope using a FITC filter. Guts of three or four individuals were examined per treatment for each species of aphid tested with two to four cryosections examined per sample. For *A. glycines* and *M. persicae*, which are relatively small in size, three or four guts were combined for cryosectioning, while the guts of *A. pisum* were examined individually for fluorescence. Images were usually captured within 24 h, but the signal retained intensity for up to 1 week. All samples were exposed for 145 ms at a magnification of 40×.

Injection of peptide–EGFP fusions into the aphid hemocoel

The peptide (GBP3.1 or C6)–EGFP fusions were injected into the aphid hemocoel as described previously (Fukatsu et al., 2001). Each aphid was injected with 100 nl of 1× PBS containing 100 ng of fusion protein by using ultra thin glass capillary tubes. The capillary was inserted into the base of the aphid hind leg and the solution injected by air pressure created with the help of a syringe. At least 10 aphids were injected with each fusion protein. Aphids were examined for fluorescence as described above.

In vitro transcription of PEMV RNA and plant inoculation

The full-length clones of PEMV RNAs 1 (pPER1) and 2 (pPER2) were linearized with *Pst* I and *Sma* I, respectively (Demler et al., 1997), and 1–2 μg of the linearized cDNA was used for *in vitro* transcription to generate infectious RNA1 and RNA2 transcripts. Capped, *in vitro* transcripts were synthesized with T7 RNA polymerase, by using the mMESSAGE mMACHINE® High Yield Transcription Kit (Ambion, Austin, TX) according to the manufacturer's directions. Seven-day-old pea seedlings were inoculated with a mixture of RNA1 and RNA2 transcripts (1 μg of each) by rubbing the inocula on to the leaves, five plants per treatment.

Interference of PEMV entry into the hemocoel

To determine the minimum time needed for detection of PEMV RNA in the hemolymph by RT–PCR, third or fourth instar aphids (20–40 per time point) that were fed on 25% sucrose for 16 h, were allowed to acquire PEMV for periods from 5 to 60 min in 5-min intervals. Following the acquisition period, hemolymph was isolated immediately (Liu et al., 2006) or aphids were frozen at −80 °C for subsequent extraction of total RNA. Hemolymph from five aphids was pooled for RT–PCR analysis.

Third and fourth instar pea aphids (30 per sachet, 3 sachets per treatment; total of 90 aphids per treatment) were fed on phage or peptide–EGFP fusions as described above. The test aphids were then transferred to PEMV infected plants (7–10 dpi) for acquisition of PEMV for variable amounts of time and then used for hemolymph isolation. To avoid variation in virus titer in different plants, which could affect the detection threshold for viral RNA in the aphid hemocoel, the same leaves of the same infected plants were used sequentially for virus acquisition by aphids in the different treatments. This transmission blocking experiment was replicated with 10 to 75 aphids tested per treatment per time point (5 aphids per RT–PCR sample, three plants per experiment).

Isolation of aphid hemolymph and detection of viral RNA

Methods for hemolymph isolation were as described previously (Liu et al., 2006). For each treatment, hemolymph was collected and pooled from five individual aphids and stored at −80 °C until use. Total RNA from whole viruliferous aphids or from hemolymph samples was extracted using Trizol® Reagent (Invitrogen) according to the manufacturer's instructions. The precipitated RNA was resuspended in 20 μl of nuclease-free water (Ambion) and stored at −80 °C.

RT–PCR was used for detection of PEMV1 RNA in viruliferous aphids. For reverse transcription of PEMV RNA1 viral RNA, 5 μl of total RNA was used, and 2 μl was used for generating the first-strand cDNA of 18S RNA as an internal control. Superscript III (Invitrogen) was used for the RT reaction. In addition to using the standard protocols provided by the manufacturer, 1 μl of RNaseOUT™ Ribonuclease (RNase) inhibitor (Invitrogen) was included in the RT reaction. The primer used for RT was RNA1 4719R (5′-TATCGTGGTCATTTCTCTC-3′) for PEMV. For PCR, the forward primer was RNA1 4123 (5′-GAGGCG-GGGGATTGAATG) and the reverse primer was RNA1 4719R. The primers used for RT–PCR amplification of pea aphid 18S rRNA were as reported previously (Liu et al., 2006).

Quantification of relative virus levels in aphid hemolymph

Aphids were membrane fed for 16 h as described above on GBP3.1–EGFP or C6–EGFP and then transferred to PEMV-infected plants. Hemolymph was extracted from five aphids after 30, 60, or 90 min of acquisition feeding on PEMV-infected plants, for each treatment. Hemolymph samples were preserved in 100 μl of TRIzol reagent (Invitrogen) and stored at −80 °C. Total RNA was isolated according to the manufacturer's directions. For a positive control, five aphids were fed on infected plants for a period of 20 min, and RNA isolated from the whole aphids. For a negative control, RNA was isolated from the hemolymph of five aphids that had fed on infected plants for a period of only 15 min.

Reverse transcription of the viral RNA was as described above. Methods for qRT–PCR with TaqMan polymerase were as described previously (Liu et al., 2006, 2009). A conserved region located between nt 3726 and 3870 in RNA1 was amplified. IQ SYBR Green Supermix (Bio-RAD) and IQ Supermix (Bio-RAD) were used for the amplification of18S and viral cDNA, respectively. PCR was performed in triplicate and analyzed on a Bio-Rad My IQ5 Optical system. Values for relative viral RNA titers were calculated and normalized with reference to 18S rRNA in the RNA samples. The experiment was replicated three times. Statistical significance in relative virus loads between treatments at each time point and across time points were compared by one-way ANOVA.

Acknowledgments

The authors thank Dr. J.K. Scott, Simon Fraser University, B.C., Canada for provision of the phage display library and K-91 *E. coli* strain, Dr. Zhaohui Wang for assistance with inoculation of plants with PEMV, and Dr. John Hill for critical reading of the manuscript. This material is based upon work supported by the Carver Trust Fund, the Iowa State University Plant Sciences Institute, and the USDA National Research Initiative grant no. USDA 2009-35302-05266 as well as Hatch Act and State of Iowa funds.

References

Baulcombe, D., 2002. RNA silencing. Curr. Biol. 12, 82–84.
Bonnycastle, L.L., Mehroke, J.S., Rashed, M., Gong, X., Scott, J.K., 1996. Probing the basis of antibody reactivity with a panel of constrained peptide libraries displayed by filamentous phage. J. Mol. Biol. 258, 747–762.
Brault, V., Van den Heuvel, J.F., Verbeek, M., Ziegler-Graff, V., Reutenauer, A., Herrbach, E., Garaud, J.C., Guilley, H., Richards, K., Jonard, G., 1995. Aphid transmission of beet western yellows luteovirus requires the minor capsid read-through protein P74. EMBO J. 14, 650–659.
Brault, V., Perigon, S., Reinbold, C., Erdinger, M., Scheidecker, D., Herrbach, E., Richards, K., Ziegler-Graff, V., 2005. The polerovirus minor capsid protein determines vector specificity and intestinal tropism in the aphid. J. Virol. 79, 9685–9693.
Brisson, J.A., Stern, D.L., 2006. The pea aphid, *Acyrthosiphon pisum*: an emerging genomic model system for ecological, developmental and evolutionary studies. Bioessays 28, 747–755.
Brown, J.K., Bird, J., 1992. Whitefly-transmitted diseases in the Americas and the Caribbean Basin: past and present. Plant Dis. 76, 220–225.
Bruyere, A., Brault, V., Ziegler-Graff, V., Simonis, M.-T., van den Heuvel, J.F., Richards, K., Guilley, H., Jonard, G., Herrbach, E., 1997. Effects of mutations in the beet western

yellows virus readthrough protein on its expression and packaging and on virus accumulation, symptoms and aphid transmission. Virology 230, 323–334.
Chay, C.A., Gunasinge, U.B., Dinesh-Kumar, S.P., Miller, W.A., Gray, S.M., 1996. Aphid transmission and systemic plant infection determinants of barley yellow dwarf luteovirus-PAV are contained in the coat protein readthrough domain and 17-kDa protein, respectively. Virology 219, 57–65.
de Zoeten, G.A., Skaf, J.S., 2001. Pea enation mosaic and the vagaries of a plant virus. Advances in Virus Research, vol. 57. Academic Press, New York, pp. 323–350.
Demler, S.A., deZoeten, G.A., 1991. The nucleotide sequence and luteovirus-like nature of RNA 1 of an aphid non-transmissible strain of pea enation mosaic virus. J. Gen. Virol. 72, 1819–1834.
Demler, S.A., de Zoeten, G.A., Adam, G., Harris, K.F., 1996. Pea enation mosaic enamovirus: properties and aphid transmission. In: Harrison, B.D., Murant, A.F. (Eds.), The Plant Viruses, Volume 5: Polyhedral Virions and Bipartite RNA Genomes. Plenum Press, New York, pp. 303–344.
Demler, S.A., Rucker-Feeney, D.G., Skaf, J.S., deZoeten, G.A., 1997. Expression and suppression of circulative aphid transmission in pea enation mosaic virus. J. Gen. Virol. 78, 511–523.
Fereres, A., Moreno, A., 2009. Behavioural aspects influencing plant virus transmission by homopteran insects. Virus Res. 141, 158–168.
Fuchs, M., Gonsalves, D., 2007. Safety of virus-resistant transgenic plants two decades after their introduction: lessons from realistic field risk assessment studies. Annu. Rev. Phytopathol. 45, 173–202.
Fukatsu, T., Tsuchida, T., Nikoh, N., Koga, R., 2001. Spiroplasma symbiont of the pea aphid, *Acyrthosiphon pisum* (Insecta: Homoptera). Appl. Environ. Microbiol. 67, 1284–1291.
Ghosh, A.K., Ribolla, P.E., Jacobs-Lorena, M., 2001. Targeting *Plasmodium* ligands on mosquito salivary glands and midgut with a phage display peptide library. Proc. Natl. Acad. Sci. U. S. A. 98, 13278–13281.
Gildow, F.E., 1993. Evidence for receptor-mediated endocytosis regulating luteovirus acquisition by aphids. Phytopathology 83, 270–277.
Gildow, F.E., 1999. Luteovirus transmission mechanisms regulating vector specificity. In: Smith, H.G., Barker, H. (Eds.), The Luteoviridae. CABI, Wallingford, pp. 88–111.
Gildow, F.E., Rochow, W.F., 1980. Transmission interference between two isolates of barley yellow dwarf virus in *Macrosiphum avenae*. Phytopathology 70, 122–126.
Gildow, F.E., Damsteegt, V.D., Stone, A.L., Smith, O.P., Gray, S.M., 2000. Virus–vector cell interactions regulating transmission specificity of soybean dwarf luteoviruses. Phytopathology 148, 333–342.
Goldbach, R., Bucher, E., Prins, M., 2003. Resistance mechanisms to plant viruses: an overview. Virus Res. 92, 207–212.
Gray, S., Gildow, F.E., 2003. Luteovirus–aphid interactions. Annu. Rev. Phytopathol. 41, 539–566.
Jacobs-Lorena, M., 2003. Interrupting malaria transmission by genetic manipulation of anopheline mosquitoes. J. Vector Borne Dis. 40, 73–77.
James, A.A., 2003. Blocking malaria parasite invasion of mosquito salivary glands. J. Exp. Biol. 206, 3817–3821.
Lee, L., Kaplan, I.B., Ripoll, D.R., Liang, D., Palukaitis, P., Gray, S.M., 2005. A surface loop of the potato leafroll virus coat protein is involved in virion assembly, systemic movement, and aphid transmission. J. Virol. 79, 1207–1214.
Leiser, R.M., Ziegler, G.V., Reutenauer, A., Herrbach, E., Lemaire, O., Guilley, H., Richards, K., Jonard, G., 1992. Agroinfection as an alternative to insects for infecting plants with beet western yellows luteovirus. Proc. Natl. Acad. Sci. U. S. A. 89, 9136–9140.
Li, C., Cox-Foster, D., Gray, S.M., Gildow, F., 2001. Vector specificity of barley yellow dwarf virus (BYDV) transmission: identification of potential cellular receptors binding BYDV-MAV in the aphid, *Sitobion avenae*. Virology 286, 125–133.
Liu, S., Bonning, B.C., Allen Miller, W., 2006. A simple wax-embedding method for isolation of aphid hemolymph for detection of luteoviruses in the hemocoel. J. Virol. Methods 132, 174–180.
Liu, S., Sivakumar, S., Wang, Z., Bonning, B.C., Miller, W.A., 2009. The readthrough domain of pea enation mosaic virus coat protein is not essential for virus stability in the hemolymph of the pea aphid. Arch. Virol. 154, 469–479.
Mayo, M.A., Ziegler-Graff, V., 1996. Molecular biology of luteoviruses. Adv. Virus Res. 46, 413–460.
Miller, L.K., Ball, L.A., 1998. The Insect Viruses. Plenum Press, New York.
Miller, W.A., Liu, S., Beckett, R., 2002. Barley yellow dwarf virus: Luteoviridae or Tombusviridae? Mol. Plant Path. 3, 177–183.
Mohan, B.R., Dinesh-Kumar, S.P., Miller, W.A., 1995. Genes and *cis*-acting sequences involved in replication of barley yellow dwarf virus-PAV RNA. Virology 212, 186–195.
Nault, L.R., Ammar, E.D., 1989. Leafhopper and planthopper transmission of plant viruses. Annu. Rev. Entomol. 34, 503–529.
Ng, J.C., Falk, B.W., 2006. Virus-vector interactions mediating nonpersistent and semipersistent transmission of plant viruses. Annu. Rev. Phytopathol. 44, 183–212.
Prins, M., Laimer, M., Noris, E., Schubert, J., Wassenegger, M., Tepfer, M., 2008. Strategies for antiviral resistance in transgenic plants. Mol. Plant Pathol. 9, 73–83.
Prüfer, D., Wipf-Scheibel, C., Richards, K., Guilley, H., Lecoq, H., Jonard, G., 1995. Synthesis of a full-length infectious cDNA clone of cucurbit aphid-borne yellows virus and its use in gene exchange experiments with structural proteins from other luteoviruses. Virology 214, 150–158.
Reinbold, C., Gildow, F.E., Herrbach, E., Ziegler-Graff, V., Goncalves, M.C., van den Heuvel, J.F., Brault, V., 2001. Studies on the role of the minor capsid protein in transport of beet western yellows virus through *Myzus persicae*. J. Gen. Virol. 82, 1995–2007.
Reineke, U., Schneider-Mergener, J., 1998. Protein mimicry: A new dimension for peptides as lead compounds? Angew. Chem. Int. Ed. 37, 769–771.
Reutenauer, A., Ziegler-Graff, V., Lot, H., Scheidecker, D., Guilley, H., Richards, K., Jonard, G., 1993. Identification of beet western yellows luteovirus genes implicated in viral replication and particle morphogenesis. Virology 195, 692–699.
Rochow, W.F., 1970. Barley yellow dwarf virus: phenotypic mixing and vector specificity. Science 167, 875–878.
Rouze-Jouan, J., Terradot, L., Pasquer, F., Tanguy, S., Giblot Ducray-Bourdin, D., 2001. The passage of potato leafroll virus through *Myzus persicae* gut membrane regulates transmission efficiency. J. Gen. Virol. 82, 17–23.
Sambrook, J., Russell, D.W., 2001. Appendix 8: commonly used techniques in molecular cloning. Molecular Cloning: A Laboratory Manual, Vol. 3. Cold Spring Harbor Laboratory Press, New York, pp. A8.1–A8.51.
Seddas, P., Boissinot, S., Strub, J.M., Van Dorsselaer, A., Van Regenmortel, M.H., Pattus, F., 2004. Rack-1, GAPDH3, and actin: proteins of *Myzus persicae* potentially involved in the transcytosis of beet western yellows virus particles in the aphid. Virology 325, 399–412.
Smith, G.P., Scott, J.K., 1993. Libraries of peptides and proteins displayed on filamentous phage. Methods Enzymol. 217, 228–257.
Targett, G.A., Greenwood, B.M., 2008. Malaria vaccines and their potential role in the elimination of malaria. Malar. J. 7, S10.
Tarn, T.R., Tai, G.C.C., Jong, H.D., Murphy, A.M., Seabrock, J.E.A., 1992. Breeding potatoes for long-day temperate climates. Plant Breeding Rev. 9, 217–332.
Tatchell, G.M., 1989. An estimate of the potential economic losses to some crops due to aphids in Britain. Crop Protection 8, 25–29.
Terradot, L., Souchet, M., Tran, V., Ducray-Bourdin, D.G., 2001. Analysis of a three-dimensional structure of potato leafroll virus coat protein obtained by homology modeling. Virology 286, 72–82.
The International Aphid Genomics Consortium, 2010. Genome Sequence of the Pea Aphid *Acyrthosiphon pisum*. PLoS Biol. 8, e1000313. doi:10.1371/journal.pbio.1000313.
Torrance, L., 1992. Analysis of epitopes on potato leafroll virus capsid protein. Virology 191, 485–489.
Tsonis, P.A., Dwivedi, B., 2008. Molecular mimicry: structural camouflage of proteins and nucleic acids. Biochim. Biophys. Acta 1783, 177–187.
van den Heuvel, J.F., Verbeek, M., van der Wilk, F., 1994. Endosymbiotic bacteria associated with circulative transmission of potato leafroll virus by *Myzus persicae*. J. Gen. Virol. 75, 2559–2565.
Veidt, I., Bouzoubaa, S.E., Leiser, R.-M., Ziegler-Graff, V., Guilley, H., Richards, K., Jonard, G., 1992. Synthesis of full-length transcripts of beet western yellows virus RNA: messenger properties and biological activity in protoplasts. Virology 186, 192–200.
Whitfield, A.E., Kumar, N.K., Rotenberg, D., Ullman, D.E., Wyman, E.A., Zietlow, C., Willis, D.K., German, T.L., 2008. A soluble form of the tomato spotted wilt virus (TSWV) glycoprotein G(N) (G(N)-S) inhibits transmission of TSWV by *Frankliniella occidentalis*. Phytopathology 98, 45–50.
Yang, X., Thannhauser, T.W., Burrows, M., Cox-Foster, D., Gildow, F.E., Gray, S.M., 2008. Coupling genetics and proteomics to identify aphid proteins associated with vector-specific transmission of polerovirus (Luteoviridae). J. Virol. 82, 291–299.