Phloem Protein Partners of *Cucurbit aphid borne yellows virus*: Possible Involvement of Phloem Proteins in Virus Transmission by Aphids

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Poleroviruses are phytoviruses strictly transmitted by phloem-feeding aphids in a circulative and nonpropagative mode. During ingestion, aphids sample virions in sieve tubes along with sap. Therefore, any sap protein bound to virions will be acquired by the insects and could potentially be involved in the transmission process. By developing *in vitro* virus-overlay assays on sap proteins collected from cucumber, we observed that approximately 20 proteins were able to bind to purified particles of *Cucurbit aphid borne yellows virus* (CABYV). Among them, eight proteins were identified by mass spectrometry. The role of two candidates belonging to the PP2-like family (predominant lectins found in cucurbit sap) in aphid transmission was further pursued by using purified orthologous PP2 proteins from *Arabidopsis*. Addition of these proteins to the virus suspension in the aphid artificial diet greatly increased virus transmission rate. This shift was correlated with an increase in the number of viral genomes in insect cells and with an increase of virion stability *in vitro*. Surprisingly, increase of the virus transmission rate was also monitored after addition of unrelated proteins in the aphid diet, suggesting that any soluble protein at sufficiently high concentration in the diet and acquired together with virions could stimulate virus transmission.

The plant vascular tissue is a crucial compartment that poleroviruses (members of family *Luteoviridae* and genus *Polerovirus*) exploit both to multiply and to invade distant parts of the plant. Virus replication takes place in the nucleus-containing phloem parenchyma and companion cells that surround the

enucleate sieve elements (SE). Long-distance movement of virions occurs via the sieve tubes formed by connecting SE (Taliany and Barker 1999). Virus particles have been observed in the branched plasmodesmata connecting companion cells and SE (Esau and Hoefert 1972; D'Arcy and De Zoeten 1979; Shepardson et al. 1980; Mutterer et al. 1999), suggesting that virions are the infectious entity moving over long distances. For their transmission from plant to plant, virus particles are taken up exclusively from SE by the phloem-feeding aphids during sap ingestion. Members of the *Luteoviridae* family (called hereafter luteovirids) are transmitted in a persistent and circulative mode by aphids, and successively cross the intestinal and accessory salivary gland cell epithelia by transcytosis, before being released into a healthy plant together with saliva during a subsequent feed (Gildow 1999; Brault et al. 2007). Luteovirids have icosahedral particles which enclose a single-stranded RNA genome of approximately 6 kb. The virions are composed of two structural proteins: multiple copies of the major coat protein (CP) and a few copies of a minor component (readthrough [RT] protein). The RT protein is a fusion protein containing the CP at its N terminus and a RT domain at its C terminus. Both proteins are required for aphid transmission and are also involved in virus movement in plant (Brault et al. 1995, 2003; Chay et al. 1996; Peter et al. 2008). The RT protein has also been implicated in limitation of the virions to the vascular tissue (Peter et al. 2009).

Research in recent years has identified a plethora of small molecules in phloem sap, including sugars, amino acids, and phytohormones, as well as macromolecules such as RNA and proteins (Lough and Lucas 2006; Kehr and Buhtz 2008; Turgeon and Wolf 2009). The non-cell autonomous nature of these macromolecules has been the subject of particular recent attention, because it has been demonstrated that some of them are able to move over long distances in sieve tubes (Golecki et al. 1999; Aoki et al. 2005), potentially acting as regulators in distant cells or as shuttles for any interacting molecules. Several hundred proteins have been identified in the sap of different plant species (Fisher et al. 1992; Nakamura et al. 1993; Walz et al. 2002, 2004; Barnes et al. 2004; Giavalisco et al. 2006; Lin et al. 2009). Because mature SE are deprived of functional translation machinery, the most likely synthesis sites of phloem sap proteins are either immature SE or adjacent companion

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cells. Specialized branched plasmodesmata connecting SE and companion cells display the ability to mediate trafficking of proteins of up to 67 kDa (Stadler et al. 2005), a size-exclusion limit that largely exceeds the size-exclusion limit of plasmodesmata (approximately 1 kDa) interconnecting mesophyll cells (Wolf et al. 1989). Thus, most proteins synthesized in the companion cells could diffuse through these widened plasmodesmata into the SE. Moreover, microinjection experiments have revealed that some phloem sap proteins can significantly increase the size-exclusion limit of mesophyll plasmodesmata (Balachandran et al. 1997; Ishiwatari et al. 1998). If this property can be extrapolated to plasmodesmata connecting phloem cells, phloem proteins contained in sap could further facilitate protein exchanges between SE and companion cells.

Roles have already been assigned to some phloem sap proteins. For example, an antioxidant defense mechanism has been observed in *Cucumis sativus* (cucumber) and *Cucurbita maxima* sieve tubes (Walz et al. 2002). There is evidence that two abundant phloem proteins (PP1 and PP2) associate following wounding to plug the SE (Read and Northcote 1983; Schulz 1986). Phloem proteins are also thought to play an important role in transporting RNA molecules to their destination sites (Xoconostle-Cazares et al. 1999, 2000; Yoo et al. 2004; Gomez et al. 2005). Finally, some proteins such as the protein encoded by the *FLOWERING LOCUS T* act as signals for developmental switches transmitted from the leaves to the meristems (Corbesier et al. 2007). The functions of most of the other phloem proteins are still unknown but there is speculation that many are implicated in long-distance signaling, development,

stress, and defense reactions (Ruiz-Medrano et al. 2001; van Bel 2003; Lough and Lucas 2006).

A few studies reported that phloem proteins can bind to pathogens, such as phytoviruses or viroids. The phloem protein 1-homolog from *Cucumis melo* was shown to bind to *Cucumber mosaic virus* (CMV) particles in vivo and to increase virus stability (Requena et al. 2006). Two predominant lectins in cucumber sap, belonging to the phloem-protein 2 family (PP2), were also shown to bind to viroids and virus RNA in vitro and in vivo (Gomez and Pallas 2001, 2004; Owens et al. 2001), a property that was proposed to facilitate long-distance movement of these entities. Because the SE is not only used by luteovirids to move over long distances but also contains the virus stock for phloem-feeding insects, we reasoned that phloem proteins might not only have an effect on virus movement in planta but also could play a role in virus transmission by aphids. Implication of plant proteins in the aphid transmission process is so far limited to the nonpersistently transmitted *Cauliflower mosaic virus* (CaMV). Recent work by Martinière and associates (2009) showed that specific interactions between host proteins involved in microtubule genesis, together with viral determinants, are implicated in the formation of specialized structures required for vector transmission (Drucker et al. 2002; Khelifa et al. 2007).

In the present study, we have shown that several proteins from cucumber SE can interact with *Cucurbit aphid borne yellows virus* (CABYV) (Lecoq et al. 1992). We have further observed that phloem lectins can stimulate virus transmission by aphid when mixed with virus in an artificial diet and have a protective activity of the virions against in vitro degradation.

Fig. 1. Virus-overlay assays on cucumber sap proteins separated by two-dimensional (1D) gel electrophoresis. **A** and **C**, Two independent experiments of virus-overlay assays in which 300 μ g of total sap proteins were separated by 2D gel electrophoresis, transferred onto nitrocellulose, and incubated with 20 μ g of purified *Cucurbit aphid borne yellows virus* (CABYV). Complexes between sieve element proteins and CABYV were revealed using CABYV-specific antibodies. **B** and **D**, Similar gels loaded with the same sap extracts and stained with Colloidal blue. Positions of the proteins sequenced by mass spectrometry are indicated on the stained gel; * indicates background reactions due to antibodies directed against CABYV; plain arrow indicates the more reproducible signal observed in four of five performed experiments whereas open arrows indicate less-reproducible signals. Size of the molecular markers is indicated in kilodaltons. N.I.: nonidentified by mass spectrometry.

However, this effect on virus transmission rate was not restricted to plant lectins and could be extended to various non-plant proteins, suggesting that any protein present in phloem sap in sufficiently high concentration could stimulate virus transmission by aphids.

RESULTS

Identification of phloem sap protein partners of CABYV.

Our knowledge concerning luteovirid transmission by aphids is, at present, limited to the identification of viral proteins essential for virus acquisition, retention, and transport through the aphid, and to the identification of several aphid proteins which could be involved in the transmission process (Brault et al. 1995, 2000; Gildow et al. 2000; Reinbold et al. 2001; Peter et al. 2008; Yang et al. 2008). This study was designed to identify cucumber sap proteins that can bind to purified polerovirus particles, with the idea that such proteins could be putatively involved in aphid transmission. Interaction of CABYV virions with phloem sap proteins was evaluated using a virus-overlay assay on crude sap protein extract collected by incision to the vasculature of cucumber plants. This plant species is particularly well adapted to sap collection, as highlighted by the abundant literature reporting identification of cucurbit sap proteins (Kehr 2006). Moreover, CABYV particles can be detected in phloem exudate collected from infected plants (data not shown), in agreement with previous observations of virus-like particles in SE of other plant species (Esau and Hoefert 1972; D’Arcy and De Zoeten 1979; Shepardson et al. 1980; Mutterer et al. 1999). Phloem sap proteins (300 µg) were separated by two-dimensional (2D) gel electrophoresis, prior to electroblotting and incubation with purified virions in the conditions described in Material and Methods. The blot was then incubated with CABYV-specific antiserum and the proteins exhibiting the ability to bind to purified CABYV were excised from a duplicate gel loaded in parallel with the same extract. No unique profile of spots corresponding to CABYV-interacting proteins was observed among the five independent experiments performed with phloem sap collected over a 4-month

period. However, in four of the five experiments, a very strong signal was observed that corresponded to one or several proteins migrating between 18 and 22 kDa (Fig. 1A, arrowhead indicates the most typical result). The absence of such a signal in the fifth experiment was likely due to the range of molecular weights (MW) covered by the separation in the gel (Fig. 1C). Note that the labeled spots marked with asterisks in Figure 1A and C are due to nonspecific reactions with CABYV-specific antibodies, because they were also observed when the virus overlay was omitted from the experimental protocol (Supplementary Fig. S1A). The virus-binding proteins migrating at approximately 18 to 22 kDa were extracted from a replicate 2D gel loaded in parallel and stained with Colloidal blue. The proteins corresponding to the 18- to 22-kDa strong signal exhibited a closely spaced tetrad-like arrangement that facilitated their localization and excision (Fig. 1B). After trypsin digestion, the resulting peptides were analyzed by mass spectrometry (matrix-assisted laser desorption/ionization mass spectrometry [MALDI-MS] or nano liquid chromatography tandem mass spectrometry [LC-MS/MS]) and referenced to databases (Table 1; Supplementary Table S1). Although not all the proteins of the tetrad could be identified with confidence, one spot could be assigned by nano LC-MS/MS to the 17-kDa phloem lectin from *C. sativus*. The sequencing also revealed the presence of an additional nonidentified protein. Therefore, identification of the 17-kDa protein is still hypothetical and it is indicated as nonidentified in Table 1. However, because the four proteins in the tetrad are in close proximity, the observed signal could be due to an interaction between CABYV virions and one or more unidentified proteins from the tetrad. Other signals (for instance, those corresponding to the 32- to 34-kDa or the 24- to 27-kDa proteins) (Fig. 1A and C, respectively) were less reproducibly observed (Table 1, two of five experiments performed). The three spots corresponding to 32- to 34-kDa proteins (one example, Fig. 1A, spot labeled by an open arrow) did not give any MS identification, probably because of a lack of sequence homology to proteins in the available databases, whereas, among the five signals that appeared between 24 and 27 kDa (Fig. 1C, open arrows), the cucumber phloem

Table 1. Identification of proteins from cucumber sap separated by electrophoresis on a two-dimensional gel that putatively interact in vitro with *Cucurbit aphid borne yellows virus* (CABYV) virions

Signals		Protein ^b	Organisms	Accession no.	Pep ^c	Cov (%) ^d	Theoretical		Experimental		MS ^e
Observed	Freq ^a						MW	pI	MW	pI	
18–22 kDa											
(tetrad)	4/5	No identification	...	...	...	...	...	...	18.48-20.70	7.50-7.70 ^f	...
	...		...	...	...	...	...	...	18.48-20.70	7.65-8.00	...
	...		...	...	...	...	...	...	19.68-22.70	7.50-7.70	...
	...		...	...	...	...	...	...	19.68-22.70	7.65-8.00	...
24–27 kDa	2/5	26-kDa phloem lectin ^g	<i>Cucumis sativus</i>	22023939/21952270 ^h	7–19	37–68	26.67/25.03	8.85/9.01	24.00	7.15-7.37	2
	...		...	...	11	44	...	...	27.10	6.10	...
	...	No identification	...	...	...	...	...	...	26.49-26.70	5.90-6.20	...
	...		...	...	...	...	...	...	26.49-26.70	5.40-5.60	...
	...	No signal	...	...	...	...	...	...	26.49-26.70	5.70-5.85	...
32–34 kDa	2/5	No identification	...	...	...	...	...	...	32.00	7.00	...
	...		...	...	...	...	...	...	34.00	6.00	...
	...		...	...	...	...	...	...	34.83	6.78	...

^a Frequency of observed signals.
^b Criteria to accept peptide matches is the protein score (−10 × log(*P*)), where *P* is the probability that the observed match is a random event. Only the proteins with scores greater than 78 are significant (*P* < 0.05) were considered as potential CABYV partner.
^c Peptide matches are based on matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) data.
^d Cover.
^e Number of identification of the protein by MS.
^f Identification by nano liquid chromatography tandem mass spectrometry (LC-MS/MS) was conducted on this protein spot. It reveals the presence of at least two proteins, one which could potentially be the 17-kDa phloem lectin from *C. sativus* and the other a nonidentified protein.
^g Identification by MALDI-MS.
^h Two sequences of the 26-kDa protein from *C. sativus* are referenced in databases. Only 5 of 213 amino acids are different between the two sequences and one of the sequence is lacking the first 12 amino acids.

lectin of 26 kDa was identified twice with a sequence coverage of 37 to 68% (Table 1). The signal corresponding to the 26-kDa phloem lectin appeared on two blots as a succession of three or four spots that gave similar protein identification (for example, Fig. 1C).

Identification of CABYV partners was further pursued using fractions of sap proteins obtained after size fractionation by exclusion chromatography (Fig. 2). Phloem sap proteins were collected by exudation as above, before being deposited on the exclusion chromatography column. The proteins collected in each fraction were first concentrated before being separated by one-dimensional (1D) gel electrophoresis (20 µg/lane). It is interesting to note that mixtures of proteins of different sizes were present in most of the fractions (Fig. 2B), suggesting the existence of protein complexes in sap exudates. Nine independent sap protein fractionation experiments were performed over a 5-month period and these protein extracts were then used in the virus-overlay assays. Similarly to the previous

experiments, in which the sap proteins were separated on a 2D gel electrophoresis prior to virus-overlay assay, the nine experiments performed with proteins separated on a 1D gel did not produce a unique CABYV-binding proteins profile. One example of reactions obtained after incubation of the phloem sap proteins with purified virions is shown in Figure 2A. Whereas the CABYV antiserum reacted nonspecifically with phloem sap proteins of approximately 35 kDa, 14 proteins ranging from 16 to approximately 100 kDa displayed a capacity to bind specifically to CABYV particles in the nine experiments performed (Table 2). The most reproducible interactions with the CABYV virions (signals observed in eight of nine experiments) were mainly observed in two fractions ranging from 12 to 14 with proteins exhibiting a MW between 22 and 29 kDa (Fig. 2A, plain arrows). The corresponding bands were extracted from a replicate gel and further processed for MS identification. Among the candidates, we found the lectin CsLec26 from *C. sativus* previously identified by 2D gel-over-

Fig. 2. Virus-overlay assay on cucumber sap proteins separated by one-dimensional (2D) gel electrophoresis. **A**, Example of virus-overlay assay in which 20 µg of proteins from column fractions 11 to 17 were loaded on a 12% gel for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose before being incubated with 10 µg of purified *Cucurbit aphid borne yellows virus* (CABYV). Complexes between phloem proteins and CABYV were revealed using CABYV-specific antibodies. **B**, Similar gel loaded with the same protein extract and stained with Colloidal blue. Positions of the proteins sequenced by mass spectrometry are indicated on the stained gel. The far-right lanes are from a separate gel in which proteins were separated on a 13.5% gel for SDS-PAGE; * indicates background reaction due to antibodies directed against CABYV; plain arrows represent the more reproducible signals observed in eight of nine experiments performed, whereas open arrows indicate less-reproducible signals. Molecular marker size is indicated in kilodaltons. HMT: hydroxymethyltransferase; CsLec17: 17-kDa phloem lectin from *Cucumis sativus*; CsLec26: 26-kDa phloem lectin from *C. sativus*.

Table 2. Identification of proteins from cucumber sap obtained after size fractionation, separated by electrophoresis on a one-dimensional gel that putatively interact in vitro with *Cucurbit aphid borne yellows virus* (CABYV) virions

Signals ^a		Protein identification ^b	Organisms	Accession no.	Pep ^c	Cov (%) ^d	Molecular weight		
Obs (kDa)	Freq						Theoretical	Experimental	MS ^e
16–19	3/9	17-kDa phloem lectin ^e	<i>Cucumis sativus</i>	21745315	10	60	17.60	16.00	1
		Csf-2 ^e	<i>C. sativus</i>	5762258	15	80	17.86	17.00	1
		Tumor-related protein ^{e,f}	<i>C. melo</i>	7208434	12	66	18.80	19.30	1
22–29	8/9	26-kDa phloem lectin ^e	<i>C. sativus</i>	22023939/21952270	11; 17	44; 50	26.67/25.03	22.00–26.60	2
		Cytosolic ascorbate peroxidase	<i>C. sativus</i>	1669585	19	71	27.55	23.70	1
		P0677H8.27 ^e	<i>Oryza sativa</i>	34908654	19	57	25.67	25.10–29.00	1
		No identification						24.70, 25.00, 27.00, 33.00	...
		No signal						23.11, 24.22	
53–57	3/9	Hypothetical protein ^{e,g}	<i>C. melo</i>	62149091	22	53	35.39	53.80–57.40	1
100	2/9	Lipoxigenase/13S-lipoxigenase ^{e,h}	<i>C. sativus/C. melo</i>	1017772/82547876 ^e	21; 33	17; 31	99.50/100.02	96.30–100.09	2

^a Observed signals and frequency of observed signals.

^b Criteria to accept peptide matches is the protein score ($-10 \times \log(P)$), where P is the probability that the observed match is a random event. Only the proteins with scores greater than 78 are significant ($P < 0.05$) were considered as potential CABYV partner.

^c Peptide matches are based on matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) data.

^d Cover.

^e Number of identification of the protein by MS.

^f Translationally controlled tumor-related protein.

^g Hypothetical protein similar to hydroxymethyltransferase of *Arabidopsis thaliana*.

^h Identification by nano liquid chromatography tandem mass spectrometry (LC-MS/MS).

lay assay (discussed above), an ascorbate peroxidase, and a protein with homology to a protein of unknown function from *Oryza sativa* (Fig. 2B). Additional proteins with MW between 22 and 29 kDa that interacted with CABYV could not be identified by MS, due to absence of sequence homology in the databases. Other reproducible signals (observed in three of nine experiments) occurred at a MW range of 16 to 19 kDa in fractions 16 or 17 and of 53 to 57 kDa in fractions 11 or 12 (Fig. 2B, open arrows). These spots corresponded to the lectin CsLec17, the Csf-2 protein, a translationally controlled tumor-related-protein, and a hypothetical protein from *C. melo* similar to a hydroxymethyltransferase from *Arabidopsis thaliana*. This last protein exhibited a higher MW than the homologous protein in *C. melo* (35 kDa). An additional less-reproducible band was observed at approximately 100 kDa in fraction 12 (in two of nine experiments) and could be assigned by MS to a lipoxygenase (Table 2).

Effect of phloem sap proteins on CABYV transmission.

To analyze the effect of phloem sap components on virus transmission by aphids, purified CABYV preparations were supplemented with phloem exudates collected from healthy cucumber. The mixture was placed between two stretched Parafilm membranes that aphids can puncture to feed and acquire the virus. Virus transmission parameters (virus concentration, acquisition access period [AAP], and number of aphids transferred per plant) were adjusted so as to obtain a basal transmission efficiency ranging from 0 to 13% when no additional substance was added (Table 3). Cucumber crude sap extract was collected from several plants, combined, and mixed with the virus preparation. In five independent experiments (Table 3, experiments 1 through 5), virus transmission rates of the purified CABYV alone varied from 0 to 12% (average 6%), whereas the transmission rates shifted to 28 to 93% (average 60%) after addition of sap content. When sap was collected from individual plants and mixed with purified virus, the stimulatory effect of phloem exudates varied considerably between plants (Table 3, experiments 6 through 9). These variations could be due to the position on the plant from which sap was collected because, depending on the rate of exudation flow, sap was collected at different sites along the cucumber stem in different plants. We have found that protein concentration in SE varies considerably depending on the leaf position from which sap was collected (Supplementary Fig. S2). However, no obvious difference in protein composition was observed (data not shown). Although we cannot exclude an additional effect of protein content, protein aggregation state, or presence of other sap compounds, variation in protein concentration

could explain the difference in the transmission rates among the different plants.

To further evaluate the role of sap protein composition on CABYV transmission, phloem sap proteins were fractionated by size-exclusion column chromatography and concentrated. Three separate fractionation experiments were conducted and 30 to 40 µg of proteins of each fraction were added to the purified virus before being delivered to aphids. Note that the protein concentration in the aphid diet in these experiments was much lower than that used in the previous transmission experiments, with whole-sap extracts in which approximately 150 to 200 µg of sap proteins were added in the diet.

Half of the protein fractions of experiment 1 (8 of 18 fractions tested) had a mild to strong stimulatory effect on virus transmission compared with the “NP” sample containing only purified virus (Fig. 3), whereas the remaining protein fractions did not alter the transmission rate. This effect may be attributed to the presence of specific sap proteins in certain fractions. This was particularly striking with proteins contained in fraction 17 of two independent experiments, which shifted the aphid transmission rate from 0 to 29% (ex-

Fig. 3. Aphid transmission of purified *Cucurbit aphid borne yellows virus* (CABYV) after addition of cucumber sap proteins fractionated by exclusion chromatography. After a 24-h acquisition access period on artificial medium containing CABYV-purified virus (50 µg/ml) and supplemented or not with cucumber sap proteins (30 to 40 µg) from concentrated fractions obtained after size exclusion chromatography, 20 *Myzus persicae* were transferred to healthy *Montia perfoliata* seedlings for a 4-day inoculation access period. Four to nine test plants were inoculated with the viruliferous aphids for each condition in each of the three independent experiments. NP: no protein added to the virus.

Table 3. Aphid transmission of purified *Cucurbit aphid borne yellows virus* (CABYV) after addition of cucumber phloem exudates^a

CABYV supplemented with	Experiments 1 to 5	Experiment 6	Experiment 7	Experiment 8	Experiment 9
NP	3/49 (6%)	1/8 (13%)	0/7 (0%)	0/7 (0%)	1/8 (13%)
Cucumber sap ^b	31/52 (60%)	...	...	...	...
Sap from plant 1 ^c	...	1/8	3/8	4/5	7/8
Sap from plant 2	...	2/8	1/8	0/5	0/6
Sap from plant 3	...	2/7	1/8	0/4	0/3
Sap from plant 4	...	6/8	1/6	0/4	0/4
Sap from plant 5	...	1/6	2/6	1/5	...
Sap from plant 6	...	...	4/8	...	...
Total from plants 1 to 6	...	12/37 (32%)	12/44 (27%)	5/23 (22%)	7/21 (33%)

^a Number of plants infected (determined by double-antibody sandwich enzyme-linked immunosorbent assay 3 weeks after virus inoculation) compared with number of aphid-inoculated plants after a 24-h acquisition access period on artificial medium containing CABYV-purified virus (25 µg/ml) and supplemented or not with cucumber sap exudates (15 µl of phloem sap containing 150 to 200 µg of protein was added to the virus suspension). After being allowed to feed on the virus suspensions, groups of 20 *Myzus persicae* were transferred to healthy *Montia perfoliata* seedlings for a 4-day inoculation access period. NP: no sap proteins added to the purified virus.

^b Phloem exudates collected from several individual plants were mixed in the diet.

^c Phloem exudates were collected from individual plants (plants 1 to 6).

periment 2) or 71% (experiment 1). In experiment 3, on the other hand, the stimulatory effect was mainly elicited by proteins contained in fractions 12, 16, and 20 (Fig. 3). Analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the protein composition of the three sap fractions that predominantly increased transmission efficiency revealed the presence of low MW proteins (less than 17 to 33 kDa) in fractions 16 and 17 and additional proteins of higher MW, including a major band at approximately 100 kDa, in fraction 12 (Supplementary Fig. S3). It is interesting to note that the experimental MW of more than half of the proteins identified by virus-overlay assay that exhibited the ability to bind to CABYV ranged between 17 and 24 kDa and that a lipoxigenase of approximately 100 kDa was also identified by virus-overlay assay (Tables 1 and 2).

Role of phloem proteins in CABYV transmission by aphids.

To confirm a stimulatory effect of some phloem proteins on viral transmission, we selected the two phloem lectins CsLec17 and CsLec26, identified as CABYV potential partners by virus-overlay assays (discussed above), for additional experimentations. These two proteins belong to the PP2 family (Dinant et al. 2003). Their close orthologs in *A. thaliana*, AtPP2-A1 and AtPP2-A2, display the same domain organization and share over 38% similarity. The transcription pattern of *AtPP2-A1* and *AtPP2-A2* genes is restricted to the companion cell-SE complexes as shown for CsLec26 and CsLec17 (Dinant et al. 2003), and AtPP2-A1 exhibits lectin activity identical to its orthologous protein in cucumber (Beneteau et al. submitted). Together, these data provide strong evidence that both cucumber and *Arabidopsis* PP2 proteins share identical functions in the phloem.

To test the effect of the *A. thaliana* PP2 lectins on CABYV transmission, we purified AtPP2-A1 and -A2 proteins from re-

combinant *Escherichia coli* (Beneteau et al. submitted). The recombinant AtPP2-A1 and -A2 proteins contained a six-histidine C-terminal extension and were purified on a nickel affinity column. As previously, the transmission parameters were fixed to obtain a basal virus transmission rate (2.7%) in the absence of any compound added to the virus solution (virus concentration of 50 µg/ml, 24-h AAP, and 10 aphids transferred per plant). When these parameters were modified to 100 µg/ml for the virus concentration, 48 h for the AAP, and 20 aphids per plant, the transmission efficiency raised to 80% (Table 4). In the low-transmission conditions, addition of 30 µg of either AtPP2-A1 or -A2 to the virus in the aphid diet increased the transmission rate to 70.5 and 90%, respectively (Table 4). This enhancement in the virus transmission rate was similarly observed when AtPP2-A1 was added to a purified suspension of *Turnip yellows virus* (TuYV), another polerovirus transmitted by aphids and also able to infect *A. thaliana* (Table 4). Finally, to establish whether this effect on virus transmission is specific to phloem proteins, different purified proteins from various origins were added to the aphid diet. Virus transmission rate also increased when bovine serum albumin (BSA), casein, lysozyme, cytochrome C, or carbonic anhydrase were mixed to the virus (Table 4). In contrast, addition of two other lectins from *Lens culinaris* (LcH) or from *Maclura pomifera* (MPA) did not induce a stimulation of virus transmission by aphids. This experiment suggests that the enhancement of transmission by added proteins may be more complex than originally thought.

Despite the apparent nonspecific enhancement of transmission provided by different proteins, we performed additional experiments with AtPP2-A1. First, we evaluated the effect of this protein on the stability of the virions. Viral particles mixed with AtPP2-A1 were submitted to an RNase treatment using increasing concentrations of RNase A (Fig. 4). This treatment should induce a partial degradation of any viral RNA that is poorly protected by the capsid. The protected RNA was extracted and amplified by reverse-transcription polymerase chain reaction (RT-PCR). We observed that the PCR-amplified virus fragment was more abundant when AtPP2-A1 was mixed with the virus prior to RNase addition. Therefore, incubation of purified CABYV particles with AtPP2-A1 seems to diminish the accessibility of the encapsidated RNA to RNase A in vitro, suggesting that interaction with this protein masks accessible sites on the virus particles.

Table 4. Aphid transmission of purified *Cucurbit aphid borne yellows virus* (CABYV) and *Turnip yellows virus* (TuYV) after addition of purified proteins^a

Virus source ^b	No. of infected/aphid-inoculated (%) ^c
CABYV ₅₀	2/72 (2.7) ^d
CABYV ₁₀₀	24/30 (80) ^{e,f}
CABYV ₅₀ + PP2-A1	50/75 (70.5) ^g
CABYV ₅₀ + PP2-A2	18/20 (90)
CABYV ₅₀ + BSA	28/42 (66.7) ^f
CABYV ₅₀ + casein	10/10 (100)
CABYV ₅₀ + lysozyme	9/10 (90)
CABYV ₅₀ + Cyto C	8/10 (80)
CABYV ₅₀ + Carb Anh	10/10 (100)
CABYV ₅₀ + LcH	0/9 (0)
CABYV ₅₀ + MPA	0/9 (0)
TuYV ₁₀	1/15 (6) ^h
TuYV ₁₀ + PP2-A1	15/15 (100) ^h

^a After a 24-h acquisition access period (AAP) on artificial medium containing CABYV (at 50 or 100 µg/ml [CABYV₅₀ or CABYV₁₀₀]) or TuYV (at 10 µg/ml [TuYV₁₀]) purified viruses supplemented or not with purified proteins (30 µg), 10 *Myzus persicae* were transferred to healthy *Montia perfoliata* seedlings for a 4-day inoculation access period.

^b PP2-A1 and PP2-A2: purified protein from *Arabidopsis thaliana*; BSA: bovine serum albumin acetylated; Cyto c: cytochrome C; Carb Anh: carbonic anhydrase; LcH: lectin from *Lens culinaris*; MPA: lectin from *Maclura pomifera*.

^c Number of plants infected (determined by double-antibody sandwich enzyme-linked immunosorbent assay 3 weeks after virus inoculation) compared with number of aphid-inoculated plants.

^d Results combine eight independent experiments.

^e For these experiments, AAP was extended to 48 h and 20 aphids were transferred on each test plant.

^f Results combine three independent experiments.

^g Results combine five independent experiments.

^h Results combine two independent experiments.

Fig. 4. RNaseA sensitivity assay of *Cucurbit aphid borne yellows virus* (CABYV) particles. Purified CABYV particles (210 ng, equivalent to 42 ng of RNA) mixed with purified AtPP2-A1 (10 µg) in TN buffer (0.1 M Tris HCl, pH 7.5, and 15 mM NaCl) were treated with RNase A (1×10^{-2} , 6×10^{-3} , and 3×10^{-3} µg/µl). After 30 min of incubation at 37°C, RNA was phenol extracted and reverse transcribed, followed by polymerase chain reaction (PCR). Primers complementary or identical to nucleotides 4,088 to 4,105 and 3,503 to 3,520, respectively, of the CABYV genome were used. Reverse-transcription (RT)-PCR products were analyzed by electrophoresis in 1% agarose gel and were viewed after ethidium bromide staining. C-: RT-PCR negative control (water); C+: RT-PCR positive control (CABYV virions without RNase A treatment). This experiment was carried out twice and gave similar results.

We further investigated whether the enhancement in transmission efficiency was due to an increase in the amount of virus retained in the aphids. To this aim, using quantitative (Q)-RT-PCR, we evaluated virus accumulation in aphids fed on purified preparations of CABYV supplemented with AtPP2-A1 by comparing it with a reference scale of quantified CABYV RNA genomes. Before extracting RNA from the viruliferous aphids for quantification, aphids were transferred for 4 days on healthy plants to empty the digestive tract lumen of free virions. Therefore, the number of virus genomes estimated by Q-RT-PCR represents the number of internalized virions.

A significant increase (2.5-fold) of the number of viral genomes internalized when AtPP2-A1 was present in the aphid diet is revealed in Figure 5: 3.5×10^5 virus genomes/ng of total RNA in aphids after acquisition of CABYV alone (cycle threshold [Ct] value of 24.63 ± 0.17) versus 8.8×10^5 virus genomes after addition of AtPP2-A1 (Ct value of 23.04 ± 0.15). The increase in transmission efficiency observed when purified AtPP2-A1 was added to the virus in the aphid diet may be explained, at least in part, by the higher number of viral particles present within the aphid.

DISCUSSION

CABYV protein partners in sieve elements: a role in plant defense or a function in virus long-distance movement?

As noted in the introduction, poleroviruses such as CABYV are strictly confined to the phloem compartment. Therefore, it is reasonable to hypothesize that interactions between virions and phloem-specific host proteins could play a role in the viral infection cycle. In this study, we have used virus-overlay assays of sap extracts to detect a number of host phloem proteins which can bind in vitro to CABYV. Concerning the possible significance of these interactions, at least three types of situation can be envisaged: i) the interaction could be involved in a host anti-virus defense mechanism; ii) alternatively, the interaction could represent a situation in which the virus hijacks that machinery for its own ends; and iii) we can by no means rule out the possibility that at least some of the interactions have no effect one way or the other.

Some of the virus–host phloem protein interactions reported here were reproducibly observed, although protein identification did not lead to unique candidates. Other interactions were found less frequently and the protein identification remained hypothetical (Tables 1 and 2). Overall, the experiments performed clearly showed that CABYV virions could bind to sap proteins but the interacting proteins could not be identified with confidence. Several reasons could explain the low reproducibility of some interactions, such as a weak affinity between virus particles and these particular phloem proteins, experimental variations in protein composition of cucumber sap, or more basic technical problems related to proper identification and excision of spots.

Among the proteins extracted from the gel that could bind to CABYV virions were two lectins, CsLec 17 (Dinant et al. 2003; Walz et al. 2004; Gomez et al. 2005) and CsLec26 (Dinant et al. 2003; Walz et al. 2004). These proteins, known to be the most abundant species in the sap of cucurbits, are poly-GlcNAc-binding lectins belonging to the PP2 family and are widespread among the angiosperms (Dinant et al. 2003). They have been suggested to function in plant defense responses by recognizing invading pathogens (Read and Northcote 1983). Recently, it was shown that *Arabidopsis* PP2-A1 phloem lectin binds to different classes of glycans, including N-glycans (Beneteau et al. submitted). They could potentially recognize glycosylated virus particles. However, a recent reinvestigation

did not provide any evidence for the presence of sugar residues on structural proteins of poleroviruses (Revollon et al. in press) as had been previously suggested (Seddas and Boissinot 2006).

Most of the other identified putative CABYV partners can also be linked directly or indirectly to the stress and defense response pathway. This probably reflects, at least to some extent, the abundance of stress-related proteins in the phloem sap (Kehr 2006; Dafoe et al. 2009). Among the interacting proteins, we found a protein of the antioxidant defense pathway (ascorbate peroxidase) and a lipoxygenase involved in the jasmonic acid biosynthesis pathway dedicated in the peroxidation of membrane lipids and synthesis of signaling molecules (Siedow 1991). Lipoxygenase expression has been reported to be induced after injury of plants by feeding aphids (Fidantsef et al. 1999; Voelckel et al. 2004). A hydroxymethyltransferase was also found among the candidates. This enzyme is involved in the photorespiratory chain pathway. In *Arabidopsis*, one isoform was shown to minimize the oxidative damage caused by biotic and abiotic stresses (Moreno et al. 2005). A similar isoform was highly abundant in pumpkin. Conversely, implication of the other two candidates in plant defense mechanisms was not established: Csf-2 is a plant factor whose expression was previously shown to be induced by ripening in cucumber (Suyama et al. 1999) and the translationally controlled tumor-related protein was characterized in human as IgE-dependent histamine releasing factor (MacDonald et al. 1995). Note that all the candidates identified have already been found in phloem exudates of different plant species (Aoki et al. 2005; Kehr 2006; Dafoe et al. 2009), except the protein similar to an *O. sativa* protein of unknown function (Table 2).

The potential of a number of phloem proteins to move over long distances has been demonstrated. For instance, in addition to trafficking in both directions between companion cells and SE, PP2 proteins can also move into SE by following assimilate transport from source to sink (Golecki et al. 1999). It is conceivable that CABYV and other poleroviruses could take advantage of the ability of these phloem proteins to traffic to distant locations by forming complexes with them. The rootward movement of *Cucurbita maxima* PP16-1 was shown to depend on its interaction with other specific phloem proteins,

Fig. 5. Accumulation of *Cucurbit aphid borne yellows virus* (CABYV) genomes in aphids measured by quantitative reverse-transcription polymerase chain reaction. Number of CABYV genomes accumulated in aphids which have acquired CABYV virions supplemented or not with 30 µg of purified AtPP2-A1. Batches of 10 aphids were analyzed in duplicate after being transferred on healthy plants for 4 days to empty the lumen of the digestive tract of virions.

including a translationally controlled tumor-related protein (Aoki et al. 2005) identified in this study as a putative CABYV partner. Similarly, Csf-2, another potential CABYV partner, has recently been shown to be part of a multiprotein complex with RBP50 (Ham et al. 2009), which is able to move over long distances. Moreover, such a complex is likely to play an essential role in long-distance transport of several messenger RNAs in phloem sap. Interaction of Csf-2 with CABYV virions raises the possibility that this protein could bridge viral particles to the aforesaid complexes and, thus, be involved in addressing viruses to specific distant tissues.

Virus binding to phloem proteins could also be an important step in virus exit from SE. Virus interaction with parietal proteins could, for instance, favor capture of virions from sap flow, therefore promoting their transport into companion cells through plasmodesmata. This mechanism would share similarities with virus uptake in aphid intestinal cells, which is thought to rely on the presence of receptors on the apical plasma membrane that would capture virions from the food bolus (Reinbold et al. 2001; Seddas et al. 2004; Yang et al. 2008). Trafficking through plasmodesmata could also be facilitated by certain phloem sap proteins, which have been reported to modify the size-exclusion limit of the plasmodesmata connecting adjacent cells (Balachandran et al. 1997; Ishiwatari et al. 1998). By sequentially interacting with these protein types (parietal and plasmodesmatal), the virus could usurp cellular trafficking mechanisms to enter uninfected companion cells.

On the other hand, virus interaction with parietal proteins could function as a defense mechanism against viruses by sequestering the virions and, therefore, limiting their systemic movement. Conversely, the virus could make use of the interaction with phloem proteins, independently of their mobility, to phenotypically mask particles and protect them from plant defense mechanisms. It is noteworthy that CABYV and TuYV particles can bind to a bacterial endosymbiotic protein (symbionin) in the aphid hemolymph, with consequent protection of virions from recognition by the insect immune system (van den Heuvel et al. 1997). The identification of phloem CABYV-partners remains hypothetical; therefore, further studies are required to validate the interactions observed and to understand their role in the virus cycle.

Purified proteins can stimulate CABYV transmission by aphids.

When a crude sap extract was added to purified virus, a stimulatory effect on virus transmission by aphids was observed in our experiments. Implication of proteins present in SE in the aphid transmission process is further suggested by the results obtained when using purified phloem lectins from *A. thaliana* (AtPP2-A1 and -A2) and expressed in *E. coli*. Moreover, enhancement of virus transmission after addition of these proteins to the virus in the aphid diet was directly correlated with an increase of virus accumulation in the aphid's body. Unfortunately, the ability of these specific *A. thaliana* proteins to bind to CABYV could not be assessed in a virus-overlay assay because of a nonspecific reaction between the purified proteins and the serum directed against CABYV (data not shown). Therefore, we could not determine whether the increase in virus transmission efficiency was directly related to the capacity of the lectins to bind to CABYV virions.

Surprisingly, a stimulatory effect on virus transmission was also recorded when several purified proteins of nonplant origin were added to the aphid diet, although the two lectins from *L. culinaris* or *M. pomifera*, which are probably toxic for the aphids (Murdock and Shade 2002), did not have a stimulatory effect. This finding suggests that virtually any protein that has no harmful effect on aphids could stimulate virus transmission.

However, we did not observe such an increase in virus transmission rate with all the proteins present in the different enriched sap fractions obtained after size-exclusion chromatography of sap exudate. Because most proteins present in the purified sap fractions appear to be associated in complexes (Fig. 2B), it is possible that only partially uncomplexed proteins have the ability to increase virus transmission rate. It is likely that the concentration of uncomplexed proteins in all the enriched sap fractions is different from the protein concentration used in the artificial aphid diet in the control experiments, which could explain why not all the sap protein fractions had the capacity to increase virus transmission rate. If virtually any protein can have significant effect on virus transmission rate at sufficiently high concentration, then the increase of virus transmission observed when using total phloem sap exudate could be the effect of the sum of small "nonspecific" contributions from many proteins in relatively low concentration.

Purified phloem proteins can protect CABYV particles from degradation.

Addition of *A. thaliana* lectin AtPP2-A1 to virions can stabilize CABYV particles in an in vitro assay (Fig. 4). In the aphid digestive tube, the presence of a membranous cathepsin-L-like cysteine proteinase in *Acyrtosiphon pisum* and *Aphis gossypii* midgut has been reported (Cristofolletti et al. 2003; Deraison et al. 2004). Virions complexed with sap proteins could potentially be protected from in vivo degradation. Such protection could also occur in the phloem compartment, although no proteases have been identified so far in SE of different plant species (Kehr 2006). Protection of virus particles by a phloem sap protein was also reported for CMV, where resistance to ribonuclease treatment was monitored in vitro following interaction with a 48-kDa protein from cucumber (homologous to the pumpkin PP1 protein) (Requena et al. 2006). However, effect of addition of the phloem protein to CMV on the aphid transmission rate was not evaluated. Finally, the action of plant proteins could also operate at the hemocoel level. Indeed, Powell and associates (1998) reported that, after oral uptake, some lectins could be recovered from the insect's hemolymph. The presence of phloem proteins could prevent an insect immune response by masking viral antigens. Thus, the site of the putative protective effects of proteins may not be strictly limited to the aphid gut.

Despite the fact that many of the virus partners could not be identified with confidence, the work presented here represents an extensive analysis of phloem sap proteins that can bind to a phloem-limited virus. This study constitutes the first report of plant proteins that may enhance the circulative transmission of virus by aphids. Additional studies will be required to determine i) whether the effects on virion stability observed with AtPP2-A1 are also monitored for the non-phloem proteins that are able to stimulate virus transmission by aphids and ii) whether the increase in the amount of virus retained in the aphids is also observed for these non-phloem proteins. Importance of the protein status could also be more carefully addressed by adding complexing agents to the protein suspension to see whether the stimulatory effect on virus transmission is maintained. In addition, experiments like co-immunoprecipitation of proteins complexed to virions in SE, coupled to protein identification, would help to unravel the mode of action of these factors in the transmission process.

MATERIALS AND METHODS

Cucumber sap collection and virus strains.

Seedlings of *Cucumis sativus* cv. Genereux and Marketer were grown in the greenhouse. Aliquots of phloem exudates were collected from 4-week-old cucumber plants after section-

ing petioles. After the first droplet was discarded, the remaining exuded phloem sap was immediately diluted in a buffer appropriate to the type of experiment (discussed below). Depending on the exudate flow and the volume of sap required, sap was collected from two to eight sectioned petioles from the same plant or from different plants.

CABYV and TuYV (formerly referred to as *Beet western yellows virus*) were purified from agroinfected *Montia perfoliata* (Leiser et al. 1992; Prüfer et al. 1995) according to the method of van den Heuvel and associates (1991).

Size-exclusion chromatography and preparation of protein extracts for electrophoresis.

Sap proteins were fractionated by gel-exclusion chromatography. Aliquots of 500 µl of cucumber sap were mixed with three volumes of ice-cold citrate buffer (0.5 M sodium citrate, pH 6.0; 0.3 M NaCl; 5 mM dithiothreitol [DTT]; and 1 mM 4-[2-aminoethyl]-benzenesulfonyl fluoride hydrochloride) (anti-protease, Pefablock C; Roche, Meylan, France). Gel-exclusion chromatography was performed on an AKTA purifier system (GE Healthcare, Velizy, France). The sample was filtered through a 0.2-µm Millipore membrane before being loaded onto a HiPrep 16/60 Sephacryl S-200 high-resolution column (Amersham Biosciences, Europe GmbH, Freiburg, Germany) pre-equilibrated with citrate buffer (also used as elution buffer). Absorption of elutes was monitored at 280 nm and fractions of 5 ml were collected. After addition of antiprotease (Pefablock C; Roche) to 10 µM final concentration, each sample was concentrated 100-fold using centricon-3 microconcentrators (Millipore Corp., Amicon, Bedford, MA, U.S.A.).

For 1D gel electrophoresis, sap protein fractions were diluted in an equal volume of Laemmli 2× buffer (Laemmli 1970) before denaturation at 90°C for 10 min. For 2D gel electrophoresis, the protein extraction method was as described by Giavalisco and associates (2006), with modifications. Crude sap protein extract was successively sedimented into cold acetone and a mixture of methanol/ammonium acetate at 0.1 M (vol/vol). The pellet was finally suspended in 2 M thiourea, 7 M urea, 4% CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate), and 10 mM DTT. Protein concentrations were determined using the Bradford protein assay kit (Bio-Rad, Marnes La Coquette, France) with BSA as a standard.

Aphid transmission experiments using diets supplemented with crude cucumber sap or purified proteins.

Virus-free colonies of *Myzus persicae* were reared on caged pepper (*Capsicum annuum*) seedlings at 20°C with a 16-h photoperiod. Purified CABYV suspensions were mixed with sap extracts prepared as follows. One volume of crude sap (150 to 200 µg of proteins) was diluted in three volumes of aphid artificial diet MP148 (Bruyère et al. 1997) containing 2.5 mM DTT. Purified virus was also added to sap protein fractions collected after gel-exclusion chromatography. Sucrose was added to each fraction to a final concentration of 20%. Finally, purified virus was also mixed with *A. thaliana* recombinant proteins (30 µg of AtPP2-A1 or -A2) presenting a C-terminal six-histidine-tag extension. Recombinant AtPP2-A1 was produced in *E. coli*, purified on a nickel affinity column (Beneteau et al. submitted), and then lyophilized. The purification of the recombinant AtPP2-A2 followed a similar procedure, except that solubilization of the protein before nickel affinity column was done in 50 mM Tris HCl, 0.2% SDS, 10 mM imidazole, pH 8.0 (E. Douville, unpublished data). AtPP2-A1 and -A2 purity was estimated by gel electrophoresis to be over 90 and 80%, respectively. Purified AtPP2-A1 and -A2 were directly suspended in aphid artificial diet before being sonicated for 5 min in an ultrasound water bath to dissolve the protein. Aphid

artificial diet was also supplemented with 30 µg of additional purified proteins such as BSA acetylated (BSA, Promega, Charbonnières-Les-Bains, France), casein (Sigma, St. Quentin Fallavier, France), cytochrome C from equine heart (Sigma), carbonic anhydrase from bovine erythrocytes (Sigma), LcH and MPA lectins (Sigma), and lysozyme (Sigma).

Third- and fourth-instar nymphs or adults were given a 24-h AAP on the different virus sources, supplemented or not with recombinant proteins, before being transferred to *M. perfoliata* test plants for a 4-day inoculation access period (Bruyère et al. 1997). Test plants were assayed by double-antibody sandwich enzyme-linked immunosorbent assay 3 to 4 weeks later (Herrbach et al. 1991) using a CABYV-specific antiserum (Sediag, Longvic, France) or TuYV-specific antibodies (Loewe, Ulm, Germany).

Protein separation by gel electrophoresis.

For 1D SDS-PAGE, 20 µg of crude phloem protein extract or size-fractionated proteins were diluted in one volume of 2× Laemmli buffer. Protein samples were incubated at 90°C for 10 min before being loaded on a 12% SDS-PAGE gel (Laemmli 1970).

For 2D gel electrophoresis, the first dimension was performed in 17-cm-long nonimmobilized NL gradient (IPG) Drystrips, pH 3 to 10 (Bio-Rad). Proteins (300 µg) were mixed with isoelectric focusing (IEF) buffer and applied on Drystrip for active rehydration overnight at 50 V at room temperature before being focused on an Protean IEF cell (Bio-Rad) for 60,000 Vh, with amperage limited to 50 mA. After IEF, the strips were equilibrated for 15 min in 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (vol/vol) glycerol, and 1% (wt/vol) SDS supplemented with 65 mM DTT, followed by a second 15-min equilibration in the same buffer containing 240 mM iodoacetamide instead of DTT and 0.05% bromophenol blue. Strips were then sealed onto 12% 2D SDS-PAGE gels (24.3 by 24 cm). Electrophoresis was carried out with running buffer in a Protean II XL cell tank (Bio-Rad) at room temperature at 30 V for 1 h followed by 100 V overnight (limited to a maximum of 24 mA per gel).

Virus-overlay assay and Western blotting.

For virus-overlay assay, proteins present in cucumber sap were separated by electrophoresis on either a 1D SDS-PAGE or 2D gel before being transferred onto a nitrocellulose membrane (Millipore Corp.) by liquid transfer (Brault et al. 1995) or by electroblotting in a Milliblot apparatus (W.E.P. Company, Washington, U.S.A.) for 45 min at 80 V and 2.5 mA cm⁻² of membrane. The membranes were first saturated with phosphate-buffered saline with 1% Tween 20 (TPBS) containing 5% skimmed milk powder before being incubated overnight with 20 µg of purified CABYV for 24 h at 4°C in TPBS buffer. The membranes were successively incubated with primary (CABYV-specific) antibodies and secondary antibodies (goat anti-rabbit antibody coupled either to peroxidase [Invitrogen, Cergy-Pontoise, France] or to alkaline phosphatase [Sigma] prepared in TPBS containing 5% skimmed milk powder. Depending on the enzyme coupled to the secondary antibody, the complexes were revealed using the Lumi-light Western blotting substrate (Roche) or the Immun-Star Chemiluminescent protein detection systems (Bio-Rad). In control experiments, Western blotting was performed following a similar procedure, except that incubation with purified virus was omitted.

Protein identification by MS and database analysis.

MS was conducted at the Proteomic Platform hosted at the Institute of Molecular and Cellular Biology of Strasbourg (France).

In-gel digestion. For protein identification, stained protein bands were excised from a duplicate gel loaded in parallel with the one used in the virus-overlay experiment and stained with colloidal blue 0.1% (Serva Blue G) prepared in 17% (NH₄)₂SO₄, 0.5% acetic acid, and 34% methanol. After an incubation overnight at room temperature, the gel was destained by successive washes in H₂O. The spots in the gel were located with regard to their position relative to the MW markers on the virus-overlay autoradiography. The nitrocellulose membrane was also stained with 0.1% Ponceau S Red solution (Sigma) to visualize the abundant proteins transferred onto the membrane to provide additional reference points to collect protein spots. The gel digestion procedure was carried out as described by Rabilloud and associates (2001). Selected bands were washed twice with 100 µl of 25 mM NH₄HCO₃/100 µl of acetonitrile (ACN)/dehydration cycles. Between the two wash cycles, reduction was achieved by 45 min of treatment with 10 mM DTT in NH₄HCO₃ buffer (100 µl) at 56°C, and the alkylation reaction was performed by addition of 100 µl of 25 mM iodoacetamide in 25 mM NH₄HCO₃ buffer for 45 min at room temperature. All treatments were performed under continuous agitation. The final dried spots were rehydrated with three volumes of trypsin (V5111; Promega) at 12.5 ng/µl in 25 mM NH₄HCO₃ buffer (freshly diluted) and the digestion was performed at room temperature overnight. Finally, 5 µl of 35% H₂O/60% ACN/5% HCOOH was added and the mixture was vigorously shaken for 15 min to extract tryptic peptides.

MALDI MS. MALDI mass measurements were carried out with a Biflex III (Bruker-Daltonik GmbH, Bremen, Germany) MALDI time-of-flight (MALDI-TOF) mass spectrometer used in reflector positive mode. A saturated solution of α -cyano-4-hydroxycinnamic acid in 50% water/50% acetonitrile was used as a matrix. Mass spectra were internally calibrated with trypsin autolysis peaks (m/z = 842.510 and m/z = 2211.105). Monoisotopic peptide masses were assigned and the peak list transferred through the MS BioTools program (Bruker-Daltonik GmbH) as input to search against preferentially National Center for Biotechnology Information cucurbits or *A. thaliana* taxonomic subdatabases of nonredundant protein sequences. Variable modifications (methionine oxidation and cysteine carbamidomethylation) were taken into account and the peptide mass error was limited to 50 ppm.

Nano LC-MS-MS.

Analysis of the digested proteins was performed using a CapLC capillary LC system (Waters, Altrincham, U.K.) coupled to a hybrid quadrupole orthogonal acceleration TOF-MS/MS (Q-TOF Micro, Waters). Diluted sample (5 µl) was first loaded, concentrated, and cleaned up on a C18 PepMap precolumn cartridge (LC Packings, Dionex, France) and then separated online by an analytical reversed-phase capillary column (NanoEase C18, 75-µm i.d., 15-cm length; Waters) using a linear gradient of 200 µl min⁻¹ flow rate. Internal calibration was provided by the Lockspray module (Waters) during the acquisition run and the spray system (liquid junction) was used at 3.6 kV. Mass data acquisitions were piloted by MassLynx 4.0 software (Waters). Nano LC-MS/MS data were collected by data-dependent scanning; that is, automated MS to MS/MS switching. Four ion precursors were allowed to be fragmented at a time. Mass data collected during a nano LC-MS/MS analysis were processed automatically with the ProteinLynx Process (Waters) module. Data analysis was performed with MASCOT (Matrix Science Ltd., London) against all nonredundant sequences databases with 0.25-Da mass error, one miss cleavage, and taking into account modifications such as carbamidomethylation and oxidation.

Q-RT-PCR.

For Q-RT-PCR analysis, aphids were allowed to acquire purified CABYV (50 µg/ml) for 24 h before being transferred to healthy *Montia perfoliata* for 4 days to empty the digestive tract. A similar virus acquisition was performed after incubating CABYV virions with 30 µg of *A. thaliana* PP2-A1. Pools of 10 aphids were then subjected to total RNA extraction on microcolumns (RNeasy Plant Mini kit; Qiagen, Montreuil-sous-Bois, France), followed by elution with 50 µl of RNase-free water. RNA was quantified with the Nanodrop ND-100 instrument (Nanodrop Technologies, Wilmington, DE, U.S.A.) and stored at -20°C for subsequent Q-RT-PCR assays. Primers listed below were designed manually to amplify a specific 180-bp CABYV sequence of the CP gene (from nucleotides 3,149 to 3,328). The forward CABYV primer (5'-ATACATG GCTATGAACCGGAAT-3') and the reverse CABYV primer (5'-GGATAGTGTCTTTACTACTCC-3') were analyzed for the absence of possible hairpins and secondary structure with the Beacon Designer 2.0 software (Biosoft International, Palo Alto, CA, U.S.A.). Total RNA (12.5 ng) in a 25-µl reaction were subjected to one-step RT-PCR using the Quantitect SYBR Green RT-PCR kit (Qiagen) containing two reverse transcriptases (Omniscript and Sensiscript) and a HotStartTaq DNA polymerase according to the manufacturer's recommendations, with final primer concentrations of 0.5 µM. Q-RT-PCR was performed with the iCycler IQ system (Bio-Rad, Paris). PCR cycles were as follows: 1 cycle of 30 min at 50°C and 15 min at 95°C, followed by 40 cycles each of 15 s at 94°C, 30 s at 55°C, and 30 s at 72°C. A dissociation curve was produced at the end of the cycling phase to ensure that a single PCR product was produced with no primer dimers. Absolute quantification was realized by using a dilution series of 10³ to 10⁸ viral RNA copies obtained from RNA extracted from purified CABYV virions. The Ct were plotted proportionally to the logarithm of the input RNA copy numbers to generate the standard quantification curve. The correlation value was 0.998 and amplification efficiency was 0.953, calculated by the formula $E = [10^{-1/\text{slope}}] - 1$ with a slope of -3.440. No fluorescent signal was obtained with nonviruliferous aphids and each analysis was carried out in duplicate.

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