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Rack-1, GAPDH3, and actin: proteins of *Myzus persicae* potentially involved in the transcytosis of beet western yellows virus particles in the aphid

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Abstract

Beet western yellows virus (BWYV) is a *Polerovirus* that relies on the aphid *Myzus persicae* for its transmission, in a persistent-circulative mode. To be transmitted, the virus must cross the midgut and the accessory salivary glands (ASG) epithelial barriers in a transcytosis mechanism where vector receptors interact with virions. In this paper, we report in vitro interaction experiments between BWYV and aphid components. Using the *M. persicae* clone from Colmar, we showed that a set of aphid polypeptides, separated by SDS-PAGE or 2D electrophoresis (2DE), can bind in vitro to purified wild type or mutant particles. Using subcellular fractionation, we showed that the 65-kDa polypeptide identified as symbionin is a soluble protein whereas the other polypeptides seem to be associated more or less strongly to the membrane. We hypothesize that three polypeptides, identified by mass spectrometry as Rack-1, GAPDH3, and actin, may be involved in the epithelial transcytosis of virus particles in the aphid vector.
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Keywords: Receptor; Aphid transmission; *Luteovirus*; BWYV; *Myzus persicae*; Membrane; Rack-1; GAPDH3; Symbionin; Actin

Introduction

Beet western yellows virus (BWYV) is a member of the genus *Polerovirus* (family *Luteoviridae*) (Mayo and D'Arcy, 1999). Particles of BWYV are icosahedral, 25 nm in diameter, containing a monopartite-positive RNA of 5.6 kb (Mayo and Ziegler-Graff, 1996).

BWYV relies on *Myzus persicae* (*Homoptera*, *Aphididae*), its main vector species, for its horizontal transmission. As for all luteoviruses, BWYV and the vector interact according to the persistent-circulative and nonpropagative mode. In this highly specific interaction, virions are transported across two cellular barriers, the gut and the accessory salivary glands (ASG) epithelia. At each barrier, the transcytosis (endocytosis/exocytosis) event is believed to be based on a receptor-mediated mechanism (Gildow, 1987, 1999; Gray and Gildow, 2003; Roth, 1993).

Studies on other members of the *Luteoviridae*, including the species *Barley yellow dwarf virus*-MAV and -PAV (BYDV-MAV and -PAV) and *Cereal yellow dwarf virus* (CYDV-RPV = formerly BYDV-RPV), indicate that these viruses are acquired into the aphid vector through the hindgut tissues (Gildow, 1987). Recently, Reinbold et al. (2003) have shown that the *Cucumber aphid borne yellows virus* (CABYV) is acquired by *M. persicae* through the hindgut whereas, in contrast, *Potato leafroll virus* (PLRV) and BWYV are acquired by *M. persicae* through the midgut (Garret et al., 1993; Reinbold et al., 2001). Particles of BWYV are first transported across midgut cells into the haemocoel and then across ASG cells for delivery to the plant via saliva (Gildow, 1999; Reinbold et al., 2001). The ability of luteoviruses to cross the gut wall is a trait generally unrelated to vector specificity (Gildow, 1999; Rochow et al.,

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1975) because particles of many luteovirus species can be transported across the gut wall of different aphid species, whether they are efficient vectors or non-vectors (Gildow, 1999). However, it has been reported that the gut wall may affect transmission efficiency (Rouzé-Jouan et al., 2001).

Once released into the haemocoel, virions are thought to bind to symbionin, a major protein produced and released by the major endosymbiotic bacteria of the genus *Buchnera* (Bauman et al., 1995; Filichkin et al., 1997). The role of symbionin in transmission if any would be to protect the virions from degradation in the hostile haemocoel environment.

In contrast to the gut barrier, the ASG barrier appears to be virus-specific and to account for most of the transmission specificity between vector and virus species (Rochow et al., 1975). Moreover, this barrier was shown to function at two levels, that is, the basal lamina and the plasmalemma, which are independently involved in the recognition and transmission of luteoviruses (Gildow, 1993; Gildow and Gray, 1993; Peiffer et al., 1997). Some luteoviruses can penetrate the basal lamina of certain nonefficient vector species (Peiffer et al., 1997). However, the underlying basal plasmalemma can be crossed by virus only in efficient vectors (Gildow and Gray, 1993; Gildow and Rochow, 1980). Thus, the plasmalemma permeability dictates vector specificity (Peiffer et al., 1997).

The viral determinants of the transmission specificity are localized on the capsid of BWYV, which is composed of the major coat protein (CP) of 22 kDa, encoded by ORF 3, the minor readthrough (RT) protein of 74 kDa, encoded by ORF 3 and the downstream ORF 5, which is expressed by a stop codon readthrough mechanism (Mayo and Ziegler-Graff, 1996). The RT protein plays a role in virus accumulation in planta and is important for virus–vector interactions. This protein is required for stability of virions in the haemocoel, due to binding to *Buchnera* GroEL (Van den Heuvel et al., 1997), or for their transport to the ASG (Brault et al., 1995, 2000; Bruyère et al., 1997; Chay et al., 1996). Bruyère et al. (1997) showed that the C-terminal part of the RT protein is not necessary for transmission of BWYV, since C-terminal deleted mutants or purified particles, in which the RT protein is truncated, are still transmissible by *M. persicae*. The exact molecular mechanism of virus recognition and the role of CP and the N-terminal part of the RT protein have yet to be determined. Reinbold et al. (2001) showed that the RT protein of BWYV does not seem to be required for the transport of virions across midgut cells, although the presence of RT protein greatly enhances the efficiency of this process. Surprisingly, ultrastructural examination of aphids revealed that ingested RT-minus virus-like particles (VLPs) prepared from PLRV were observed in ASG cells and in salivary ducts (Gildow et al., 2000), whereas in contrast, no particles of a mutant lacking the RT protein were detected in ASG cells, even after microinjection into the haemocoel (Reinbold et al., 2001).

The specific receptors that mediate endocytosis of BWYV in *M. persicae* remain to be determined. A common strategy to study vector components that interact with virus particles is based on the far-Western blot methodology. The far-Western blot approach has been used successfully to detect proteins that are potential virus receptors in the body of aphids and other vector taxa. Several proteins within *M. persicae* have been found to bind in vitro to PLRV (Van den Heuvel et al., 1994). Li et al. (2001) showed that two proteins, SaM35 and SaM50, in the vector aphid *Sitobion avenae*, bind specifically to purified BYDV-MAV particles. These proteins are considered as potential ASG-borne receptors for BYDV-MAV in *S. avenae*. In other virus/vector models, vector proteins interacting with virus particles have been determined. Zhou et al. (1999) showed the existence of a 32-kDa membrane protein, a potential receptor of *Rice ragged stunt virus* (*Oryzavirus*) in the leafhopper *Nilaparvata lugens*, via the 39-kDa viral spike protein. A 50- and 94-kDa protein of the thrips vector, *Frankliniella occidentalis*, have been shown to bind in vitro to particles of *Tomato spotted wilt virus* (*Tospovirus*) (Bandla et al., 1998; Kikkert et al., 1998; Medeiros et al., 2000). The 94-kDa protein may represent a receptor protein involved in virus circulation through the vector, but probably not in viral uptake in the midgut (Kikkert et al., 1998), whereas the 50-kDa protein has been localized in the midgut brush border of thrips (Bandla et al., 1998).

In this paper, we show that a set of *M. persicae* proteins bind in vitro to purified wild type (BWYV-WT) or BWYV-mutant particles in far-Western blot experiments. Using subcellular fractionation of *M. persicae*, we discriminated between soluble and membrane-associated aphid proteins that interact specifically with BWYV particles and identified five of these proteins using mass spectrometry. Among these proteins, we propose that Rack-1 may belong to a membrane complex (possibly including GAPDH3) and that it probably facilitates transcytosis of BWYV-WT (but not of BWYV-mutants) by interacting with actin in aphid epithelial cells. We hypothesize also that endocytosis of BWYV may occur via macropinocytosis by direct interaction between BWYV and actin.

Results

Nature of mutations in BWYV-6.4 and BWYV-5.123L mutants and their effects on aphid transmission

We used two BWYV-mutants. BWYV-6.4 mutant, which is deleted of the total RT domain, is capable of crossing the midgut epithelial barrier with a low frequency compared to BWYV-WT and is not aphid transmitted (Brault et al., 1995). BWYV-5.123L mutant, which possesses three-point mutations in the RT domain [E(59)→A, D(60)→A, and P(32)→L], is unable to cross the midgut epithelial barrier although it is aphid-transmitted after microinjection into the haemocoel of the aphid vector (Brault et al., 2000). Subsequently, in several electron microscopy experiments, we

have demonstrated that this mutant is actually capable of crossing the midgut barrier with the same frequency as for the BWYV-6.4 mutant and that this mutant is aphid-transmitted (unpublished results). Nevertheless, the efficiency of BWYV-5.123L transmission by aphid is lower compared to that of BWYV-WT. Using a concentration of 25 μg ml^{-1} or 50 μg ml^{-1} of purified BWYV-5.123L for membrane feeding of aphids, 52% or 84% of aphid transmission were obtained, respectively, whereas 100% of aphid transmission was obtained with BWYV-WT used at the same concentrations.

Whole cell lysate aphid proteins separated by SDS-PAGE bind differentially to purified BWYV-WT particles and to the two mutant particles, BWYV-6.4 and BWYV-5.123L

Because the two mutants (BWYV-6.4 and BWYV-5.123L) can cross the midgut epithelium but at very low frequency as compared to wild-type virus (BWYV-WT), we determined whether the same aphid proteins bind to BWYV-WT, BWYV-6.4, or BWYV-5.123L particles.

Far-Western blot experiments were performed using whole cell lysate (WCL) proteins of luteovirus-free *M. persicae* clone from Colmar (Mp-Col) aphids and purified particles of wild type (BWYV-WT) or of the two virus mutants. As shown in Fig. 1, BWYV-WT particles were found to bind to a set of WCL proteins of Mp-Col aphids having apparent molecular weights of 65, 52, 37, 35, 32, 31, 29, 26, 22, and 21 kDa, respectively. All these proteins, except those of 65, 52, and 35 kDa were detected using either BWYV-6.4 or BWYV-5.123L purified particles. A weaker signal is observed with the mutant particles although the same quantity of aphid proteins was deposited in each lane. This is likely only due to the lower affinity of the anti-BWYV mouse polyclonal antibody used for the mutants than for the wild-type virus. Most probably, some antibodies molecules constituting the polyclonal antibody could not bind to the specific epitopes against which they are directed because the virus mutations may belong to these epitopes. However, this difference in affinity is limited as judged by quantitative ELISA (Table 1) and should not affect interpretation of the results. No signal was obtained when omitting BWYV particles (lane −) or using purified particles of GFLV, a nonrelated nepovirus that is not aphid-transmissible.

Table 1
ELISA responses of anti-BWYV mouse polyclonal antibody

Amount of protein (μg)	BWYV-WT	BWYV-6.4	BWYV-5.123L
0	0.022 ± 0.003	0.022 ± 0.003	0.022 ± 0.003
0.01	0.124 ± 0.023	0.091 ± 0.015	0.094 ± 0.013
0.1	0.547 ± 0.032	0.428 ± 0.024	0.447 ± 0.014
1	3.210 ± 0.065	2.644 ± 0.054	2.655 ± 0.042

ELISA responses of anti-BWYV mouse polyclonal antibody using purified particles of BWYV-WT, BWYV-6.4, or BWYV-5.123L after 2 h of substrate's incubation. Each value is the mean of three triplicate independent experiments.

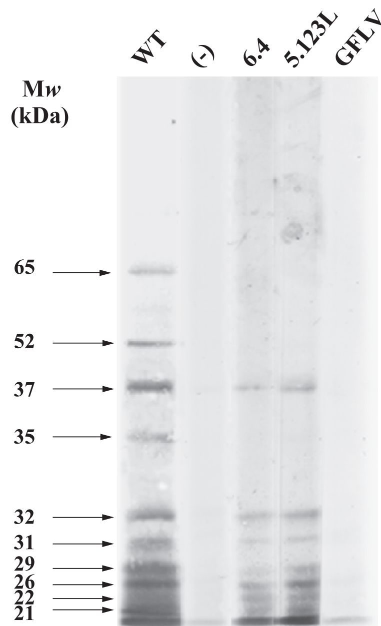

Fig. 1. Binding of purified BWYV-WT, -6.4, -5.123L mutants particles to whole cell lysate (WCL) of luteovirus-free Mp-Col aphid proteins separated by SDS-PAGE revealed by far-Western blot. BWYV-WT, BWYV-6.4, or BWYV-5.123L mutants was used. Controls were made by omitting virus (lane −) or using purified GFLV particles from the F13 isolate, a nonrelated nepovirus. Relative molecular weight markers (M_w) are indicated on the left. Eighty-microgram equivalent protein was deposited in each lane.

Binding of BWYV-WT and of mutants BWYV-6.4 and BWYV-5.123L to aphid proteins separated by 2D electrophoresis

Two-dimensional electrophoresis (2DE) analysis was carried out to evaluate the distribution of proteins contained in WCL of luteovirus-free Mp-Col aphids (Fig. 2). As judged from protein silver staining of the 2D gel, a luteovirus-free *M. persicae* whole cell extract gives rise to about 1,050 different protein spots (Fig. 2A). The molecular weight (M_w) of these proteins ranges between 388 and 3 kDa and the isoelectric point (pI) between 3.34 and 10 units, as determined by the Melanie 3.0 computer program.

To identify the proteins to which BWYV-WT purified particles bind, far-Western blot experiments were carried out. As shown in Fig. 2B, BWYV-WT particles were able to bind strongly to three aphid proteins: P37 with a pI of 7.47, P35 with a pI of 7.60, and P33 with a pI of 7.55 as determined by the Melanie 3.0 computer program. About seven other proteins of M_w ranging from 61 to 4 kDa and pI between 8.56 and 6.49, and one protein of 100 kDa and pI 5.6 were found to bind to BWYV-WT particles more weakly (Fig. 2B). Compared to WT virus, mutant

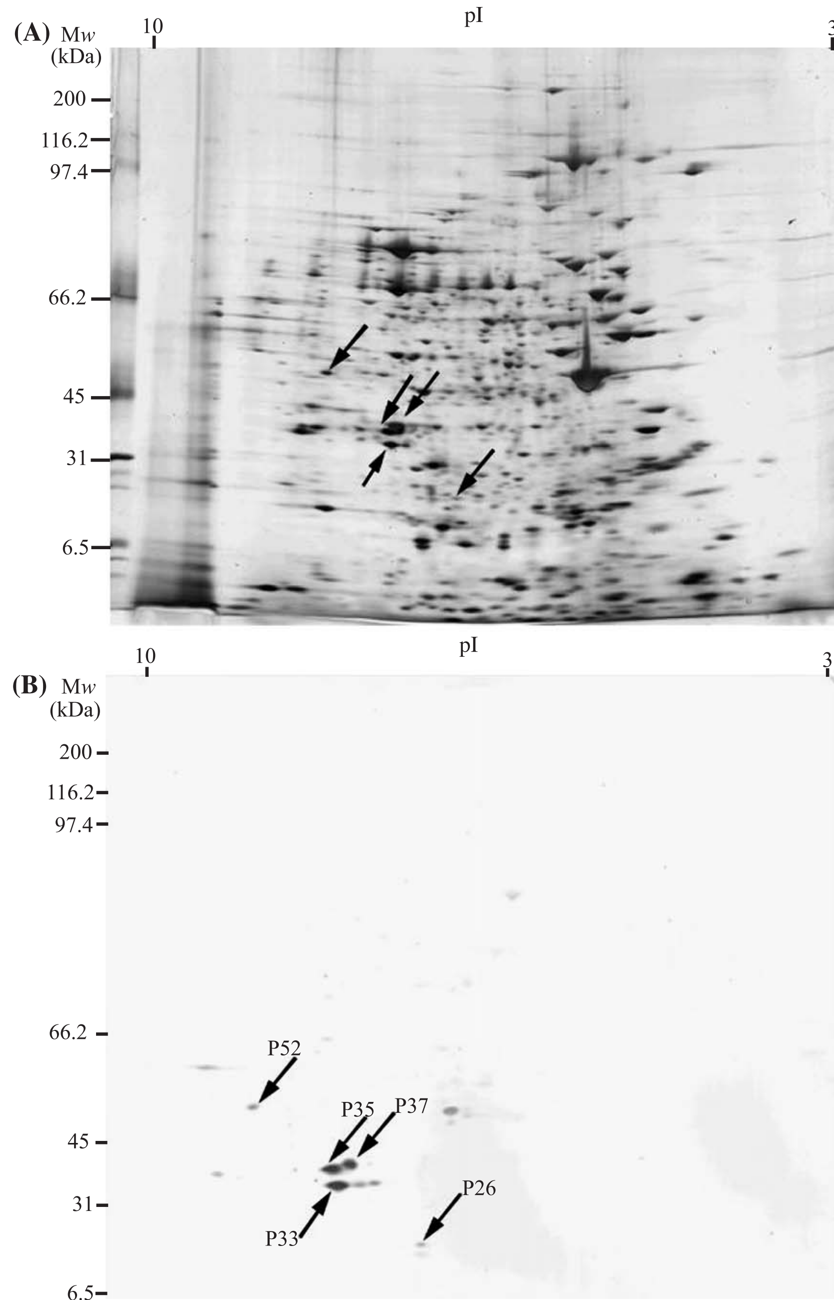

Fig. 2. Separation of whole cell lysate (WCL) proteins from luteovirus-free Mp-Col aphids by 2D electrophoresis and binding experiment with purified BWYV-WT particles. (A) Silver staining of aphid proteins. (B) Binding experiment. Relative molecular weight markers (M_w) are indicated on the left and pI ladder on the top. 2D spots indicated by an arrow have been selected for the LC-MS-MS analysis and for Edman degradation.

BWYV-5.123L and BWYV-6.4 particles were able to bind more weakly to P37 and mutant BWYV-5.123L was able to bind more weakly to P33 but mutant BWYV-6.4 was unable to bind to P33 (Fig. 3). Moreover, the two mutants were unable to bind to P35 and to P52 as shown in Fig. 3. Results for P52, P37, and P35 from these 2DE binding experiments agree with those obtained in 1D binding experiments. No binding was observed when BWYV particles were omitted (data not shown). The far-Western blot experiment after 2D electrophoresis separation of proteins could not be done with the membrane subfractions isolated from *M. persicae* because of an insufficient amount of aphid membrane proteins.

Determination of the nature of the aphid proteins that link BWYV

We performed a subcellular fractionation of *M. persicae* to obtain a low-density membrane fraction. At each step of the subcellular fractionation, we determined the percentage and the amount of proteins (Table 2). The results show that in *M. persicae*, 97.4% of the proteins contained in the WCL

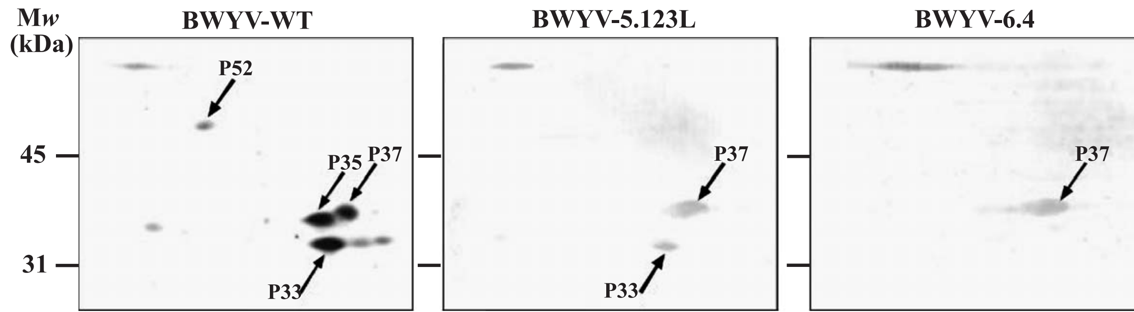

Fig. 3. Binding experiments between purified BWYV-WT, BWYV-5.123L, and BWYV-6.4 viruses and whole cell lysate (WCL) proteins from luteovirus-free Mp-Col aphids separated by 2D electrophoresis. Relative molecular weight markers (M_w) are indicated on the left.

are soluble proteins. Membrane proteins represent only 2.6% of the WCL proteins.

We isolated three microsomal subfractions by sucrose gradient centrifugation. The protein content (Table 2) and the SDS-PAGE electrophoretic profiles (Fig. 4A) of each subfraction were determined and the low-density membrane fraction was analyzed by 2DE (Fig. 5). The silver-stained electrophoretic profiles of the proteins (Fig. 4A) showed that the majority of the aphid proteins were soluble ones because the electrophoretic profiles of WCL proteins and of the soluble proteins (lane S) were nearly the same. We isolated the membrane fraction from aphid bodies containing all the membranes (lane M). These membranes were fractionated into three subfractions to obtain a fraction enriched in plasma membrane proteins (lane PM).

Using Mp-Col aphids, binding experiments were carried on SDS-PAGE-separated proteins from WCL and those contained in each step of the subcellular fractionation and purified particles of BWYV-WT (Fig. 4B). We used BWYV-WT and not BWYV-mutants because BWYV-WT detected more aphid polypeptides than the two BWYV-mutants.

Table 2
Subcellular fractionation of *M. persicae*

Step	Fraction[a]	Amount of proteins (mg)	Percentage of proteins (%)
1	whole cell lysate (WCL)	49.40	100
2	soluble proteins fraction S	48.10	97.40
3	total membrane fraction fraction M	1.30	2.60
4a	membrane subfractions	0.48	1.00
4b		0.40	0.80
4c		0.40	0.80

Amount and percentage of proteins were determined in each step of subcellular fractionation. After grinding of the aphid bodies in buffer H (step 1), proteins from WCL were separated by ultracentrifugation in fraction S containing the soluble proteins (step 2) and fraction M containing all the membranes (step 3). The different types of membranes were then separated in three subfractions by ultracentrifugation in a discontinuous sucrose step gradient [5% (w/v), 30% (w/v), and 40% (w/v)] (step 4). Subfraction 4a represents the plasma membrane proteins collected at the 5–30% sucrose interface. Subfraction 4b corresponds to microsomal enriched proteins collected at the 30–40% sucrose interface and subfraction 4c corresponds to proteins contained in the bottom of the sucrose gradient.
[a] For details, see Materials and methods.

The 65-kDa polypeptide that occurred in the WCL was only detected in the fraction S, but not in the fractions containing membrane proteins (lanes M, BSG, PM, and Mi). The 52-kDa polypeptide was detected in the WCL, in the fraction S, in the fraction M, and in the plasma membrane fraction (lane PM). The polypeptides of 42, 37, 35, 32, 31, 29, 26, 22, and 21 kDa were clearly detected in the plasma membrane fraction (lane PM). All these polypeptides, except the 42-kDa one, which is only detected in the plasma membrane fraction, were detected more weakly in the WCL and in the microsomal membrane fraction (lane Mi). Note that the 26, 22 and 21 kDa polypeptides are not detected as well in WCL in Fig. 4B (lane WCL) whereas they are clearly visible in Fig. 1 (lane WT). This is probably due to the eight times lower quantity of proteins deposited in Fig. 4B compared to Fig. 1. Moreover, the polypeptides of 37 and 35 kDa were also detected in membrane proteins from the gradient step centrifugation pellet fraction (lane BSG). The 35-kDa polypeptide was found in the fraction S and in the fraction M, whereas the 37-kDa polypeptide was only found in the fraction M. Whereas the same protein quantity was deposited on each lane, fraction PM showed the highest labeling indicating that a higher concentration of proteins of interest was present in this plasma membrane fraction.

The low-density membrane fraction was also analyzed by 2D electrophoresis (Fig. 5). After silver staining of the gel, a total of 298 different spots were visible. The proteins had relative molecular weights between 232 and 20 kDa and isoelectric points (pI) between 9.11 and 4.21 (Fig. 5).

Identification of aphid proteins

Selected protein spots from 1D or 2D gels (indicated by an arrow in Fig. 2) were subjected to in-gel trypsin digestion. Generated peptides were analyzed by LC-MS-MS on a Q-TOF 2 hybrid quadrupole/time-of-flight mass spectrometer. Identified proteins are listed in Table 3 and include symbionin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH3), the receptor for activated C kinase 1 (Rack-1),

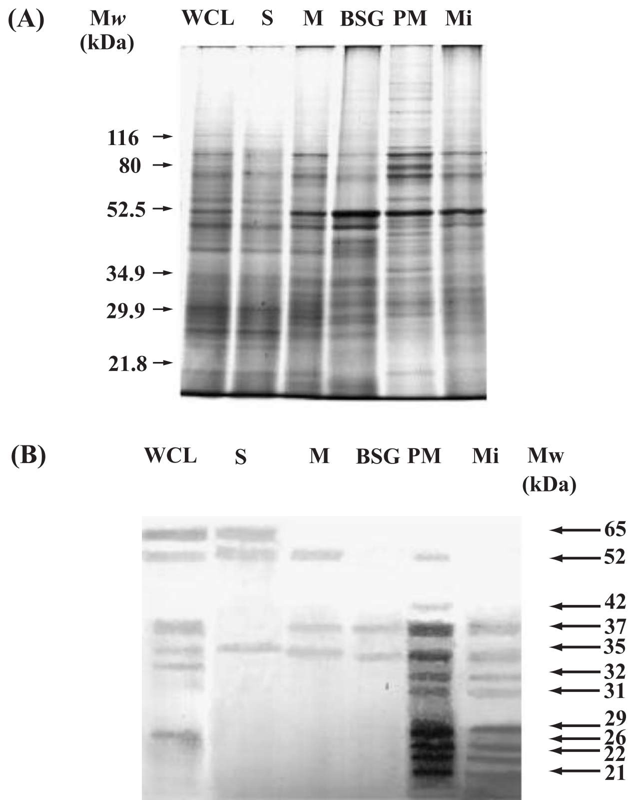

Fig. 4. Binding of purified BWYV-WT particles to proteins from subcellular fractions: (A) Luteovirus-free Mp-Col protein patterns from subcellular fractions after silver staining of SDS-PAGE. (B) Binding experiments with purified BWYV-WT particles by far-Western blot. Whole cell lysate, WCL; soluble proteins, fraction S; total membrane proteins, fraction M; bottom of the sucrose gradient, BSG; 5–30% sucrose interface (plasma membranes, PM); or 30–40% sucrose interface (microsomal membranes, Mi). Relative molecular weight markers (M_w) are indicated in each figure. Ten-microgram equivalent protein was deposited in each lane.

cuticular protein, and actins. The experimentally observed pI values were in good agreement with the calculated values reported in the NCBI database for Rack-1 but not for GAPDH3 and cuticular protein. Moreover, a discrepancy was also observed among relative molecular weights of symbionin, actin and GAPDH3, and the corresponding calculated molecular mass from NCBI sequences (Table 3). These discrepancies could be due in part to steric influences, partial fragmentation of proteins, or to conformational effect, but the LC-MS-MS data confirm the identifications.

The set of protein spots identified in WCL 2D gel (Fig. 2B) were also subjected to Edman degradation. Only one sequence from P26 could be determined. This sequence (P G N G L L E T [S,V] P N X V I [V,N]) does not match with any protein sequence in databases.

Discussion

Insect transmission of plant viruses involves complex interactions between viral proteins and vector-associated compounds (Gray and Banerjee, 1999; Gray and Gildow, 2003). Transmission by aphids is one of the most conserved features of viruses of the family *Luteoviridae*. Luteoviruses have a very high level of vector specificity and each luteovirus is efficiently transmitted by one or a few aphid species (Herrbach, 1999). Evidence suggests an intimate association between a luteovirus and its vector in which interactions occurring at cell membranes or basal lamina of specific aphid tissues regulate virus transmission (Gildow, 1999; Gray and Gildow, 2003). Although multiple domains of both the CP and the RT proteins of *Luteoviridae* appear to be involved in virus transmission (Brault et al., 2000), little

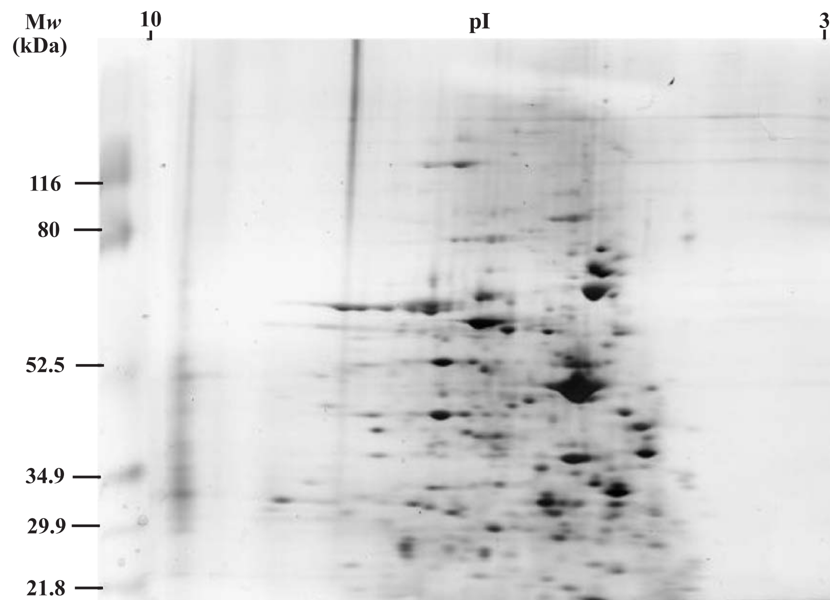

Fig. 5. Two-dimensional electrophoresis experiments. Silver staining of low-density membrane proteins from luteovirus-free Mp-Col aphids. Relative molecular weight markers (M_w) are indicated on the left and pI ladder on the top.

Table 3
Identification of aphid proteins using LC-MS-MS

Spots	Identified proteins	Score	Coverage (%)	NCBI code	NCBI calculated M_w (Da)	Experimentally derived values for M_w (Da)	NCBI calculated pI	Experimentally derived values for pI
P65	symbionin from *Buchnera aphidicola* (*Myzus persicae*)	183	70	gi|14030559	57,885	65,000	5.19	nd
P52	symbionin from *Buchnera aphidicola* (*Myzus persicae*)	1,200	52	gi|14030559	57,885	52,000	5.19	8.31
P42	actin from *Bombyx mori*	355	42	gi|113231	41,776	42,000	5.29	nd
P37	GAPDH3 (homologue of *Drosophila melanogaster*)	295	27	gi|84977	35,361	37,000	8.75	7.47
P35	RACK1 (homologue of *Drosophila melanogaster*)	177	26	gi|2290597	35,695	35,000	7.64	7.60
P33	cuticular protein (from *Myzus persicae*)	81	12	gi|2290597	23,445	33,000	6.07	7.55
P32	actin from *Artemia* sp.	369	58	gi|113255	41,811	32,000	5.30	nd
P31	actin from *Anser anser*	249	43	gi|627304	41,851	31,000	5.39	nd

Selected protein spots were subjected to in-gel trypsin digestion. Protein fragments were then separated by mono HPLC before being processed for MS and MS-MS using a Q-TOF 2 hybrid quadrupole/time-of-flight mass spectrometer. Data analyses were done with Global Server software and Mascot against NCBInr database. The score is significant if it is higher than 69. nd means nondetermined.

is known about the aphid contribution to transmission specificity.

SDS-PAGE and 2DE coupled to mass spectrometry allow the identification of proteins that interact with the BWYV

The use of virus overlay studies to investigate binding of virus to vector proteins is a straightforward method. In spite of the shortcoming, overlay studies have yielded important results in luteovirus research. Van den Heuvel et al. (1994) showed that purified PLRV particles had some affinity for five proteins of *M. persicae* of M_w approximately of 84, 78, 63, 50, and 49 kDa. The most abundant protein of 63 kDa with a pI 5.8–6.0 was identified as symbionin (MpSym), a prokaryotic protein coded for and synthesized in vivo by the major bacterial endosymbiont *Buchnera aphidicola* in *M. persicae* (Ishikawa, 1982).

It is clear however that these in vitro studies may have excluded certain interactions that occur in vivo which require particular conditions of pH and salt concentration (Kikkert et al., 1998; Roivanen et al., 1993). The environment within the gut or haemocoel of a vector is probably significantly different from that within a plant cell or present in vitro. Perhaps conditions within the haemolymph alter the conformation of the luteovirus particle to expose different regions of the RT and CP proteins on the surface of the virion and to allow interactions with the aphid ASG. Moreover, binding of the virus particles to the symbionin in the haemocoel may produce conformational changes in the virion structure. It is also not known whether the virus particles bind the ASG receptor(s) alone or still associated with the symbionin.

Nevertheless, using this in vitro approach, Li et al. (2001) showed that two proteins, SaM35 and SaM50, in the vector aphid *S. avenae*, bind specifically to purified BYDV-MAV particles but the nature of these proteins was not identified.

Using mass spectrometry, we have identified five aphid proteins including Rack-1, GAPDH3, and actin that bind in vitro to BWYV particles. In addition, the amino acid sequence, which was determined by Edmann sequencing, did not correspond to any sequence in the current databases. The MS-MS analysis allows unambiguous identification of proteins provided that relevant homologs are present in the current databases. Generated peptides could be de novo sequenced to find sequences suitable for Blast research. This feature is important for studies of novel biological samples such as aphids, and in particular *M. persicae*, whose genome has not yet been sequenced. The advantages of the MS methodology were clearly demonstrated in this study because it allowed the identification of unexpected proteins: GAPDH3, Rack-1, and actins. It is worth pointing out that combination of 1D or 2D electrophoresis was essential for this study. Due to the limited amount of membrane proteins available and the well-known problems associated with membrane proteins migration in 2D gels, 1D electrophoresis can provide suitable material for MS-MS analysis of membrane fractions.

Symbionin, cuticular protein, Rack-1, GAPDH3, and actins as identified proteins interacting with BWYV

We found in our experiments that BWYV-WT particles bind to P65 present in WCL and in the fraction containing the soluble proteins, whereas both BWYV-6.4 and BWYV-5.123L mutant particles did not bind to this polypeptide. Using mass spectrometry, we have identified this protein as symbionin from *B. aphidicola*. P52 that is present in the soluble and membrane fractions has also been identified as symbionin of *B. aphidicola*. This is nonrelevant for the entry of the BWYV into aphid but our result confirm those of Van den Heuvel et al. (1994) and validate our approach. P52 is certainly a fragment of P65; but, whereas P65 is only present

in the soluble fraction, P52 is present in both soluble and membrane fractions. This result would suggest that part of symbionin, having been synthesized by *B. aphidicola*, could be associated to membranes. Our results confirm those of Brault et al. (1995, 2000) and Bruyère et al. (1997) who have shown by ELISA that MpSym can bind in vitro to purified BWYV-WT particles but not to BWYV-6.4 particles. Our results obtained with BWYV-5.123L suggest that amino acids E(59), D(60), and P(32) of the BWYV RT protein could be involved in MpSym direct attachment or that they could participate to a 3D particular structure of the virus capsid that can bind symbionin. The association between aphids and bacteria of the genus *Buchnera* is a "mycetocyte symbiosis" (Douglas, 1998). MpSym probably plays a crucial role in virus transmission by preventing virus particles from being proteolytically degraded in the haemocoel of the aphid or by favoring the transport of the viral particles to the ASG of the aphid (Gray and Banerjee, 1999; Van den Heuvel et al., 1994), although it is not involved in vector specificity (Van den Heuvel et al., 1997).

The cuticular protein that we have identified does not probably play a role in the transmission mechanism of BWYV but can be implicated in noncirculative transmission of plant viruses (Palacios et al., 2002). The interaction between the cuticular protein of *M. persicae* and BWYV could be a less efficient remnant BWYV transmission mode. Our results suggest that the RT protein is the viral protein interacting with this cuticular protein because both the wild type and the BWYV-5.123L mutant particles bind to this aphid protein but not the RT-deleted mutant (BWYV-6.4).

P35, which has been found to bind only to BWYV-WT, has been determined as the Rack-1 homologue of *Drosophila melanogaster*. In animals, Rack-1 is a multifunctional, WD motif-containing protein (Neer et al., 1994) important in regulating several cell surface receptors and intracellular protein kinases (Choi et al., 2003). Rack-1 has been described as an interacting-protein with the insulin-like growth factor 1 receptor (Hermanto et al., 2002) and is known to bind to β subunits of receptors (Il5, Il3, GM-CSF, Geijsen et al., 1999) and to integrins, which can interact with viruses (Albinsson and Kidd, 1999), and which are components of the extracellular matrix basal lamella of invertebrates such as aphids (Pederson, 1991). Rack-1, a homolog of the β subunit of G proteins (Ron et al., 1995), binds to activated protein kinase C (PKC), acting as an intracellular receptor to anchor the activated PKC to the cytoskeleton and to target the enzyme to the membrane (McCahill et al., 2002; Ron and Mochly-Rosen, 1994, 1995; Ron et al., 1995, 1999). Rack-1 produces signals required for the organization of actin in the cytoskeleton (Buensuceso et al., 2001; Liliental and Chang, 1998). In addition to its role as a major signaling event in the cell, PKC can regulate virus endocytic trafficking by phosphorylation (Sieczkarski and Whittaker, 2002). Rack-1 is localized at internal leaflet of membranes and on cytoskeleton elements (Ron and Mochly-Rosen, 1995; Ron et al., 1999). Our results agree with the localization of Rack-1 either in the cytosol or associated with membranes. Rack-1 is a cargo protein that may belong to a membrane complex (Chang et al., 1998; Liliental and Chang, 1998; Wang et al., 2002). It is a scaffold protein that physically connects various signal transduction components into stable complexes (Wang et al., 2002) and directs "cross-pathway-control" by integrating communication from different signaling pathways (such as Ser/Thr kinases A and C and cAMP signaling cascades) through the orchestration of protein–protein interactions (Liedtke et al., 2002; McCahill et al., 2002). Rack-1 is well conserved in all plants, animals, and invertebrates (Kuo et al., 1995; Kwon et al., 2001, McCahill et al., 2002), suggesting that it is essential for cellular functions. The deduced amino acid sequences of the coding region of Rack-1 homologues from vertebrates such as porcine, rat, mouse, human, chicken, zebrafish, *Tilapia nilotica* share nearly 100% identity whereas sequences from other sources such as *Drosophila* (Vani et al., 1997), Chlamidomonas, Neurospora, *Lycopersicon esculentum*, yeast, tobacco, *Arabidopsis thaliana*, rice, and *Trypanosoma brucei* show 76–55% identity (Chou et al., 1999).

Due to its localization at the inner membrane leaflet, Rack1 is clearly not the BWYV extracellular receptor. But the clear interaction detected between the wild-type virus and Rack1 and the absence of interaction with the two mutants, which display altered efficiency in endocytosis/transcytosis (Reinbold et al., 2001), strongly suggest that Rack1 is a key element of the transcytosis mechanism that enhances the efficiency of the transcytosis of wild-type particles compared to mutants, and is in direct contact with the virus at some stage of the process. Our results also suggest that RT protein is the viral protein interacting with Rack1.

P37, which binds to wild type and to the two mutant BWYV particles, has been determined as the GAPDH3 homologue of *D. melanogaster*. In animals, GAPDH3, besides playing an important role in glycolysis in the cytosol, has diverse cellular functions depending on its localization in each subcellular compartment. When being phosphorylated by PKC (Tisdale, 2002), GAPDH3 binds to membranes and regulates endocytosis (probably by promoting bundles and formation of microtubule network, Sirover, 1997, 1999; Somers et al., 1990; Volker et al., 1995) and exocytosis (by catalyzing membrane fusion activity, Glaser and Gross, 1995). A role has been attributed to this enzyme in vesicular transport and apical organelles biogenesis (Daubenberger et al., 2003). Glaser et al. (2002) have suggested a coordinated mechanism through which membrane trafficking and cellular signaling can be integrated with glycolytic flux. GAPDH3 has a weak association with the cytoskeleton and the association may strengthen in epithelial cells (Cao et al., 1999). GAPDH3 has been found sometimes accessible at cell surface (Alvarez et al., 2003; Delgado et al., 2001; Fernandes et al., 1992; Gil-Navarro et al., 1997; Goudot-Crozel et al., 1989; Gozalbo et al., 1998; Pancholi and Fischetti, 1992). This enzyme binds different proteins such as fibronectin (Alvarez et al., 2003; Gozalbo et al., 1998), which is a

component of basal lamella of invertebrate cells (Pederson, 1991). Schmitz and Bereiter-Hahn (2002) supported the idea of a specialized function for the interaction of GAPDH3 and cytoskeletal elements such as actin filaments. In our experiments, BWYV-WT does not bind to GAPDH3 in the fraction containing soluble proteins. GAPDH3 can be present in very small quantity in its soluble form compared to its membrane form in *M. persicae*. A different conformation of GAPDH3 in the cytosol compared to the membrane-associated form cannot be excluded. We hypothesize that GAPDH3 could be part of the BWYV receptor in aphids if localized at the outside leaflet of the plasmalemma of midgut and ASG cells, or in the basal lamella of the ASG cells where it could be associated with fibronectin.

The majority of virus families utilize endocytosis as a means to penetrate into cells. Besides clathrin-mediated endocytosis, which is the primary route for internalization into cells, viruses can be internalized through macropinocytosis. Macropinocytosis requires actin-driven membrane ruffling with a strict requirement for actin (Sieczkarski and Whittaker, 2002). Actin microfilaments are well known to play a critical role in endocytosis at the apical of polarized epithelial cells by providing the energy required for converting a membrane invagination or a pit into an endocytic vesicle within the cytoplasm (Gottlieb et al., 1993). Endocytosis is regulated by PKC activity and is tightly controlled by the dynamic organization of actin cytoskeleton (Deckert et al., 1996). Although viral interactions with the cytoskeleton are well documented, essentially for viruses that replicate in the cell, few specific viral proteins have been shown to bind directly to cytoskeletal proteins; and where it has been shown, the functional significance of these interactions has not been always well understood. The colocalization of actin with the viral glycoproteins in *Pseudorabies Virus* has been correlated with the internalization process of the virions (Van de Walle et al., 2001). *Autographa californica* M nucleopolyhedrovirus capsid can induce polymerization of actin cables, which, in conjunction with a myosin-like motor, facilitates its transport in the cell (Lanier and Volkman, 1998). Our results demonstrate a direct interaction of BWYV capsid with actin but its biological significance remains to be elucidated.

Conclusion

Although the true receptor for BWYV has not yet been identified, our results suggest that the protein Rack-1 may be involved in the transcytosis mechanism of BWYV particles through direct interaction between Rack-1 and a specific motif on the viral capsid RT protein, enhancing the polarized transport of BWYV-WT particles in the epithelial cells. The low efficiency of crossing the midgut barrier for BWYV-6.4 and BWYV-5.123L is probably due to the absence of this specific RT domain, inhibiting the direct binding of these particles to Rack-1. The capture of the viral particles could be mediated by a receptor, perhaps including GAPDH3 that may belong to a membrane complex together with Rack-1 in interaction with actin to facilitate transcytosis of BWYV in the aphid body.

The direct binding between BWYV-WT and Rack-1/GAPDH3/actin in epithelial cells would also suggest that BWYV particles need to escape from the classical route of clathrin-mediated endocytosis (Gildow, 1987; Reinbold et al., 2001). BWYV particles could be taken up by Rack-1, GAPDH3, and actin via macropinocytosis and their polarized transport across the epithelial cells may use cytoskeleton element trails. This hypothesis is supported by the fact that, in midgut cells, clathrin-coated vesicles, containing BWYV particles, that emerge from the apical plasma membrane are rarely seen but numerous uncoated vesicles, containing BWYV particles, are often detected (unpublished results). No immuno-electron microscopy has been carried out yet for studying transport of BWYV in midgut cells (Gray and Gildow, 2003). Immuno-electron microscopy did not succeed in establishing that PLRV particles are always surrounded by a membrane in midgut epithelial cells (Garret et al., 1993). Moreover, whereas BWYV-6.4 particles can cross midgut epithelium, the virions are not detected in membrane vesicles from epithelial cells (Reinbold et al., 2001). Virions could only be detected in the lumen or between the basal plasmalemma and the basal lamella of midgut cells.

It is obvious that the proteins identified in this study represent only a fraction of the aphid components that may bind to BWYV particles during its life in the insect vector. Nevertheless, the current investigation identified a variety of novel as well as unexpected aphid components that bind BWYV. The results obtained open up a new avenue to directly characterize the interactions between BWYV particles and Rack-1, GAPDH3, and actins. This should ultimately lead to an understanding of the molecular mechanisms responsible for aphid transmission of BWYV and especially for the enhanced transmission efficiency of wild-type virus compared to mutant viruses.

It will be important to reveal the true nature of vector determinants which produce the transcytosis of BWYV particles because this will offer novel opportunities for disease control, including the genetic manipulation of the vector and transmission neutralization by means of recombinant proteins expressed in transgenic plants.

Materials and methods

Materials

All chemicals were obtained from Sigma Co Ltd, unless otherwise stated.

Production and purification of BWYV

Particles of a wild-type BWYV (BWYV-WT), isolate FL1 originating from lettuce, were purified from agro-infected

Nicotiana clevelandii or aphido-infected *Montia perfoliata* plants (Brault et al., 1995), according to the method developed by Van den Heuvel et al. (1991). Particles of the two BWYV-mutants (BWYV-6.4 and BWYV-5.123L) were isolated using the same method as for the BWYV-WT. The concentration of purified particles was determined by reading the optical density at 260 nm and using an extinction coefficient of 8.6. Purified particles were stored in citrate buffer (0.1 M sodium citrate, pH 6.0) at −80 °C until use.

Maintenance of insects

The luteovirus-free colony of *M. persicae* (clone Colmar, Mp-Col) was maintained on *Capsicum annuum*. Colony was reared in a growth chamber at 20 °C as previously described (Reinbold et al., 2001).

Production of BWYV-specific antibodies

Balb/c female mouse, 6 weeks old, was obtained from the Laboratoire d'immunotechnologies (Dr. P. Pothier, Faculté de Médecine, Université de Bourgogne, Dijon).

A 6-week-old female Balb/c mouse was intraperitoneally injected using 50 μg equivalent proteins of purified particles of BWYV-WT. Five injections were realized at 2 weeks interval. The first one used the complete Freund's adjuvant and the others used the incomplete Freund's adjuvant (Freund, 1956). After 1 month, a last injection without adjuvant was realized to boost the immunological system of the mouse as described by Seddas et al. (2000).

Monoclonal antibodies against BWYV particles were unsuccessfully obtained, and the specific polyclonal serum of the mouse was collected and used for further experiments.

Enzyme-linked immunosorbent assay

To characterize the mouse polyclonal antibody, we developed a TAS-enzyme-linked immunosorbent assay (ELISA) based on the DAS-ELISA currently used in the laboratory (Reinbold et al., 2001). After coating of the ELISA plate with Bio-Rad SA antibody against BWYV, different dilutions (ranging from 0.01 to 1 μg) of purified virions of BWYV-WT, BWYV-6.4, and BWYV-5.123L were deposited. The presence of BWYV was detected using the mouse polyclonal antibody diluted at 1:50,000 then alkaline phosphatase goat anti-mouse IgG diluted at 1:5,000 (Interchim) according to the manufacturer's instructions. After alkaline phosphatase substrate deposition, optical density was read at 405 nm after 1 and 2 h.

Virus transmission by aphids

All transmission experiments were done at 20 °C. Third or fourth-instar nymphs or adults were given a 24-h acquisition access period (AAP) on different concentrations of purified virus suspension through a stretched parafilm membrane. Purified virus particles were prepared in 20% (w/v) sucrose in the artificial diet MP148 (Harrewijn, 1983). After AAP, aphids were transferred onto luteovirus-free *M. perfoliata* seedlings to assess their capacity to transmit the virus. Eight aphids were placed on each test plant for a 3-day inoculation access period (IAP). The plants were then tested for BWYV infection 4 weeks later using DAS-ELISA and the reactives commercialized by Bio-Rad SA., as described previously (Reinbold et al., 2001).

Subcellular fractionation of M. persicae

About 1 g of *M. persicae* individuals was crushed in 4 ml buffer H [1 mM ethylene glycol-bis(β-aminoethyl ether)-tetraacetic acid (EGTA), 3 mM ethylene diamine tetraacetic acid (EDTA), 0.5 mM $MgCl_2$, 100 mM 2-[*N*-morpholino] ethanesulfonic acid (MES), NaOH, pH 6.4] at 4 °C (Drücker et al., 1993) containing 1 mM de Pefabloc. Exoskeletons were eliminated by centrifugation (3,000 × *g*, 10 min, 4 °C), then the cellular debris contained in the supernatant were discarded by a second centrifugation (40,000 × *g*, 30 min, 4 °C). The membranes were then pelleted by ultracentrifugation (130,000 × *g*, 70 min, 4 °C). After resuspension of the membranes in 600 μl buffer H, the different types of membranes were separated by ultracentrifugation (100,000 × *g*, 40 min, 4 °C) in a discontinuous sucrose step gradient [5% (w/v), 30% (w/v), and 40% (w/v)].

After ultracentrifugation, the membranes contained in the pellet and in the two interfaces, 5–30% and 30–40%, were collected and washed with buffer H to eliminate sucrose. The two subcellular membranes were then pelleted by ultracentrifugation (130,000 × *g*, 70 min, 4 °C). After resuspension of each membrane fraction in 100 μl buffer H, protein content was estimated. Membranes were re-concentrated before either being stored at −80 °C until use, or directly solubilized for 2DE or SDS-PAGE.

Protein content

The protein content of each fraction was determined using the dye-binding colorimetric method developed by Bradford (1976), using bovine serum albumin as a standard.

SDS-PAGE of proteins from M. persicae

One hundred-microgram proteins of *M. persicae* (WCL, soluble or membrane proteins) were processed for solubilization: after addition of an equivalent volume of Laemmli buffer, the proteins were solubilized during 10 min at 100 °C (Laemmli, 1970) before being processed for SDS-PAGE, as described by Dozolme et al. (1995).

SDS-PAGE was done according to the method of Laemmli (1970). The polyacrylamide gels were prepared as previously described (Dozolme et al., 1995), except that

the slab gels were 1 mm thick and consisted of a 10–15% (w/v) gradient acrylamide resolving gel and a 4% (w/v) acrylamide stacking gel with 10 wells for analytical purpose (subfractionation experiments) or one continuous well for far-Western blot experiments.

Polypeptides in the resolving gels were either directly fixed in 30% (v/v) ethanol, 5% (v/v) acetic acid for silver staining according to Blum et al. (1987), or washed three times in water before being stained with Dodeca silver (Bio-Rad) for mass spectrometry analysis, or used for far-Western blot experiments. Apparent molecular masses of aphid polypeptides were calculated based on the mobility of prestained protein standards. Marker proteins used were: rabbit muscle phosphorylase b, 116 kDa; bovine serum albumin, 80 kDa; ovalbumin, 52.5 kDa; bovine carbonic anhydrase, 34.9 kDa; soybean trypsin inhibitor, 29.9 kDa; and lysozyme, 21.8 kDa (Bio-Rad).

Two-dimensional PAGE

Two-dimensional (2D) gel electrophoresis was optimized according to Lutomski et al. (1996).

After 2D electrophoresis, the protein profile was directly fixed in 30% (v/v) ethanol, 5% (v/v) acetic acid for silver staining (Blum et al., 1987), or washed three times in water before being stained with Bio-Safe Coomassie (Bio-Rad), or used for far-Western blot experiments.

The silver-stained gels were analyzed using the Melanie computer program (Bio-Rad). Apparent molecular masses of aphid polypeptides were calculated based on the mobility of non-pre-stained protein standards. Marker proteins used were: rabbit myosin, 200 kDa; β-galactosidase, 116.2 kDa; rabbit muscle phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; bovine carbonic anhydrase, 31 kDa; bovine aprotinine, 6.5 kDa (Bio-Rad).

Far-Western blot experiments

After separation of *M. persicae* proteins by SDS-PAGE or 2D electrophoresis, the protein profile was transferred onto Immobilon-P (Millipore) membranes using a Millipore semidry transfer apparatus (80 V, 2.5 mA cm^{-2} for 45 min at room temperature) according to the manufacturer's instructions. Nonspecific protein binding sites were blocked with 2% (w/v) Tween 20 in Tris-buffered saline (TBS) (200 mM NaCl, 20 mM Tris–HCl, pH 7.6) for 15 min at room temperature. The membrane was then incubated for 16 h at 20 °C with 4 μg of purified BWYV particles diluted in TBST [TBS containing 0.05% (w/v) Tween 20]. Control experiments were conducted omitting BWYV particles or using 4 μg of purified GFLV particles. After five washings with TBST, the presence of BWYV particles bound to aphid proteins was detected using mouse polyclonal antibody directed against BWYV particles. These antibodies were used at 1:10,000 dilution in TBST and were incubated at room temperature during 1 h. To detect GFLV particles (GFLV-F13 isolate), monoclonal antibody was used at 1 μg ml^{-1} dilution in TBST during 1 h at room temperature according to Van Regenmortel (1984). After five washings with TBST and after 2 h of incubation with phosphatase alkaline-goat anti-mouse IgG polyclonal antibodies, the presence of the immune complexes was revealed using 5 mg α-naphtyl acid phosphate, 0.04% (w/v) fast blue RR salt (4-benzoylamino-2,5-dimethoxybenzenediazonium chloride hemi[zinc chloride] salt), and 10 mM $MgCl_2$ in 80 mM Tris–HCl, pH 8.6.

To select spots from 1D or 2D gels for identification by LC-MS-MS, parallel experiments were carried out including a step of staining of the electrophoretic profile of aphid proteins using amido black [0.1% (w/v) naphtol blue black in 7% (v/v) acetic acid and 45% (v/v) methanol] or 0.2% (w/v) Ponceau Red after transfer onto Immobilon-P (Millipore) or nitrocellulose membranes, respectively. The stained electrophoretic profiles of aphid proteins were analyzed using the Melanie computer program (Bio-Rad). After destaining and blocking of the nonspecific protein binding sites, far-Western blot experiments were carried out as previously described. Comparisons between the stained electrophoretic profiles of aphid proteins in gel and after transfer onto membrane, and results of far-Western blot experiments allow the unambiguous selection of spots for LC-MS-MS analysis from 1D or 2D gels.

In-gel trypsin digestion and nano HPLC

Selected protein spots from 1D or 2D gels were excised from the gel using a razor blade (1DE) or a pipette tip (2DE), then subjected to in-gel trypsin digestion (Promega V5111).

A CapLC (Micromass Ldt., Manchester, UK) system was used for sample injection and preconcentration. The sample preconcentration and desalting was done on a precolumn cartridge packed with a 5 μm, 100 Å C18 PepMap stationary phase (LC-Packings) with length of 1 mm and an ID of 300 μm at 30 μl min^{-1} during 3 min. The loading solvent for sample preconcentration and clean-up consisted of 0.1% (v/v) formic acid in water.

After clean-up, the preconcentration system was switched (Stream Select) and the precolumn placed on-line with the analytical column. Bound peptides were backflushed eluted from the precolumn onto the analytical column (15 cm × 75 μm), packed with 3 μm, 100 Å C18 PepMap stationary phase (LC-Packings).

Mobile phase A consisted of 0.1% (v/v) formic acid in water and mobile phase B of 0.1% (v/v) formic acid in acetonitrile.

The elution was performed at a flow rate of 200 nl min^{-1} using a 5–45% gradient (mobile phase B) over 35 min, followed by a 95% (solvent B) over 5 min. The re-equilibration of the column was done during 20 min by 100% of mobile phase A.

Mass spectrometry

The MS and MS-MS mass measurements were performed with a Q-TOF 2 hybrid quadrupole/time-of-flight mass spectrometer (Micromass Ltd., Manchester, UK) equipped with a Z-spray ion source and the liquid junction. The instrument consisted of an electrospray ionization source, a quadrupole mass filter operating as a variable bandpass device, a hexapole collision cell, and an orthogonal acceleration time-of-flight (TOF) mass analyzer. The TOF mass analyzer was used to acquire data both in MS and MS-MS modes.

Nano LC-MS-MS data were collected using data-dependent scanning, that was, automated MS to MS-MS switching. The data-dependent scanning used was one collision energy for each precursor, with the collision energy used based on the charge state and the *m*/*z* of the precursor ion. The spray system (liquid junction) was at 3.5 kV.

Data processing and data analysis

Data processing of LC-MS-MS data was done automatically with the ProteinLynx Process (Micromass) module. Data analysis was done with Global Server (MicroMass, Ldt., Manchester, UK) software and Mascot (Matrix Science Ldt., London, UK) against NCBInr database.

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