The helper-component protease transmission factor of tobacco etch potyvirus binds specifically to an aphid ribosomal protein homologous to the laminin receptor precursor

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INTRODUCTION

Natural dissemination of most plant viruses is mediated by insect vectors, in either circulative or non-circulative processes, with aphids (Hemiptera, Aphididae) being the most common vectors (Pirone & Perry, 2002; Ng & Falk, 2006; Hogenhout et al., 2008). Whilst the viral components implicated in transmission are frequently well known, there is substantially less knowledge about the insect counterparts, and only a few published studies have identified insect products with the capacity to interact with virions or viral components that might play a role in plant virus transmission. Using far-Western blotting or similar methodologies, studies with circulative plant viruses have found proteins interacting with virions at key points of the pathways followed by these viruses inside their insect vectors. Examples include a thrips-transmitted tospovirus (Medeiros et al., 2000) and several aphid-transmitted members of the family Luteoviridae (van den Heuvel et al., 1997; Li et al., 2001; Seddas et al., 2004). A promising approach for further advances in this field is represented by the recent adoption of combined genetic and proteomic tools in the analysis of vector specificity in luteovirids (Yang et al., 2008).

In the case of non-circulative plant viruses, the insect vector factors implicated in transmission remain largely unknown. The genus Potyvirus, the largest group of plant viruses transmitted by aphids (López-Moya et al., 2009), is a good example. Potyvirus particles are thought to associate specifically with the lining of the anterior alimentary tract or mouthparts of aphids during transmission (Ammar et al., 1994; Wang et al., 1996). Correlation with successful transmission strongly suggests that the...
virus-retention sites are located on the distal region of the aphid stylets, where food and salivary canals merge (Wang et al., 1996). Indeed, this site is compatible with the evidence, which indicates that, after acquisition, non-persistent viruses are subsequently released and inoculated upon salivation (Martin et al., 1997), and it overlaps with the retention sites of other plant viruses (Uzest et al., 2007). An interesting peculiarity of the transmission process of potyviruses is the involvement of a helper factor in addition to virions (Govier & Kassanis, 1974a, b; Govier et al., 1977). This factor is a virus-encoded multifunctional protein known as helper-component protease (HCPro). For successful transmission, aphids must have access to HCPro before or at the same time as access to virus particles, and there must be a physical interaction between HCPro and the virion-forming coat protein (CP) (reviewed by Pirone & Blanc, 1996; Raccah et al., 2001).

Assistance by an auxiliary factor during transmission is a strategy adopted by many different plant viruses (Froissart et al., 2002). Despite the intense research efforts made since the first identification of these transmission factors of plant viruses, many details concerning their mechanisms of action are still obscure. In the case of potyviruses, the study is complicated by the multiple functions played by HCPro during the virus cycle in addition to aphid transmission, including proteolysis of the viral polyprotein (Carrington et al., 1989), genome amplification and expression of symptoms (Atreya et al., 1992; Atreya & Pirone, 1993) and virus movement in the host plant (Cronin et al., 1995; Kaschau et al., 1997; Rojas et al., 1997). In addition, HCPro acts as a potent suppressor of RNA silencing (Lakatos et al., 2006). Structurally, self-aggregated forms of the transmission-active HCPro have been reported (Plisson et al., 2003; Ruiz-Ferrer et al., 2005). Variants of HCPro that are non-functional for transmission due to mutations in a conserved KITC motif have been described (Atreya et al., 1992). In the case of tobacco etch virus (TEV), this motif has been implicated in binding to aphid stylets (Blanc et al., 1998).

The number of virus particles involved in transmission has been estimated to be quite low in the case of potyviruses (Moury et al., 2007), which may explain why it has been so difficult to identify putative receptors in aphids. Overcoming this hindrance, a study using a far-Western blotting approach in the aphid Myzus persicae (Sulzer) produced a set of cuticular proteins that were able to interact with the HCPro of zucchini yellow mosaic virus (ZYMV) (Dombrovsky et al., 2007). In this study, the absence of interaction with a mutated version of HCPro unable to assist ZYMV transmission by a change in a KLSC motif (similar to the KITC motif of TEV) suggested a correlation between binding to cuticle proteins and transmission.

The most striking recent advances in the study of transmission of non-circulative plant viruses have been obtained with cauliflower mosaic virus. In this virus, particles form a stable complex with the viral P3 protein, which is then retained in the aphid vector stylets via an interaction with the aphid transmission factor P2 (Leh et al., 1999; Plisson et al., 2005; Hoh et al., 2010). Taking advantage of the ability to label the P2 protein in vivo with GFP, retention of P3–virion complexes was shown to take place by protein–protein interactions near the tip of the stylet (Uzest et al., 2007), a site where a new anatomical structure named the ‘acrostyle’ was recently described (Uzest et al., 2010).

Identification of candidate receptors for viruses in their vectors is one of the aspects that might help us to understand the transmission processes, and eventually might serve to design novel virus-control strategies. With this objective in mind, the present paper describes a proteomics-based search for candidate proteins interacting with the helper factor of a non-persistently transmitted plant virus. Proteins extracted from the heads of M. persicae, a highly efficient vector of potyviruses, were submitted to overlay assays with the HCPro of TEV, and HCPro-interacting proteins were selected and analysed by mass spectrometry (MS), taking advantage for their identification of aphid sequence databases derived from genome sequencing initiatives (Tagu et al., 2008; The International Aphid Genomics Consortium, 2010). Among the HCPro interactors characterized, a putative ribosomal protein S2 (RP2) was selected for further analysis and its putative involvement in potyvirial transmission is discussed.

RESULTS

Binding of TEV HCPro to proteins extracted from M. persicae heads and separated by two-dimensional (2D) electrophoresis

To characterize proteins from aphid heads that bound to TEV HCPro, 2D electrophoresis separation and far-Western blotting analysis were carried out. As shown in the protein silver stain of a representative 2D gel (Fig. 1a), about 1400 different protein spots were detected in extracts obtained from M. persicae heads, with molecular masses ranging from 7 to 203 kDa, and distributed by isoelectric points (pIs) between 3.5 and 11, as determined by PDQuest software (Bio-Rad).

To perform binding experiments, aphid head proteins after 2D electrophoresis were transferred to nitrocellulose membranes, which were later incubated with purified His-tagged TEV HCPro (hereafter referred simply as HCPro). Bound HCPro was then detected using either a commercial monoclonal anti-penta-His antibody or a polyclonal anti-HCPro-specific antibody. Two versions of TEV HCPro rabbit polyclonal antisera were tested, the first kindly provided by T.P. Pirone (University of Kentucky, USA) and the second obtained in house with His-tagged HCPro protein purified from infected plants. Binding assays were repeated at least three times for each antibody,
and different biological replicates (three or more) were also used for each repeat. Although basically the same set of interacting spots was observed independently of the chosen antibodies, the polyclonal antibodies produced more background and a few non-specific interactions (not shown); thus, further experiments were performed using the anti-penta-His monoclonal antibody. Repeats made with this antibody revealed only minor differences in the number and intensities of the cross-reacting spots. TEV HCPro was found to bind predominantly to about ten proteins, with estimated molecular masses ranging from 23 to 75 kDa (Fig. 1b). Only one spot (indicated by an asterisk in Fig. 1b) was found to interact directly with the anti-penta-His antibody when transferred membranes were incubated without interposed HCPro protein (not shown). Marks that did not coincide with silver-stained proteins or that were not present in repetitions of the experiment were discarded. In this way, nine spots, labelled 1–7 and I and II (according to their later identification, see below), were selected for matrix-assisted laser desorption/ionization (MALDI-MS/MS) analysis.

Identification of aphid proteins interacting with TEV HCPro

Selected protein spots were isolated and digested with trypsin, and peptides resulting from the digestion were analysed by MALDI-MS/MS on a two-hybrid quadrupole/time-of-flight (Q-TOF) mass spectrometer. Protein spot selection and digestion were repeated three times, each time from a different 2D gel.

Seven spots (1–7 in Fig. 1b) gave significant scores that allowed identification (Table 1), whilst two spots (I and II in Fig. 1b) could not be properly identified, due to the absence of recognizable peptide sequences deriving from them. The list of peptides used for identification included those derived from de novo sequencing of selected tryptic fragments in the case of spots 2 and 6. Sequence information for peptides was used by the MASCOT search engine, which was set up to query the NCBI database to predict scores for each of the matched proteins. Only one protein was identified per spot.

As summarized in Table 1, the seven identified spots comprised domains of putative adenylate kinase (spot 1), elongation factor 1α (spot 2), lysozyme precursor (spot 3), acyl-CoA dehydrogenase (spot 4), ribosomal protein (spot 5), peptidase (spot 6) and enolase (spot 7). However, in some cases (spots 2, 3, 5 and 7), important differences between calculated and experimentally derived molecular masses existed, probably reflecting the evolutionary distance between aphids and the other organisms considered (Table 1), although we cannot exclude other reasons such as extensive post-transcriptional modifications. To refine these preliminary identifications, we
### Table 1. Preliminary identification of *M. persicae* protein spots interacting with TEV HCPro

<table>
<thead>
<tr>
<th>Spot</th>
<th>Peptide‡</th>
<th>Preliminary protein identification according to MASCOT‡</th>
<th>Observed Mr (Da)</th>
<th>Predicted Mr (Da)</th>
<th>Protein name (source)</th>
<th>GenBank accession no.</th>
<th>Score</th>
<th>Coverage (%)</th>
<th>NCBI calculated Mr (kDa)</th>
<th>Experiment-derived Mr (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GFLIDGYPR</td>
<td>Adenylate kinase isoenzyme 1 (rabbit)</td>
<td>1037.51</td>
<td>1036.53</td>
<td>P00569</td>
<td>54</td>
<td>4.64</td>
<td>21.8</td>
<td>23.0</td>
<td>21.8</td>
</tr>
<tr>
<td>2</td>
<td>THINIVVGHVDKG</td>
<td>Elongation factor 1α (Bombyx mori)</td>
<td>–</td>
<td>–</td>
<td>P29520</td>
<td>798</td>
<td>3.24</td>
<td>50.4</td>
<td>25.0</td>
<td>25.0</td>
</tr>
<tr>
<td>3</td>
<td>WESGYNTR</td>
<td>Lysozyme precursor (human)</td>
<td>1012.45</td>
<td>1011.45</td>
<td>AAA36188</td>
<td>96</td>
<td>13.5</td>
<td>17.0</td>
<td>30.0</td>
<td>30.0</td>
</tr>
<tr>
<td>4</td>
<td>ENVLTGEGAGFK</td>
<td>Acyl-CoA dehydrogenase, mitochondrial precursor (human)</td>
<td>1221.56</td>
<td>1220.56</td>
<td>P41367</td>
<td>139</td>
<td>12.4</td>
<td>46.4</td>
<td>45.0</td>
<td>45.0</td>
</tr>
<tr>
<td>5</td>
<td>FPAHTGATPIAGR</td>
<td>Hypothetical protein 14, ribosomal protein S2 (Lononimia obliqua)</td>
<td>1295.68</td>
<td>1294.68</td>
<td>AAV91367</td>
<td>153</td>
<td>11.7</td>
<td>29.3</td>
<td>47.0</td>
<td>47.0</td>
</tr>
<tr>
<td>6</td>
<td>GDTGLWGFYFVCDK</td>
<td>Peptidase M16 (Anopheles gambiae)</td>
<td>–</td>
<td>–</td>
<td>XP_309120</td>
<td>626</td>
<td>8.24</td>
<td>49.4</td>
<td>49.0</td>
<td>49.0</td>
</tr>
<tr>
<td>7</td>
<td>NGWGMVMVHR</td>
<td>Enolase (Drosophila affinis)</td>
<td>1144.53</td>
<td>1143.53</td>
<td>AAX13041</td>
<td>173</td>
<td>22.3</td>
<td>38.9</td>
<td>54.0</td>
<td>54.0</td>
</tr>
</tbody>
</table>

*Spot numbers correspond to the numbers indicated in Fig. 1.
†Identified peptides for each protein are indicated, including those obtained after de novo sequencing (spots 2 and 6). Observed and predicted relative molecular masses (Mr) are indicated.
‡MASCOT analyses were performed with the NCBI database. Scores higher than 50 were considered significant. Experimentally derived values for Mr were calculated using PDQuest. Discrepancies in Mr probably reflect the distance between the aphid proteins and these preliminary identifications in the NCBI database.
decided to perform further searches centred on aphid-derived sequences.

Search for putative HCPro receptors in specific aphid libraries

Peptide sequences obtained for each spot were used to perform searches in databases of aphid libraries available at http://urgi.versailles.inra.fr/GnpSeq (Tagu et al., 2008), focusing on contigs derived from *M. persicae*, but also searching different aphid species, in particular *Acyrthosiphon pisum* (Harris). Contigs with sequences that showed homology to the peptides belonged to libraries derived from different samples and tissues, such as head, digestive tract and whole aphid. For every analysed spot except for spot 3, at least one contig containing the sequence of a possible translated aphid protein was found (Table 2).

*M. persicae* predicted protein sequences were used to search further for the presence of characteristic domains in other aphid and insect proteins. This homology search confirmed the matching of sequences with annotated orthologues from other species for the putative proteins obtained by MASCOT analysis, including confirmation of the previously presumed identities of spots 1, 2 and 4–7. Among these, spot 5 was shown to contain a ribosomal protein domain characteristic of S2 proteins, so is hereafter referred to as *M. persicae* RPS2 (MpRPS2). This protein was selected for further investigation for the reasons explained below.

Sequence of MpRPS2

The sequence of the expected protein RPS2 identified to correspond to spot 5 was determined in our clonal population of *M. persicae*, using information corresponding to contig CTG_MP_636.2-34598577. The coding sequence of MpRPS2 was deduced from two independent bacterial clones obtained after RT-PCR amplification with specific primers, ligation of the corresponding fragment to a plasmid vector and transformation of *Escherichia coli*. Total nucleic acids extracted from *M. persicae* individuals of our colony were used as template for the amplification. The sequence of the 930 nt corresponding to the ORF of the expected gene was identical for the two bacterial clones analysed, and only three differences were observed with the sequence deduced from CTG_MP_636.2-34598577, which resulted in a single amino acid difference at position 240 (Ser→Pro). This difference might reflect natural variability between our *M. persicae* clone and that used to construct library 18800 from where this contig was derived, a library originally obtained by G. Jander at the Boyce Thompson Institute for Plant Research (Cornell University, NY, USA). Interestingly, Ser residues were also present in this position in homologous proteins derived from other aphids, such as *Acyrthosiphon pisum* and *Aphis gossypii*, as shown in an alignment comparing MpRPS2 with selected homologue proteins (Fig. 2). The paired identities between the proteins of the three aphid species were in the range of 97% (*Aphis gossypii* and *M. persicae*) to 98% (*Acyrthosiphon pisum* and *M. persicae*). When the *M. persicae* protein (310 aa) was compared with its homologues from mammals, for instance with the human protein known as ribosomal protein RPS2/67 kDa laminin receptor (295 aa), identity was 60% (71% similarity) for the whole product, with most divergences in the C-terminal portion, reaching 75% identity (86% similarity) in the first 210 aa.

Heterologous expression of MpRPS2 and binding assays with a wild-type and mutated version of HCPro

After heterologous expression in *E. coli* and purification by affinity chromatography of the MpRPS2 protein in two fusion constructs with MalE and GST, binding to TEV HCPro was assayed by far-Western blotting. Fig. 3 shows the results of a representative experiment of the several repetitions performed. A strong interaction was observed in all cases between HCPro and the fusion products containing MpRPS2 (Fig. 3, upper panel, lanes 2 and 3). Two different fusions were tested to reduce the possibility of interference with the binding assay by the carrier proteins (MalE and GST). Interactions were observed between HCPro and a single band of the expected size corresponding to the fusion product (~60 kDa) in the case of GST–MpRPS2, and between HCPro and MalE–MpRPS2, although in this case a doublet band appeared (~74–76 kDa), which might represent different reduced forms or partial degradation of the product. Interestingly, when a variant of HCPro non-functional for transmission with a mutation in the conserved KITC motif, referred to as the EITC variant (Blanc et al., 1998), was tested in parallel with the same samples described above, the mutant protein failed to show an interaction with either of the MpRPS2 fusion products (Fig. 3, lower panel, lanes 2 and 3). As controls, samples of purified HCPro (lane 4) and of virion particles (lane 5) were included in the experiment, showing direct detection by the antibody in the first case, or binding between the viral CP and the overlaid HCPro protein (lane 5, upper panel) or the mutant HCProEITC variant (lane 5, lower panel). This experiment indicated that the HCProEITC variant failed to bind to MpRPS2, although its capacity to interact with TEV CP was not affected by the mutation.

DISCUSSION

The aim of this study was to analyse putative interactions between aphid proteins and the HCPro protein of a non-persistent transmitted potyvirus (TEV), using a far-Western blotting strategy to identify hypothetical receptors that might be involved in the transmission process. The use of overlay assays has resulted in important advances in the study of transmission of circulative viruses (Hogenhout et al., 2008), and was also used with another potyvirus,
Table 2. Matches of peptides of selected *M. persicae* proteins with sequence information from libraries constructed with samples of the same species and of *Acyrtosiphon pisum*, and refinement of the protein identification using a domain-based search

<table>
<thead>
<tr>
<th>Spot*</th>
<th>Peptide sequence†</th>
<th>Aphid libraries‡</th>
<th>Protein identification§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>M. persicae</em></td>
<td><em>A. pisum</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Amino acid</td>
<td>Amino acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sequence identity</td>
<td>sequence identity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Contig</td>
<td>Contig</td>
</tr>
<tr>
<td>1</td>
<td>GFLIDGYPR</td>
<td>9/9 (100 %)</td>
<td>9/9 (100 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CL_MP_833.2-34598723</td>
<td>CTG_AP_158.5-ApAL3SD-XIX-F7</td>
</tr>
<tr>
<td>2</td>
<td>THINIVGVHGDSKG</td>
<td>14/15 (93 %)</td>
<td>14/5 (93 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTG_MP_194.2-34597458</td>
<td>CTG_AP_3.5-ApAL3SD-I-C11 ( +3)</td>
</tr>
<tr>
<td>3</td>
<td>WESGYNTR</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>ENVLTETEGAGFK</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>FPAHTGTPIAGR</td>
<td>11/13 (84 %)</td>
<td>10/12 (83 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTG_MP_636.2-34598577 ( +1)</td>
<td>CTG_AP_856.5-ApHL3LD-II-C2</td>
</tr>
<tr>
<td>6</td>
<td>GDITLWGYFVCDK</td>
<td>–</td>
<td>10/12 (83 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>11/13 (84 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>18/18 (100 %)</td>
</tr>
<tr>
<td>7</td>
<td>NGWGTMVSHR</td>
<td>10/10 (100 %)</td>
<td>10/10 (100 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTG_MP_426.2-34600104</td>
<td>CTG_AP_138.5-ApAL3SD-XIII-D2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30/30 (100 %)</td>
<td>30/30 (100 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CL_MP_2507.2-34597760</td>
<td>CTG_AP_138.5-ApAL3SD-XIII-D2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17/22 (77 %)</td>
<td>21/22 (95.5 %)</td>
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<tr>
<td></td>
<td></td>
<td>CL_MP_2181.2-34601567</td>
<td>CTG_AP_138.5-ApAL3SD-XIII-D2 ( +1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16/18 (88 %)</td>
<td>16/18 (88 %)</td>
</tr>
</tbody>
</table>

*Spot numbers correspond to numbers indicated in Fig. 1 and Table 1.
†Identified peptides for each protein are indicated, including those obtained after de novo sequencing (spots 2 and 6).
‡Libraries of *M. persicae* and *Acyrtosiphon pisum* aphid species were searched (http://urgi.versailles.inra.fr/GnpSeq). When matches were found, the amino acid sequence identity for each peptide is indicated. When more than one contig was matched, the best hit is shown and the number of other contigs is indicated in parentheses.
§Protein identity after domain-based identification comparison with insect proteins. *M. persicae* predicted protein sequences were used to search further for the presence of characteristic domains in proteins from other insects. The refined identification assigned to each protein spot is indicated.
ZYMV, to identify HCPro interactors in the cuticle of an aphid vector (Dombrovsky et al., 2007). A similar approach was adopted in this research using purified transmission-active TEV HCPro protein as bait. Following reports describing the retention site for transmission of non-persistent viruses as the distal tip of the maxillary stylets (Wang et al., 1996; Martin et al., 1997), the interaction with HCPro was assayed with extracts obtained from aphid heads, after 2D separation of the proteins. To maximize the chance of detecting new products, a different extraction procedure compared with that used in the study with ZYMV (Dombrovsky et al., 2007) was adopted, as our purpose was to expand the range of potential receptors.

At least nine *M. persicae* proteins were observed to bind specifically to TEV HCPro in our assays. Such a relatively high number of interactions with cellular components, even from a non-host organism of the virus (an insect), might reflect the multifunctionality of HCPro in many important processes. Interactions between HCPro and cellular proteins have been described in other works, including eight proteins from tobacco plants shown to bind TEV HCPro (Anandalakshmi et al., 2000). The HCPro of other potyviruses has also been shown to interact with different plant products (Guo et al., 2003; Ballut et al., 2005; Jin et al., 2007a, b; Cheng et al., 2008). Together, these reports suggest that our knowledge of the HCPro interactome is still limited, and

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**Fig. 2.** Alignment of the protein sequence corresponding to the MpRPS2 gene identified as a TEV HCPro interactor in our study with the homologous ribosomal proteins of *M. persicae* (deduced from the sequence of contig CTG_MP_636.2-34598577), *Acyrthosiphon pisum* (deduced from the sequence of contig CTG_AP_155.5-ID0AA10CH02RM1), *Aphis gossypii* (deduced from the sequence of contig CTG_AG_34.2-29816266), *Anopheles gambiae* (GenBank accession no. XP_320736), *Penaeus monodon* (GenBank accession no. DT044263) and the human ribosomal protein/laminin receptor (GenBank accession no. AAB22299). Sequences were obtained from the databases http://urgi.versailles.inra.fr (in the case of aphids) or http://www.ncbi.nlm.nih.gov (for other species), aligned in CLUSTALW format using T-Coffee and displayed by BoxShade, using tools available at http://www.ch.embnet.org. Gaps in the alignment are indicated by hyphens, whilst identical and similar residues are indicated by black and grey shading, respectively. An asterisk indicates the S/P polymorphism at position 240 between MpRPS2 identified in this study and the *M. persicae* deduced sequence.

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<table>
<thead>
<tr>
<th>M. persicae</th>
<th>A. pisum</th>
<th>A. gossypii</th>
<th>P. monodon</th>
<th>H. sapiens</th>
</tr>
</thead>
<tbody>
<tr>
<td>YSIKTTAATNED</td>
<td>YSIKTTAATNED</td>
<td>YSIKTTAATNED</td>
<td>YSIKTTAATNED</td>
<td>FTATQPSDVSLPGQDQV</td>
</tr>
</tbody>
</table>
Among the other candidates, we decided to focus our attention on MpRPS2, due to its homology with cellular receptors of viruses and prions (Wang et al., 1992; Gauczynski et al., 2001; Tio et al., 2005; Akache et al., 2006; Senapin & Phongdara, 2006). Homologues of this ribosomal protein have been described in all types of organism (Nelson et al., 2008). In mammals, RPS2 has a 37 kDa precursor that dimerizes to form a mature receptor of 67 kDa, with both cytoplasmic and cell-membrane locations (Nelson et al., 2008). Localization of MpRPS2 in cell membranes would explain its role as a receptor in the transmission process. Interestingly, an equivalent RPS2 product was identified in cast cuticles from Anopheles gambiae after moulting (He et al., 2007), a fact that provides at least preliminary evidence for its presence in insect cuticles. Further work will be needed to confirm whether this is also the case for aphids, and particularly for the structures considered essential for virus transmission that have been described recently in aphid stylets (U zest et al., 2010). Our results also raise an interesting question from an evolutionary perspective, that of how an insect ribosomal protein could become a specific receptor for a plant virus. Considering the cross-species conservation of ribosomal proteins, the recent finding of a functional relationship between ribosomal proteins and several plant viruses, including a potyvirus (Yang et al., 2009), may be relevant. Nevertheless, at this point we cannot rule out the possibility that the interaction between HCPro and MpRPS2 might participate in other processes.

Understanding the transmission process of plant viruses is a challenging issue. Recent results with caulimoviruses have revealed details of a tightly regulated process that might have evolved to enhance the chances of transmission (Martiniere et al., 2009), although it is not clear whether this could be extended to other virus–host vector pathosystems. In any case, identifying elements in the vector that participate in the transmission process is a prerequisite for a better comprehension of the mechanisms governing dissemination of plant viruses. The present work is a start in this direction, aiming to expand the list of aphid proteins interacting with the potyviral HCPro, in addition to other proteins identified previously (Dombrovsky et al., 2007). The fact that, in both studies, HCPro mutants failed to interact with aphid proteins might indicate that more than one protein could be involved in the transmission process. On the other hand, we cannot rule out the possibility that the protein structure (and thus the binding properties) of the HCProEITC variant might be altered by the mutation. Further structural studies will be required to address this point.

Taken together, our results are compatible with a role for MpRPS2 during transmission of TEV by M. persicae.

**METHODS**

**Virus and viral protein purification.** A TEV variant that incorporates an N-terminal His-tagged HCPro protein, TEV HCH10 (Blanc et al., 1999), was used to infect Nicotiana tabacum...
L. cv. Xanthi nc plants. Leaves from infected tobacco plants were used to purify virion particles (Murphy et al., 1990) or HCPo protein (Ruiz-Ferrer et al., 2005). HCPo protein was also purified from leaf tissue of Nicotiana benthamiana Domi. plants after agroinfiltration with constructs that allowed transient expression of the two first cistrons of TEV (Goytia et al., 2006). A mutated version of the TEV HCPo with an EITC motif instead of the KITC motif, HCPoEITC variant (with a K→E mutation at position 358 of the viral polyprotein; numbering according to Allison et al., 1986) was generated using a QuickChange Site-directed Mutagenesis kit (Stratagene) with the oligonucleotides 5′-TCACGTGATTGAGGTAAGA-TCACATGAAACGTTGC-3′ and 5′-GGACACATTTGCATGTGA-TCTACACACCGTGTA-3′. Purified TEV HCPo proteins (either wild type or EITC variant) were analysed by SDS-PAGE and stored at −70 °C until use.

**SDS-PAGE and Western blot analysis.** Protein samples were separated on discontinuous 12.5–5 % SDS-PAGE (Laemmli, 1970) and stained with Coomassie blue. For Western blot analysis, separated proteins were electroblotted onto nitrocellulose membranes (Schleicher & Schuell) and revealed using appropriate specific antibodies, followed by incubation with the corresponding HRP-conjugated anti-aphid antisera and ECL (Amersham Biosciences) or colorimetric (4-chloronaphthol) substrate, following the manufacturers’ instructions.

**Aphid rearing and dissection of heads.** A clonal population of *M. persicae*, previously used in our laboratory for transmission studies (Fernández-Calvino et al., 2006), was reared on tobacco plants. Adult apterous aphids were collected and their heads separated with a sterile needle in PBS buffer containing 1 mM EDTA and 2 mM PMSF and supplemented with Complete Mini EDTA-free protease inhibitor cocktail (Roche Applied Sciences).

**Extraction of aphid head proteins.** For 2D gel separation of aphid proteins, 250 aphid heads stored on ice were crushed in 100 µl 8.4 M urea containing 2.4 M thiourea, 5 % CHAPS and 50 mM DTT, and submitted to repeated freeze–thaw cycles. After a clarification step in a microcentrifuge (~16000 g for 10 min at 4 °C), total protein concentration in the final supernatant was measured with Bradford reagent (Bio-Rad) so that equivalent amounts could be loaded on the gel (typically 90 µg).

**2D electrophoresis with immobilized pH gradient (IPG) strips.** 2D electrophoresis was performed following standard methods (Rabilloud, 1998; Gorg et al., 2004), using pre-cast IPG strips [18 cm, pH 3–11 nonlinear (NL); Amersham Biosciences] in the first dimension (isoelectric focusing). Samples that had previously been rehydrated with 450 µl of solution containing 7 M urea, 2 M thiourea, 2 % CHAPS, 0.75 % IPG buffer pH 3–11 NL, 100 mM DTeStreak (Amersham Biosciences) and trace amounts of bromophenol blue were applied by cup-loading to the IPG strips. Focusing was carried out with an IGPhor II (Amersham Biosciences) apparatus by voltage stepwise increases (300 V for 3 h, 300–1000 V gradient for 4 h, 1000–8000 V for 2 h and constant 8000 V until a steady-state was reached). The second-dimension separation was performed in 1 mm thick, 26 × 22 cm, 12.5 % homogeneous polyacrylamide gels, with an Ettan DALTSix large vertical system (Amersham Biosciences) at 25 °C and constant current (2 mA per gel for 45 min and 20 mA per gel for about 4 h). Gels were stained using a Silver Staining kit (Amersham Biosciences) according to the manufacturer’s instructions, with minor modifications to improve subsequent MS compatibility (Yan et al., 2000). A commercial protein ladder (Bio-Rad) was incorporated in the 2D gel, and the molecular mass of each spot was calculated using PDQuest software.

**HCPo–aphid proteins binding assays.** After electrophoresis, unstained SDS-PAGE gels were electroblotted onto nitrocellulose membranes. Membranes were blocked for 1 h in PBST/milk (PBS containing 0.05 % Tween 20 and 5 % skimmed milk) and incubated overnight at 4 °C with purified TEV HCPo at 0.2 mg ml−1 in PBST/milk. After three 10 min rinses with PBST, membranes were incubated first in PBST/milk with monoglucal anti-penta-His BSA-free antibody (Qiagen) diluted 1:500, or with specific TEV HCPo polyclonal antibody diluted 1:400, and then with HRP-conjugated anti-mouse or anti-rabbit IgG (1:15 000 and 1:10 000 dilutions, respectively). Bound secondary antibodies were detected using a commercial ECL detection system (Amersham Biosciences) or 4-chloronaphthol substrate.

**In-gel digestion of proteins.** Protein spots were excised manually from the 2D gels and digested automatically using a Proteineer DP Protein Digestion Station (Bruker Daltonics), with minor variations on the usual protocol (Shevchenko et al., 1996), including treatments in 50 mM ammonium bicarbonate (99.5 % purity; Sigma Chemical Co.) with 10 mM DTT (Amersham Biosciences) and with 55 mM iodoacetamide (Sigma Chemical Co.). Gel pieces were rinsed with 50 mM ammonium bicarbonate and acetonitrile (gradient grade; Merck) and dried under a stream of nitrogen. Modified porcine trypsin (sequencing grade; Promega) was added to a final concentration of 13 ng µl−1 in 50 mM ammonium bicarbonate and digested at 37 °C for 6 h. Finally, 0.5 % trifluoroacetic acid (99.5 % purity; Sigma) was added for peptide extraction.

**MALDI-MS/(MS) analysis and database searching.** An aliquot (1 µl) of digestion solution was mixed (1:1) with 2-cyano-4-hydroxycinnamic acid (Bruker Daltonics) in 33 % aqueous acetonitrile and 0.1 % trifluoroacetic acid, deposited onto a 600 µm AnchorChip MALDI probe (Bruker Daltonics) and dried at room temperature. MALDI-MS/(MS) data were obtained using an Ultraflex TOF mass spectrometer (Bruker Daltonics) equipped with a Lift-MS/MS device (Suckau et al., 2003). Spectra were acquired in the positive-ion mode at 50 Hz laser frequency, and the mean of 100–150 individual spectra was determined. For fragment ion analysis in the tandem TOF mode, precursors were accelerated to 8 kV and selected in a timed ion gate. Fragment ions generated were further accelerated by 19 kV in the LIFT cell and their masses analysed using post-LIFT metastable suppression. Peptide mass mapping data were analysed using flexAnalysis software (Bruker Daltonics). Internal calibration of MALDI-MS and MS/MS device were achieved with trypsin autolysis ions or proton adducts of a peptide mixture. Chemical sequencing was also applied to certain peptides. All peptide sequences derived from the data were combined through the MS BioTools program (Bruker Daltonics) to search the NCBI nr database using Mascot (Matrix Science) software (Perkins et al., 1999). The same peptides were also used to identify contigs in aphid sequence libraries (http://urgi.versailles.inra.fr/GnpSeq).

Experimental and theoretical values for molecular masses and isoelectric points of proteins were calculated using PDQuest and tools available at the ExPASy Proteomics Server (http://www.expasy.ch).

**Cloning and expression of the aphid RPS2.** The expected mRNA sequence of *M. persicae* RPS2 (MpRPS2) deduced from contig CTG MP 636.2-34578577 was used to design specific oligonucleotides covering the coding region: 5′-GCCGAAATCTTAGCTGGGAGGACG-TAGATGATTTGAGGACGACTA-3′ (sense) and 5′-CGATGATGATTTGAGGACGACTA-3′ (antisense). These primers amplified a single fragment after RT-PCR using total RNA extracted from individuals of our *M. persicae* clonal population as template. After digestion with the flanking EcoRI and Xbal sites incorporated into primers (underlined above), the fragment was cloned into a pMAL-p2X vector (New England Biolabs). Sequences of two independent pMAL-MpRPS2 clones were obtained to confirm the integrity of the constructs for expression. A similar strategy was followed to clone the...
coding region in a pGEX-4T-1 expression vector (GE Healthcare Life Sciences), after digestion with EcoRI and SalI and insertion into the corresponding sites. The fusion products of MpRPS2 with maltose-binding protein (Malted–MpRPS2) or with glutathione S-transferase (GST–MpRPS2) were expressed using standard methods after transformation of plasmids into *E. coli* BL21 cells, growth and induction with IPTG (0.3 mM). Purification was performed by affinity chromatography after cell lysis, and the final protein concentration was measured using Bradford reagent.

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**REFERENCES**


