Transcriptomic analysis of intestinal genes following acquisition of pea enation mosaic virus by the pea aphid *Acyrthosiphon pisum*

Véronique Brault,[1] Sylvie Tanguy,[2] Catherine Reinbold,[1] Gaël Le Trionnaire,[2] Joel Arneodo,[1]† Stéphanie Jaubert-Possamai,[2] Grégory Guernec[3] and Denis Tagu[2]

[1]INRA, UMR 1131 SVQV, 28 rue de Herrlisheim, 68021 Colmar, France

[2]INRA, UMR 1099 BiO3P, Domaine de la Motte, 35653 Le Rheu, France

[3]INRA, SCRIBE Campus de Beaulieu, Bâtiment 16A, 35042 Rennes Cedex, France

Correspondence
Véronique Brault
veronique.brault@colmar.inra.fr

Received 22 April 2009
Accepted 12 November 2009

Viruses in the family *Luteoviridae* are strictly transmitted by aphids in a non-propagative, circulative and persistent mode. Virions ingested by aphids successively cross the gut and the accessory salivary gland epithelia before being released, together with saliva, into the plant vasculature. Virion transport through aphid cells occurs by a transcytosis mechanism. This study conducted a transcriptomic analysis of intestinal genes of the pea aphid *Acyrthosiphon pisum* following uptake of pea enation mosaic virus. Among the 7166 transcripts analysed, 128 were significantly regulated (105 genes downregulated and 23 upregulated). Of these genes, 5 % were involved in intracellular trafficking, endocytosis and signal transduction, three important steps in the internalization and transport of virions. The limited levels of downregulation (maximum of 3.45-fold) and upregulation (maximum of 1.37-fold) suggest that the virus hijacks a constitutive endocytosis–exocytosis mechanism without heavily perturbing cell metabolism. Although limited to about 20 % of the pea aphid genes, this work represents the first large-scale analysis of aphid gene regulation following virus acquisition. A better knowledge of this virus–vector interaction will be possible only when tools representing the complete genomic capacity of the aphid become available.

INTRODUCTION

In order to survive, plant viruses must escape the plant before it dies. Several means of plant-to-plant transport are used efficiently by plant viruses, and transmission by aphids is one of the most prevalent. Members of the family *Luteoviridae* are transmitted by aphids in a manner that is non-propagative (no virus replication in the insect), circulative (virus transport across epithelial cells) and persistent (long-term virus persistence in the vector). Virus particles acquired by aphids when feeding from phloem tissue of infected plants are transported into the aphid body and must successively cross two different membranous barriers, the intestinal and the accessory salivary gland epithelia (Brault *et al.*, 2007; Gildow, 1999), before being released into the plant with the saliva. Transport of virus particles across these different types of cells operates by an endocytosis–exocytosis mechanism, which has been analysed extensively by ultrastructural observation of viruliferous aphids using transmission electron microscopy (TEM) (Brault *et al.*, 2007; Gildow, 1999). These observations have led several authors to propose a model of luteovirus particle uptake based on the presence of specific virus receptors at the intestinal apical plasmalemma of intestinal cells, as well as at the basal lamina and basal plasmalemma of the accessory salivary gland cells. Aphid receptors have not yet been identified with confidence, although some aphid proteins have been shown to exhibit *in vitro* affinity for virions (Seddas *et al.*, 2004; Yang *et al.*, 2008).

One peculiar characteristic of the transport of luteoviruses in their vector is that it is not dependent on virus replication in insect cells. Transcytosis is a natural endocytosis mechanism that can be hijacked by viruses. As well as being used by plant viruses, this mechanism is also used by a few animal viruses for their transport through specific epithelia, such as human immunodeficiency virus transport across epithelial cells (Bobardt *et al.*,

†Present address: CONICET, IMYZA-INTA, CC 25, 1712 Castelar, Argentina.

A supplementary figure showing virus transport through the intestinal cells of *A. pisum* after acquisition from purified virus suspension and a supplementary table giving details of the 128 transcripts found to be regulated in this study are available with the online version of this paper.

2008; Bomsel, 1997) and hepatitis B virus through trophoblastic cells (Bhat & Anderson, 2007). Possible effects of virus particle transport on aphid metabolism have never been analysed on a large scale. In order to identify possible host intestinal genes whose expression is significantly regulated following acquisition of viruses in the family *Luteoviridae*, we compared the transcriptome of viruliferous and non-viruliferous aphids using a cDNA chip microarray.

This project was conducted on the pea aphid, *Acyrthosiphon pisum*, an efficient vector of pea enation mosaic virus (PEMV), a complex made of two components, PEMV-1 (genus *Enamovirus*, family *Luteoviridae*; Mayo & D'Arcy, 1999) and PEMV-2 (genus *Umbravirus*). PEMV-1 is not infectious when inoculated mechanically. Association of PEMV-1 with PEMV -2 confers to PEMV-1 the ability to be mechanically transmissible to plants and to systemically infect all cellular types in the host plant (Demler *et al.*, 1996). The PEMV-1 and -2 RNA genomes are encapsidated separately in icosahedral particles with a diameter of 22–30 nm (Demler *et al.*, 1996). PEMV-1 RNA encodes the viral structural proteins – the major coat protein and the minor capsid component, which is essential for aphid transmission and is referred to as the readthrough protein (Demler & de Zoeten, 1991). PEMV-2 is deprived of a coat protein gene and relies on PEMV-1 capsid proteins for its encapsidation and its aphid transmission. PEMV-2 encodes putative movement proteins responsible for systemic infection of the plant by the two combined viruses (Demler *et al.*, 1994; Ryabov *et al.*, 2001). The pea aphid was chosen as the vector, as several important genomic tools are now available for this insect species (Tagu *et al.*, 2008) and cDNA microarrays representing several thousand transcripts are available (Le Trionnaire *et al.*, 2009).

The basic question addressed in this study was to determine whether PEMV virions employ a constitutive mechanism to enter intestinal cells without greatly perturbing aphid gene expression, or whether PEMV intestinal transcytosis would be accompanied by significant changes in gene expression. To follow gene regulation during the different steps of virus internalization, transport and release into the haemocoel, we conducted a comparative transcriptomic analysis on viruliferous versus non-viruliferous aphids.

RESULTS

Identification of aphid transmission parameters of PEMV for transcriptomic analysis

Attempts to transmit PEMV efficiently from purified virus suspension using either of the aphid species *Myzus persicae* or *A. pisum* were unsuccessful (data not shown). When aphids were fed on a purified virus suspension for a 48 h acquisition access period (AAP) before sampling the haemolymph, both virus genomes, detected by RT-PCR, were present in only one sample out of eight analysed (each sample contained haemolymph collected from five aphids; see Supplementary Fig. S1, available in JGV Online). PEMV-1 and -2 alone were detected, respectively, in two and seven samples out of eight analysed. These data may explain the absence of virus transmissibility by aphids starting with purified virions as the virus source, as both viruses are required for systemic plant infection. Even though both viruses are encapsidated in particles made of coat protein expressed from PEMV-1, the virus particles of the two viruses are morphologically different (Demler *et al.*, 1993, 1996), which could potentially induce different gene regulation for their transcytosis at the intestinal level. Therefore, we used infected plants as the virus source for aphids destined for the transcriptomic analysis. In order to select the more appropriate virus source for acquisition by aphids, we followed virus accumulation and distribution in infected plants. The kinetics of PEMV accumulation in mechanically inoculated peas were analysed by ELISA on systemically infected leaves for 5 weeks. Nearly half of the inoculated plants proved to be infected 1 week after inoculation (six infected plants out of 11 inoculated), whilst all the plants were positive in ELISA 3 weeks after inoculation and remained at this steady level for a further 2 weeks. Virus distribution in infected plants was analysed 3 weeks post-inoculation. The antigen titre was measured in four individual plants at each leaf stage above the inoculated leaves. Virus accumulation varied, depending on the leaf level, from 0 to 90 ng virus (mg leaf extract)$^{-1}$. Virus accumulation, however, was found to be consistently high in younger leaves (data not shown), and the distal part of the plant was therefore used as the virus source in aphid transmission experiments.

Previous experiments have shown that PEMV can be transmitted after a brief aphid acquisition period (from 15 min to 1–2 h) and a short inoculation period (from 7 s to 2 min) (Demler *et al.*, 1996), which suggests that PEMV inoculation can also occur in non-phloem cells during test probes. However, in order to obtain efficient virus transmission using the minimum number of aphids per plant, longer acquisition and inoculation periods were assayed. By transferring one aphid per plant after a 2 day AAP, efficient transmission was obtained after a 4–6 day inoculation access period (IAP) when the clone *A. pisum* YR2 was used (Table 1). The YR2 clone was therefore selected for the transcriptomic analysis. Most of the cDNAs spotted on the array originated from this clone (Le Trionnaire *et al.*, 2009; Sabater-Muñoz *et al.*, 2006).

In order to follow virus uptake in intestinal cells and release in the haemolymph, the presence of genomic RNA of PEMV-1 and -2 was analysed by RT-PCR in these different aphid compartments. Aphids were first fed on the upper part of infected plants before being dissected to collect intestinal tracts, which contain parts of the foregut, anterior midgut (stomach), posterior midgut and hindgut. Virus uptake in aphid intestinal cells occurred 4 h after the

Table 1. Selection of aphid transmission parameters and the aphid clone for transcriptomic analysis

Detached apexes of infected peas were used as the virus source 3 weeks after mechanical inoculation. The AAP was 2 or 6 days, whereas the IAP ranged from 4 to 6 days. One to ten aphids were deposited on each test plant. Two different aphid clones of *A. pisum* (COL or YR2) were used in the transmission assay. Results are given as the number of test plants positive in ELISA 3 weeks after aphid inoculation divided by the number of aphid-inoculated plants. The percentage of infected plants is indicated in parentheses. NT, Not tested.

AAP	IAP	Clone	No. of aphids per plant:			
			1	2	5	10
2 days	4–6 days	COL	1/20 (5 %)	1/30 (3.3 %)	8/40 (20 %)	11/30 (37 %)
		YR2	5/5 (100 %)	21/25 (84 %)	25/25 (100 %)	21/21 (100 %)
6 days	4–6 days	COL	97/144 (67 %)	58/63 (92 %)	NT	NT

start of virus acquisition, and viral RNAs persisted in intestinal cells over the entire time course of the experiment (6 days; Table 2). Whereas virus exocytosis from intestinal cells into the haemolymph was detected in 50 % of aphids tested 4 h after the start of virus acquisition, both PEMV-1 and -2 were detected in 100 and 70 %, respectively, of the sampled haemolymphs 48 h after the start of virus acquisition. This time of acquisition (48 h), which encompasses intestinal virus endocytosis, intracellular transport and exocytosis, was therefore selected for the transcriptomic analysis.

Transcriptomic analysis of viruliferous aphids

To follow the effect of plant virus internalization on pea aphid physiology, we compared the transcriptomic profiles of dissected guts from *A. pisum* fed on PEMV-infected plants or healthy plants for 2 days. The microarray contained cDNAs taken from different cDNA libraries, including gut libraries. An *in silico* expression analysis of these libraries (Sabater-Muñoz *et al.*, 2006) previously identified only two expressed sequence tags (ESTs) with a significant enrichment in gut libraries. These two sequences corresponded to unknown genes. Thus, the array contained some gut-expressed genes, but almost no specific gut-expressed genes. Microarrays were performed in four replicates with a dye-swap design resulting in eight hybridizations. Each replicate corresponded to a different RNA extraction from 100 guts. From the 7116 spotted cDNAs, 6919 (97 %) passed the quality filters (image analysis, normalization and three validated spots required per gene sample). A threshold of $P<0.01$, without correction, was finally chosen and a group of 128 differentially expressed genes was extracted from this analysis (see Supplementary Table S1, available in JGV Online). A lower threshold, corresponding to $P<0.001$, gave only 12 significantly regulated genes (the top 12 in Supplementary Table S1). In this group of 128 genes, corrected P values varied from 0.2 to 0.6 and fold changes were between 0.29 and 1.37, where values of less than 1 represent downregulated genes, whereas values above 1 corresponded to overexpressed genes. Validation of the transcriptomic analysis was performed by carrying out quantitative RT-PCR on three genes from *A. pisum* selected from the most significantly regulated genes (the second, third and fourth genes in Supplementary Table S1; the first gene on the list was avoided because it originated from *M. persicae*). The very limited downregulation of the three genes in two biological samples was confirmed (data not shown). Among the identified genes, 56 % did not show any similarity with sequences from the databases, whilst 5 % showed similarities with hypothetical proteins originating from different organisms. Thus, a putative function could only be attributed to 39 % of the genes. The protein functions of the significantly regulated genes mostly fell within general cellular processes such as metabolism (the largest category of significantly regulated genes, with 16 % of the genes), translation, protein degradation, and stress and defence reactions (Fig. 1). Of particular interest was the category of genes representing 5 % of the significantly regulated genes and involved in intracellular trafficking, endocytosis and signal transduction, three important steps in the internalization and transport of virions. Only 23 genes, most with unknown function (Supplementary Table S1), were upregulated in the gut of viruliferous aphids (maximum change 1.37-fold), whereas 105 genes were

Table 2. Time course of virus intestinal uptake and release

Kinetics of virus accumulation in *A. pisum* intestinal cells and haemolymph. After an AAP ranging from 4 h to 6 days on infected plants, aphid digestive tracts and haemolymph were collected. Viral RNA was detected by RT-PCR on total RNA extracts. Similar experiments were conducted with control aphids.

Virus	AAP					
	4 h	6–12 h	20–24 h	48 h	6 days	Control *A. pisum*
Intestinal cells						
PEMV-1	11/11	9/14	10/13	11/11	3/3	0/3
PEMV-2	4/5	11/14	9/13	9/11	3/3	0/3
Haemolymph						
PEMV-1	8/16	10/14	10/13	7/7	2/3	0/3
PEMV-2	8/16	12/14	8/13	5/7	3/3	0/3

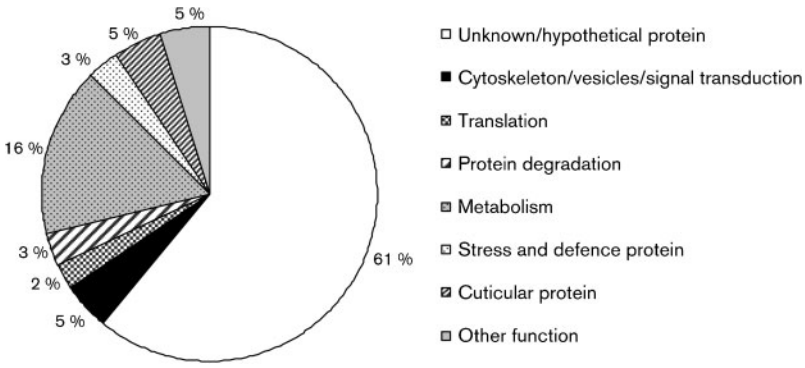

Fig. 1. Distribution of intestinal genes significantly regulated following PEMV acquisition according to functional class. The chart shows the distribution of ESTs from the transcriptomic analysis of viruliferous aphids. The functional class attributed to each EST is presented in Supplementary Table S1 (available in JGV Online).

downregulated (maximum change 0.29-fold). A statistical analysis searching for enrichment of Gene Ontology (GO) terms (corresponding to gene function) did not show any significant data ($P<0.05$), suggesting that no strong signature of specific genetic programmes was correlated with gut gene expression in viruliferous pea aphids.

Intestinal localization of PEMV in *A. pisum* by TEM

As no major modification in gene expression was observed in viruliferous aphids, we followed the fate of PEMV particles in *A. pisum* by TEM at the gut level. Observations were made after feeding aphids on infected plants for 3, 6 or 9 days and were focused on the anterior (stomach) and posterior midgut, as well as the hindgut. Except in the stomach lumen, in which a few isolated virions were seen in two out of nine aphids observed, after a 3 day AAP, no other virus-like particles were observed in the lumen or in cells of the posterior midgut (46 aphids examined) or hindgut (15 aphids examined). However, it should be noted that, although no virions could be observed inside intestinal cells, efficient transmission occurred when the aphids were transferred to test plants; indeed, after an AAP of 6 days on infected plants, 95.8 and 100 % transmission was obtained when one or two aphids, respectively, were transferred to the test plants. Intestinal cells of viruliferous aphids did not exhibit cytopathological modifications detected by TEM compared with corresponding cells in healthy aphids (data not shown).

DISCUSSION

Intestinal endocytosis and exocytosis of members of the family *Luteoviridae* are believed to rely on a sequential mechanism starting with clathrin-mediated endocytosis, followed by transport of virions enclosed in vesicles from the apical to the basal pole of the cell, and ending with fusion of virus-containing vesicles with the basal plasmalemma (Gildow, 1999). This virus transport is not accompanied by virus replication. Once inside the cell, the virions are always enclosed in vesicles and are believed not to interact with the aphid cytoplasm. Therefore, direct contact between virus capsid components and intestinal aphid proteins might be established only at the apical plasmalemma to mediate virus internalization in intestinal cells. This interaction could conceivably affect cellular gene expression, for example, by inducing production of components of a transport system that can interact specifically with the virus. Furthermore, as we cannot strictly eliminate the possibility that a few unprotected virions could be internalized into the cell and could then interact with cytoplasmic components, gene regulation could also be triggered by these putative interactions. Concerning this point, it should be mentioned that aphid proteins able to bind *in vitro* to some members of the family *Luteoviridae* have been identified (Seddas *et al.*, 2004; Yang *et al.*, 2008). However, the nature of the identified proteins is not obviously compatible with a function as virus receptors.

Among the 7166 genes assayed in the present study, only 128 (1.8 %) appeared to be differentially expressed in the presence of the virus in the epithelial cell, using a value of $P<0.01$. The only genes involved in intracellular vesicle transport or signal transduction (actin, RAS-related protein Rab-7, transmembrane transporter GA14898-PA, profilin, vacuolar protein sorting 16, S-phase kinase-associated protein) were downregulated, which suggests that the virus may avoid intracellular transport towards the lysosomal degradation pathway. Several considerations can help to explain the low number of regulated genes that we obtained. Firstly, recent estimates consider that there are about 34 000 genes present in the pea aphid genome (International Aphid Genomics Consortium, unpublished data) and therefore our array represents only about 20 % of them. Many other genes are likely to be regulated, but would not have been detected because they were absent from the array. A second possible reason for the low gene regulation observed may reside in a very limited perturbation of aphid physiology and metabolism by PEMV acquisition. This is in accordance with a previous report by Sylvester & Richardson (1966), who found that neither the longevity nor the reproductive capacity of PEMV-bearing aphids varied from that of virus-free control

aphids. In this model, PEMV virions are thus almost 'invisible' to the intestinal cell or are sufficiently adapted to the vector so as not to trigger a strong specific cellular response following virus uptake. A third possibility is that genes involved in the response to virus were not present on the array because it was based on libraries of non-viruliferous *A. pisum*. Finally, because aphids acquire plant components from infected plants together with virions, we cannot eliminate the possibility that at least part of the observed gene deregulation was induced by internalization of plant compounds produced specifically after infection.

It was puzzling to observe that efficient PEMV transmission did not correlate with high viral accumulation in intestinal cells as measured by TEM, suggesting that only a few virus particles are required for efficient transport of virions through intestinal and accessory salivary gland cells, which may also be considered a good adaptation of PEMV to its vector. The fate of PEMV particles in its vector, *A. pisum*, was studied several decades ago (Demler *et al.*, 1996; Harris & Bath, 1972; Shikata *et al.*, 1966) and the presence of virus-like particles was reported in intestinal cell cytoplasm but also in unexpected localizations such as the fat body and midgut muscles. The presence of virus-like particles in intestinal cell nuclei was also noticed, which led Harris & Bath (1972) to suggest putative virus replication in these cells. Multiplication of PEMV within its vector, however, has never been substantiated. Furthermore, virus replication would be expected to induce gene deregulation, which is also not supported by our data. The reason for the discrepancy between the previous electron microscopy experiments and our observations may be related to differences in the capacity of *A. pisum* clones to internalize and transmit PEMV virions.

A genomic-scale study of the sort reported here has been conducted on thrips (*Frankliniella occidentalis*) harbouring the propagative tomato spotted wilt virus (genus *Tospovirus*) (Medeiros *et al.*, 2004). This study analysed insect genes upregulated after virus uptake using subtractive cDNA libraries to probe DNA macroarrays. Whereas no detrimental effect of the virus on the life cycle of the insect and no cytopathological changes in infected cells have been reported, genes involved in pathogen recognition (lectins) and in the innate immune system (encoding antimicrobial peptides or Toll pathway components) were activated. However, tomato spotted wilt virus is a propagative virus, which multiplies in the vector and is therefore expected to cause more transcriptional changes in insect cells than a non-propagative virus such as PEMV.

The data presented here, although limited to approximately 20 % of the aphid genes, represent, to our knowledge, the first report of an extensive analysis of regulation of intestinal genes of a non-propagative plant virus in its invertebrate vector. A better understanding of this virus–vector interaction will be possible only when tools representing the complete genomic capacity of the aphid become available.

METHODS

***In vitro* transcription of viral RNA and mechanical inoculation of peas with viral transcripts.** Capped *in vitro* run-off transcripts were synthesized from *Pst*I- or *Sma*I-linearized full-length cDNA of PEMV-1 or -2, respectively (Demler *et al.*, 1997), using T7 RNA polymerase (Ribomax; Promega). Equal amounts of PEMV-1 and -2 transcripts were mixed and approximately 1 μg of each RNA was mechanically rubbed onto the leaf surface of 10-day-old pea seedlings (*Pisum sativum* cv. Cameor; INRA Dijon).

Aphid stock and aphid transmission experiments. Two *A. pisum* clones were used: *A. pisum* COL originating from Colmar (France) and *A. pisum* YR2 from York (UK) (Ramos *et al.*, 2003). Virus-free aphid colonies were reared on faba bean (*Vicia faba*) seedlings maintained in a controlled environment chamber at 20 °C with a 16 h photoperiod, which allowed clonal reproduction of the two genotypes. Third- or fourth-instar nymphs were allowed to acquire PEMV from the detached apexes of infected peas for an acquisition access period of 2–6 days. One to two individual aphids were deposited on each test plant (*V. faba*) for an IAP of 4–6 days. After insecticide treatment, test plants were assayed by ELISA.

Virus detection by ELISA. Infection after mechanical inoculation or aphid transmission was tested by a double-antibody sandwich ELISA (Clark & Adams, 1977) using a polyclonal antiserum (Loewe Biochemica GmbH) following the method described by Bruyère *et al.* (1997). Three to four leaf fragments from non-inoculated leaves were collected to analyse plant infection. To measure virus repartition in the plant, each pinnate leaf consisting of a pair of leaflets on each side of a common axis was sampled. Leaf extract was deposited in individual wells on the plate. Virus titre was determined by reference to a standard curve obtained using quantified amounts of purified PEMV.

Total RNA extraction and PEMV detection by RT-PCR. Haemolymph was collected by removing a mesothoracic leg with fine forceps and applying gentle pressure on the abdomen to force haemolymph droplets from the wound. The samples were collected using a fine glass capillary. Haemolymph from five specimens was pooled in each sample and total RNA was extracted using a Qiagen RNeasy Plant Mini kit (animal tissue protocol). RNA extraction was also performed on aphid digestive tracts dissected under binoculars. The digestive tracts were extracted by pulling either the aphid head (collection of part of the foregut and anterior and posterior midgut) or the cauda (collection of the hindgut and posterior midgut). For the detection of PEMV-1 or -2, five digestive tracts were combined in each sample and RNA extraction was performed using a Qiagen RNeasy Plant Mini kit (animal tissue protocol). For the transcriptomic analysis, total RNA was extracted from approximately 100 dissected guts, which were placed directly into the extraction buffer (SV Total RNA Isolation kit; Promega) and immediately subjected to extraction to reduce the risk of RNA degradation. The quality and concentrations of total RNA were checked on a Bioanalyser (Agilent). Four independent biological replicates were performed for microarray analysis and, for each four replicates, 100 guts were dissected for RNA extraction. Gut sampling was performed on stock aphids that had been deposited on the detached apexes of PEMV-infected faba bean or healthy faba bean for a 48 h period.

PEMV-1 and -2 were detected in the biological samples by RT-PCR. PEMV-1 cDNA synthesis was performed using the oligonucleotide 5′-TGAAGCTTCGCAGGCAGAGAACTC-3′ (*Hin*dIII extension) and, after 30 cycles, a 1535 bp DNA encompassing nt 3978–5512 of PEMV-1 (Demler & de Zoeten, 1991) was amplified using primer 5′-ACGGATCCCAAGACCCTCCAATAAGC-3′ (*Bam*HI extension). For detection of PEMV-2, cDNA synthesis was performed with the

oligonucleotide 5′-TGAGGAACAGGCTGAATGG-3′; in the PCR, this primer together with the oligonucleotide 5′-CTAGGACAATGG-CGGTAGG-3′ amplified a 547 bp fragment from nt 2755–3301 of PEMV-2 (Demler *et al.*, 1993).

Ultrastructural examination of virions at the gut level. For TEM observations, *A. pisum* YR2 was allowed to acquire virus on infected plants for a 3–9 day AAP. Aphids were then bisected, fixed and embedded in Epon/Araldite plastic as described previously (Reinbold *et al.*, 2001). All observations were made with a Philips EM 208 transmission electron microscope. Some aphids were transferred to healthy peas to confirm their ability to transmit the virus. These test plants were assayed for PEMV infection 3 weeks later by ELISA.

Microarray experiments. The cDNA microarray, described previously by Le Trionnaire *et al.* (2009), was constructed from 7166 cDNAs and 49 controls, spotted in duplicate to give a total of 14 430 spots. A total of 6650 cDNAs was selected after EST clustering from cDNA libraries of the antennae, digestive tract, head and salivary glands of the pea aphid (Sabater-Muñoz *et al.*, 2006). A small number of cDNAs (126) corresponded to sequences obtained after differential display or subtractive hybridization experiments (unpublished data), and 390 cDNAs were selected from a cDNA library of the green peach aphid *M. persicae*. The 49 controls consisted of 16 spots of fluorescent dyes (Cy3), three spots of buffers used for cDNA resuspension, three spots of poly(A), three spots of poly(T), three spots of polylinkers for the plasmid pDNR-lib (Clontech), three spots of polylinkers for the plasmid pTriplEX-2 (Clontech) and 18 *Arabidopsis thaliana* spike controls from the SpotReport-3 Array Validation System (Stratagene). The cDNA probes were printed on UltraGAPS II slides (Corning) using a Spotter Microgrid II (BioRobotics).

Total RNA extracted from aphid digestive tracts (1 μg) was amplified using a MessageAmp RNA kit (Ambion) prior to labelling. Fifteen micrograms of amplified RNA was labelled using the ChipShot Indirect Labelling and Clean-Up System (Promega), using CyDye (Cy3/Cy5) Reactive dye (Amersham).

Microarray hybridizations were performed with a Discovery XT System hybridization robot using a ChipMap 80 kit (Ventana) at the INRA-Scribe transcriptomic facilities (IFR GFAS, Rennes, France), as described by Le Trionnaire *et al.* (2009). Briefly, pre-hybridization was performed at 42 °C for 1 h in pre-hybridization buffer of 0.5 % BSA, 2 × SSC and 0.2 % SDS. Target labelled cDNAs were added before hybridization at 42 °C for 6 h (protocol no. 2, ALC-D60/10-H48/8; Ventana) in ChypHybe80 hybridization buffer (Ventana Medical Systems). Hybridized slides were washed twice manually with a RiboWash solution and once with 0.1 × SSC. Each wash was performed at room temperature for 2 min. Eight arrays were hybridized by combining two groups (viruliferous/non-viruliferous), a dye swap and four biological replicates.

All fluorescent images were generated using a GenePix 4000B scanner and analysed using GenePix Pro software (Axon Istruments). Raw data from two-colour microarray hybridizations were first exported from GenePix to R Software with annotation and spot types. After omitting 356 blanks from the analysis, spots with a negative flag value, a signal-to-noise ratio threshold $\leqslant 2$ and an irregular aspect were assigned a weight of 0 and did not take part in the normalization. Log_2 ratios from 7166 genes in duplicate were thus normalized using loess normalization from the Limma package (Smyth & Speed, 2003). In order to improve the assessment of group means, genes with fewer than three validated spots (out of eight) were also omitted. Finally, 6919 duplicated genes were kept to obtain the most reliable lists of differentially expressed genes by comparing the two groups of interest. Among these, 6776 corresponded to cDNA from the pea aphid. Statistical values for gene expression of sample pairs were calculated, gene by gene, after fitting the mixed model analysis of variance implemented in the Limma package (Milliken & Johnson, 1992; Smyth, 2004) with only one fixed factor of two levels: viruliferous or non-viruliferous. This model was convenient to distinguish technical from biological replicates by taking into account the correlation between dyes in the analysis. The increase in the type I error rate induced by the multiplicity of tests was controlled by the Benjamini–Hochberg correction. Finally, lists of differentially expressed genes for the two groups of interest could be obtained, with or without correction, by choosing the appropriate threshold.

The search for significant enrichment of GO terms in the regulated gene set was performed using the Babelomics platform (Al-Shahrour *et al.*, 2008). For each EST spotted on the array, the corresponding predicted genes were identified by mapping to the pea aphid genome at AphidBase (www.aphidbase.com). The corresponding *Drosophila melanogaster* homologues were retrieved for the PhylomeDB at AphidBase. The Flybase identifiers were loaded in Amigo to retrieve the corresponding GO terms. Functional enrichment analysis was performed by comparing the two lists of genes – the spotted cDNAs and the significantly regulated cDNAs – by means of Fisher's exact test at a significance level of $P<0.05$.

ACKNOWLEDGEMENTS

The authors are very grateful to W. A. Miller who provided us with the full-length infectious clones of PEMV-1 and -2. We are in debt to Danièle Giblot-Ducray who was involved at the starting point of the project. Aurélie Le Cam and Jérôme Montfort from the hybridization plate-form (INRA, UMR SCRIBE) are thanked for their help, as well as Amandine Etcheverry and Régis Bouvet at the spotting plate-form (Biogenouest, IFR GFAS, CNRS Rennes). Ken Richards and Etienne Herrbach are thanked for critical reading of the manuscript and Louis Wiss for aphid rearing. We also would like to thank the two anonymous referees and the editor Dr Gian Paolo Accotto for helpful comments on the paper.

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