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In vitro association between the helper component–proteinase of zucchini yellow mosaic virus and cuticle proteins of *Myzus persicae*

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Potyviruses, as typical non-persistently transmitted viruses, are carried within the stylets of aphids. Cuticle proteins (CuPs), which are a major component of the insect cuticle, were examined for *in vitro* binding to the potyviral helper component–proteinase (HC–Pro). Proteins in 8 M urea extracts from *Myzus persicae* were separated by SDS-PAGE, electroblotted onto membranes and identified as CuPs by using specific antibodies to *M. persicae* CuP. Blotted *M. persicae* protein extracts were overlaid with two HC–Pros, differing by the presence of K or E in the KLSC domain. The HC–Pro with KLSC, known to assist transmission, was found to bind *M. persicae* proteins, whereas the HC–Pro with ELSC, being deficient in assisting transmission, did not. To identify CuPs that react with HC–Pro, protein extracts were separated by two-dimensional gel electrophoresis. Nine proteins reacting with HC–Pro were sequenced by mass spectrometry. Sequences of peptides in four proteins, of molecular masses between 22 and 31 kDa, were identified as CuPs according to comparison with sequences in GenBank. The putative CuPs from *M. persicae* that bind HC–Pro are potentially of interest in locating receptors for virions bound to HC–Pro in aphids' stylets.

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INTRODUCTION

The non-persistent mode of transmission of viruses by aphids was defined by Watson & Roberts (1939), describing the brief retention of the viruses by vectors. Non-persistent transmission of viruses was reviewed by several authors (e.g. Pirone & Harris, 1977). Early researchers reported that the non-persistently transmitted potato virus Y (PVY) was associated with the distal parts of the aphids' stylets (Bradley & Ganong, 1955a, b). Subsequent research efforts were aimed at characterizing virus features that affected transmission by vectors. Biological and molecular approaches elucidated the role of the coat protein of non-persistently transmitted viruses: a conserved triplet of amino acids was reported for the potyviruses tobacco vein mottling virus (Atreya *et al.*, 1990) and zucchini yellow mosaic virus (ZYMV) (Gal-On *et al.*, 1992). Mutations in the coat protein also affected the transmission of a different non-persistently transmitted virus, cucumber mosaic virus (Perry *et al.*, 1994). Transmission of potyviruses by aphids requires a protein now named helper component–proteinase (HC–Pro), first described by Kassanis & Govier (1971) and whose role was summarized by Pirone & Blanc (1996) and Raccah *et al.* (2001). Govier & Kassanis (1974) proposed the so-called 'bridge hypothesis' to explain the mode of helper assistance: this hypothesis assumes that the HC–Pro molecule links the virions to the aphids' stylets. *In vitro* studies revealed an affinity between the conserved Pro–Thr–Lys (PTK) triplet in HC–Pro and virions of ZYMV (Peng *et al.*, 1998). Also, electron microscopy studies showed that virions were retained in the lumen of the food canal of aphids' stylets only if acquired with an active HC–Pro [with the wild-type Lys–Ile–Thr–Cys (KITC) motif], but not if acquired with a defective HC–Pro (with a mutation from Lys to Glu in the KITC motif) (Ammar *et al.*, 1994; Wang *et al.*, 1996; Blanc *et al.*, 1998). The food canal in the maxillary stylets of aphids is made of cuticle, which is composed of chitin (a polymer of β-1-4-linked *N*-acetyl-D-glucosamine) fibres embedded in a matrix mainly composed of cuticle proteins (CuPs) (Neville, 1975; Vincent & Wegst, 2004). Chitin is not unique to insects and arthropods; it also occurs in plants and fungi. Therefore, we suspected that the aphid-specific constituents of the cuticle that may associate with HC–Pro were the CuPs. Previously (Dombrovsky *et al.*, 2007), we isolated several genes for CuPs from *Myzus persicae* cDNA libraries and from the *M. persicae* genome (GenBank accession nos DQ108938, DQ108939, DQ108935 and DQ108936). We showed them to have the conserved amino acid sequence that was described by Rebers & Riddiford (1988) and was,

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therefore, named the 'R&R consensus'. Rebers & Willis (2001) reported that the function of the R&R consensus was to bind to chitin (chitin-binding domain). The CuPs isolated from *M. persicae* differed from one another in having different repeats at the N and C terminals. None of the CuPs that were expressed in *Escherichia coli* could interact with the HC–Pro of ZYMV. Therefore, we adopted an alternative approach of extracting proteins from *M. persicae* to ascertain an association with ZYMV HC–Pro. Reacting proteins were then identified by their reactions to antibodies and to CuPs, and by their amino acid sequence similarity to known aphid CuPs.

METHODS

Aphid species. *M. persicae* was raised on mustard (*Brassica perviridis* 'Tendergreen') in growth chambers at 25 ± 3 °C with continuous light. *Bemisia tabaci* was grown on *Datura stramonium* in cages in a greenhouse at room temperature.

Virus strains. ZYMV strains used for HC–Pro purification were obtained from our full-length clone (FLC) of ZYMV (Gal-On *et al.*, 1995). The original ZYMV FLC was engineered to encode six histidines within the gene of the HC–Pro (Kadouri *et al.*, 1998), allowing affinity purification on Ni–NTA resin. The original ZYMV FLC encoded a normal HC–Pro (with the KLSC motif). An additional strain that served as control was constructed (A. Dombrovsky, H. Huet & B. Raccah, unpublished data) to encode the mutated ELSC motif. HC–Pros with EITC or ELSC motifs (in PVY-C and ZYMV-Ct, respectively) were previously found to be deficient in assisting transmission (Thornbury *et al.*, 1990; Grumet & Fang, 1990). Herein, ZYMV strains with the normal HC–Pro and with the mutated motif are named ZYMV-KLSC and ZYMV-ELSC, respectively. For subsequent experiments, both strains were kept on squash (*Cucurbita pepo* 'Ma'ayan') in separate growth chambers.

Virus and HC–Pro purification. ZYMV was purified according to Gal-On *et al.* (1992) with minor modifications, and the HC–Pro was purified by the Ni–NTA resin method (Qiagen), based on binding and releasing His-tagged HC–Pro from the extract of virus-infected plants according to Kadouri *et al.* (1998).

Extraction of CuPs from aphids. Extraction of CuPs from aphids was based on a procedure developed for other insects (Fristrom *et al.*, 1978; Nakato *et al.*, 1990). Aphids at varying stages were collected in 5 g batches and stored at −80 °C until use. For extraction, a 5 g frozen aphid batch was ground in liquid nitrogen and then mixed with 50 ml (1 : 10, w/v) extraction buffer [20 mM Tris/HCl, 0.15 M NaCl (pH 7.5), 3 mM PMSF (Applichem), 10 mM β-mercaptoethanol (Sigma) (a low-salt mix, designated TBS-L)]. This mixture was then homogenized with a glass homogenizer and centrifuged for 5 min at 2000 ***g***. The pellet was washed repeatedly by vortexing with 200 ml TBS-L for 2–5 s and centrifuged at 2000 ***g*** for 10 min after each wash. This was repeated five to seven times until the supernatant cleared, after which 200 ml high-salt TBS (TBS-H) [20 mM Tris/HCl, 0.6 M NaCl (pH 7.5), 3 mM PMSF, 10 mM β-mercaptoethanol] was added to the pellet, vortexed for 2–5 s and centrifuged for 10 min at 3000 ***g***. Following these washes, the pellet was collected, transferred to a 30 ml tube and vortexed once with 20 ml TBS-L, as above.

The clear pellet was vortexed twice with 10 mM Tris/HCl (pH 7.0) and centrifuged at 3000 ***g*** for 10 min. The resulting pellet was extracted using 5 vols 8 M urea in 10 mM Tris/HCl (pH 7.0). The mixture of aphid pellet with 8 M urea was shaken for 2 h at room temperature, after which the supernatant was purified by two additional 5 min centrifugations at 12 000 ***g***. The resulting *M. persicae* protein extracts are designated MpPE for simplicity.

Antibodies against CuP. Two genes for CuPs, Mpcp5 with an RR-1 consensus (GenBank accession no. DQ108939) and Mpcp2 with an RR-2 consensus (GenBank accession no. DQ108935), served for expression in *E. coli* (Dombrovsky *et al.*, 2007). They were engineered to encode six histidine residues at the C terminal of the CuP. The identity of expressed proteins was confirmed by amino acid sequencing (Dombrovsky *et al.*, 2007). Expressed proteins were purified by the Ni–NTA method and separated by SDS-PAGE (12 % gels). Protein bands excised from the gels were ground in 0.05 M phosphate buffer (pH 7.2). Preimmune blood was taken from rabbits and they were then injected with either Mpcp2 or Mpcp5. Four sequential injections were maintained: the first used 1 ml 0.05 M phosphate buffer (pH 7.2) containing 1 mg protein and 1 ml incomplete adjuvant; the next was after 3 weeks and the third and fourth were at 2 week intervals. The second, third and fourth injections used 0.5 mg CuP in 1 ml 0.05 M phosphate buffer (pH 7.2) and 1 ml incomplete adjuvant. Blood was collected from immunized rabbits 1 week after the third injection (10 ml) and 1 week after the fourth injection (25 ml). Serum was separated from blood cells and the immunogenic reaction was determined by Western blotting against CuPs.

Electrophoretic analysis of CuPs from *M. persicae*. Supernatant containing MpPE at 4.5 mg ml^{-1} (total protein estimated by the Nanodrop system) was added to an equal volume of Laemmli buffer and boiled according to Laemmli (1970). The mixture was separated by SDS-PAGE (12 % gels). The proteins were electroblotted onto a nitrocellulose membrane for 45 min at 240 mA on a semi-dry transfer blot apparatus (Bio-Rad). Blotted membranes were Ponceau-stained for 2 min, washed with double-distilled H_2O and photographed. The membranes were blocked with PBS (pH 7.4) containing 3 % non-fat milk powder for 3 h at room temperature. Anti-*M. persicae* CuP antibodies (MpCuPAb; 1 : 1500) were used to identify the CuPs and to estimate their proportion in the total protein extract

Testing the binding of ZYMV HC–Pro to MpPE. After blocking, the membranes were washed twice with 0.3 M K_2HPO_4 (pH 8.8) and three times with PBS–Tween (PBS-T). Membranes blotted with MpPE were overlaid in PBS containing the normal ZYMV-KLSC or the defective ZYMV-ELSC HC–Pros (250 µl containing 200 µg purified HC–Pro was added to 5 ml PBS). The membranes were agitated slowly overnight at 4 °C to allow interaction with HC–Pro. Additional controls, to ascertain non-specific binding, used membranes blotted with healthy plant extract. After three washings with PBS-T, the membranes were challenged, under shaking, with commercial mAbs to six histidine residues (α-His mAb; 1 : 4000; Sigma) for 3 h. After additional washings with PBS-T, the alkaline phosphatase (AP) conjugate of antibodies to mouse (Sigma) (α-mouse–AP) was added at 1 : 30 000 and incubated for 1 h at room temperature with shaking. Following washes, the proteins were visualized by adding the substrate (NBT, BCIP; Promega). As controls to identify non-specific reactions, α-His mAbs were overlaid against blotted MpPE followed by α-mouse–AP, as well as overlaying α-mouse–AP alone (without previous overlaying with α-His mAb).

Two-dimensional (2-D) gel electrophoresis. In order to obtain single proteins for amino acid sequencing, MpPE was separated by 2-D gel electrophoresis. Proteins were immobilized on IPG 13 cm strips (Amersham Biosciences) at a pH gradient from 3 to 10, then re-hydrated overnight with 250 µl rehydration solution with IPG buffer [8 M urea, 3 % CHAPS, 0.5 % Triton X-100, 2 % (v/v) IPG buffer, 0.3 % (w/v) dithiothreitol and 0.002 % bromophenol blue] containing 200 µg protein at room temperature. Separation in the

first dimension, based on isoelectric focusing, used the Pharmacia Multiphor II system at 18 °C. Separation parameters were 100 V for 8 h, 300 V for 15 min, 500 V for 15 min, 1000 V for 15 min, 1500 V for 15 min, 2000 V for 15 min, 2500 V for 15 min, 3000 V for 15 min, and 3500 V for 4 h. Strips with focused protein extract were dipped in 10 ml equilibration solution for 15 min. The first equilibration was in 50 mM Tris/HCl (pH 8.8), 6 M urea, 30 % (v/v) glycerol, 2 % (w/v) SDS, 0.002 % bromophenol blue and 2 mM tributylphosphine (TBP). The second phase used the above solution with 2.5 % (w/v) iodoacetamide in place of TBP. Second-dimension separation was by SDS-PAGE (12 % gels) with an SE 600 series vertical slab gel unit (Hoefer Scientific Instruments). Proteins seen in analytical gels were stained and visualized with colloidal Coomassie blue G-250. For calibration, five gels were used to analyse the number and distribution of the MpPE spots.

Analysis of the reaction of MpPE to ZYMV HC–Pros. MpPE was separated on four gels and then challenged with purified HC–Pros. The proteins separated on one gel were overlaid with the defective ZYMV-ELSC HC–Pro; those on the other three gels were overlaid with the normal ZYMV-KLSC HC–Pro. Nine proteins on one of the three gels were excised for amino acid sequencing. The selected protein spots were separated from the others. The sequence of amino acids was determined by the HPLC/mass spectrometry/mass spectrometry (LC-MS/MS) method and electrospray ion-trap analysis of proteins (DecaXP; ThermoFinnigan). LC-MS/MS was performed at the Smoler Proteomics Center, Technion, Haifa, Israel, and in the Protein Sequencing Unit of the Medical School of the Hebrew University of Jerusalem, Israel. Colloidal Coomassie blue-stained proteins were excised and trypsinized in gel (Shevchenko *et al.*, 1996). The amino acid sequence was compared with those of proteins deposited in the GenBank nr database with the Pep-Miner software (Beer *et al.*, 2004).

RESULTS

Extraction of CuPs from *M. persicae*

The CuPs expressed by *E. coli* (Dombrovsky *et al.*, 2007) were tested, without success, for *in vitro* binding to the ZYMV-KLSC HC–Pro (data not shown). Therefore, we adopted the alternative approach of extracting proteins from whole aphids. Extraction of *M. persicae* with either 8 M urea or 1 % calcofluor yielded similar protein profiles (data not shown). However, as 8 M urea extracted a greater quantity of protein with a wider range of molecular masses (MM), it was preferred in subsequent extractions. As seen in Fig. 1, the MM of proteins in the MpPE ranged from 15 to 100 kDa. A comparison between the proteins in the Coomassie blue lane (total proteins in the MpPE) and in the α-CuP lane (MpPE bands reacting to MpCuPAb at the 1 : 1500 dilution) in Fig. 1 shows that proteins reacting to antibodies against CuPs mainly have MMs between 18 and 60 kDa. No reaction was seen when the preimmune antibodies were tested against blotted MpPE (Fig. 1, Preimmune).

Comparison of the HC–Pro of two ZYMV strains for the ability to assist aphid transmission

A mutation in the KLSC motif of the HC–Pro of ZYMV rendered it defective in assisting transmission (Grumet & Fang, 1990). Therefore, we constructed two ZYMV strains with normal and mutated KLSC motifs in the HC–Pro (see above). The Ni–NTA-purified, His-tagged HC–Pros from either the normal ZYMV-KLSC or mutated ZYMV-ELSC were tested for their ability to assist transmission of ZYMV virions by aphids. As expected, HC–Pro of ZYMV-KLSC assisted transmission of ZYMV virions (43/51; 84.3 ± 3.23 %), whereas the defective HC–Pro of ZYMV-ELSC did not (0/50; 0 %).

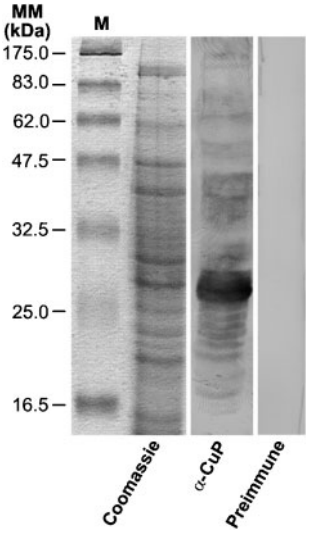

Fig. 1. Protein profile of the 8 M urea extract from *M. persicae*. Lane 'Coomassie': protein extracts were separated by SDS-PAGE (12 % gel) and stained with Coomassie blue. Lane 'α-CuP', Western blot reaction to antibodies against the CuPs of *M. persicae* (1 : 1500). Lane 'Preimmune', reaction of blotted protein extracts to preimmune antibodies (1 : 1000). MM, Molecular mass marker.

Analysis of the *in vitro* binding of the ZYMV HC–Pro to MpPE

Serological tests showed that the antibodies to Mpcp2 (with an RR-2 consensus) reacted against CuPs with either the RR-1 or the RR-2 consensus. On the other hand, antibodies to Mpcp5 (with an RR-1 consensus) reacted only against CuPs with the RR-1 consensus (data not shown). Therefore, antibodies to Mpcp2 were used in the present study (designated here as MpCuPAb).

The overlay approach (Blanc *et al.*, 1997; Peng *et al.*, 1998) was adopted to determine the *in vitro* binding of the ZYMV HC–Pro to electroblotted MpPE. His-tagged HC–Pro bound to the blotted MpPE was visualized in Western blots by using an α-His mAb. MpPE was separated in six lanes of a one-dimensional (1-D) SDS–12 % polyacrylamide gel stained with Ponceau and, after electroblotting, each separate lane was overlaid with one of the following: purified ZYMV-KLSC HC–Pro (K), purified ZYMV-ELSC

HC–Pro (E), extract from a plant infected with ZYMV-KLSC (KP), extract from a plant infected with ZYMV-ELSC (EP) and extract from a virus-free plant (H). Lane α-CuP was overlaid with MpCuPAb to identify the CuP bands in the MpPE. MpPE separation, electroblotting and overlays were repeated three times with different extracts. A representative of such a separation is shown in Fig. 2. Fig. 2(a) shows the profile of the proteins in the MpPE. In Fig. 2(b), a positive reaction to HC–Pro is seen in lanes K and KP, and no binding of HC–Pro to MpPE can be seen in lanes E, EP or H. The reaction of MpPE to ZYMV-KLSC was seen in all three repetitions (four or five protein bands in each repetition). The approximate MMs of the proteins in the reacting bands ranged from 18 to 32 kDa (see arrows in Fig. 2b). Two proteins with MMs of 27 and 30 kDa were consistently present in all three repeats. Certain proteins in lane α-CuP – reacting with MpCuPAb – were of MM similar to that of proteins reacting to HC–Pro. In controls

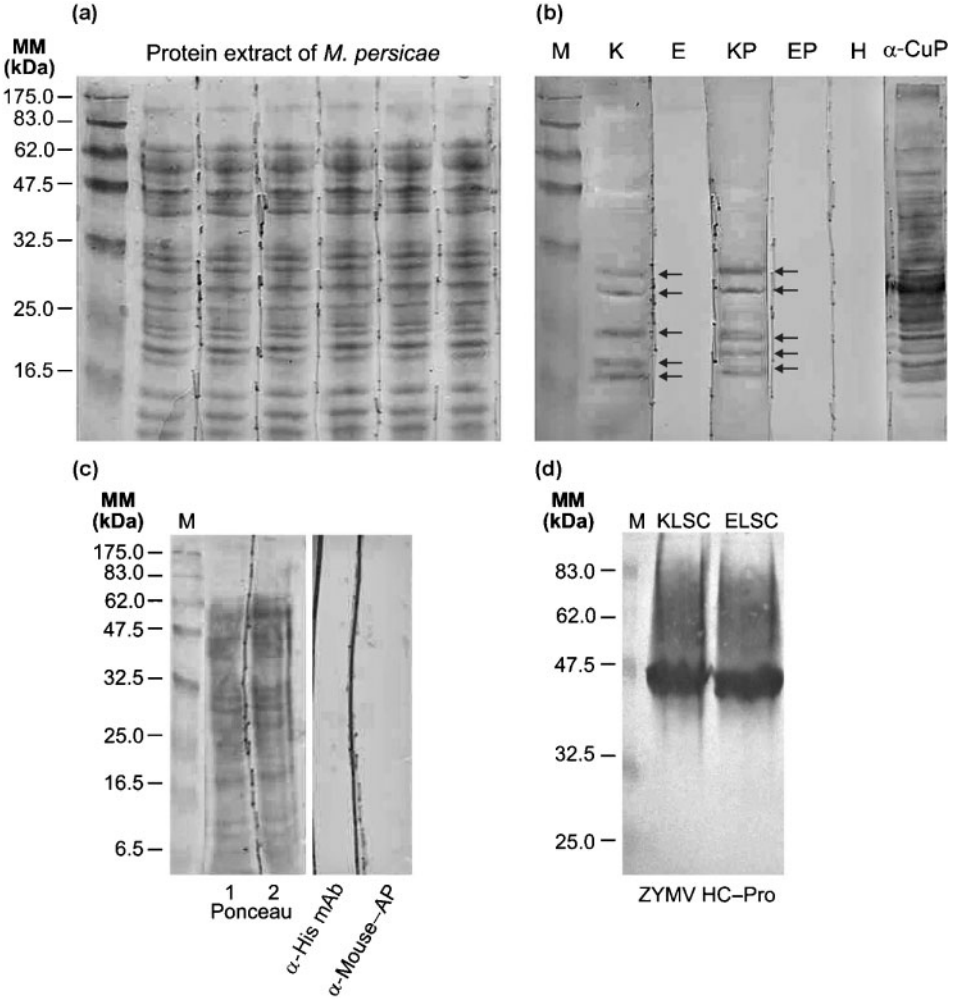

Fig. 2. Determination of HC–Pro bound to 8 M protein extracts of *M. persicae.* (a) MpPE was separated in six lanes by SDS-PAGE (12 % gels), electroblotted onto nitrocellulose membranes and stained with Ponceau. (b) Different lanes electroblotted with the protein extract in (a) were overlaid with the following treatments: K, ZYMV-KLSC HC–Pro; E, ZYMV-ELSC HC–Pro; KP, crude extract from plants infected with ZYMV-KLSC; EP, crude extract from plants infected with ZYMV-ELSC; H, crude extract from virus-free plants; α-CuP, reaction of blotted CuPs to antibodies against the CuPs of *M. persicae* (1 : 1500). The presence of HC–Pro was visualized by using anti-His monoclonal antibodies (1 : 4000) and then adding AP-conjugated α-mouse antibodies (1 : 30 000). (c) Ponceau 1 and 2: MpPE separated by SDS-PAGE (12 % gels), electroblotted onto nitrocellulose membrane and stained with Ponceau. α-His mAb: the lane of Ponceau 1 overlaid with mAbs against histidine residues (1 : 4000) and then adding AP-conjugated α-mouse antibodies (1 : 30 000). α-Mouse–AP: the lane of Ponceau 2 overlaid with AP-conjugated α-mouse antibodies (1 : 30 000) (without HC–Pro or α-His mAb). (d) Histidine-tagged ZYMV-KLSC HC–Pro and ZYMV-ELSC HC–Pro was purified by Ni–NTA resin, separated by SDS-PAGE (12 % gels), electroblotted onto nitrocellulose membranes and identified with mAbs to histidine residues (1 : 4000), then AP-conjugated α-mouse antibodies (1 : 30 000) were added. MM, Molecular mass marker.

to negate non-specific binding of either the α-His mAb or the α-mouse–AP conjugate to electroblotted MpPE, a membrane was electroblotted with MpPE and stained with Ponceau (Fig. 2c, Ponceau). As seen in Fig. 2(c) (α-His mAb), no reaction occurred when the membrane was overlaid with α-His mAb (without HC–Pro) and processed as in Fig. 2(b). No reaction was seen in Fig. 2(c) (α-mouse–AP) when the blotted MpPE was overlaid with α-mouse–AP (without HC–Pro or α-His mAb). Finally, to compare the two HC–Pros used for the overlay, ZYMV-KLSC or ZYMV-ELSC was purified from the same amount of plant tissue, separated, electroblotted and overlaid with α-His mAb. As seen in Fig. 2(d), the two HC–Pro preparations yielded comparable Western blot reactions.

In order to rule out a non-specific reaction, MpPE and an 8 M urea protein extract from the whitefly *B. tabaci* (a non-vector for potyviruses) were electroblotted on membranes. Fewer proteins were seen in Coomassie blue-stained gels for *B. tabaci* than in those for *M. persicae* (Fig. 3, left). MpPE reacted to MpCuPAb (Fig. 3, centre, *Mp*), whereas the protein extract from *B. tabaci* did not (Fig. 3, centre, *Bt*). Membranes electroblotted with proteins extracted from both insects were overlaid with purified HC–Pro of ZYMV-KLSC. At least three proteins (MMs of approximately 27, 30 and 32 kDa) from *M. persicae* reacted with the ZYMV HC–Pro (see arrows in Fig. 3, *Mp*). The proteins from *B. tabaci* did not react (Fig. 3, right, *Bt*).

The overlay results shown in this section suggested that a CuP component in MpPE showed *in vitro* binding to the normal ZYMV HC–Pro.

Lack of reaction of *M. persicae* CuPs against the ZYMV coat protein

The 'bridge hypothesis' proposed by Govier & Kassanis (1974) suggested that HC–Pro serves as a link between the virions and the stylets. However, Salomon & Bernardi (1995) favoured the possibility of direct binding of virions to the stylets. We tested whether purified virions alone would bind to CuPs contained in MpPE. MpPE was separated by SDS-PAGE (12 % gels), electroblotted onto membranes and stained with Ponceau (Fig. 4a). The presence of bound virions was visualized with antibodies to ZYMV. The various lanes (Fig. 4b) were treated as follows: lane 1, overlaid with a mixture of virions and HC–Pro; lane 2, overlaid with HC–Pro alone; lane 3, overlaid with virions alone. As seen in Fig. 4(b), virions bound to the blotted proteins were recorded only in lane 1. Stronger protein bands were seen (see arrows) at MMs of 27 and 30 kDa (similar to those in Fig. 2 for bound HC–Pros). The finding that the virions of ZYMV bound to MpPE only in the presence of HC–Pro is consistent with the 'bridge hypothesis' of Govier & Kassanis (1974).

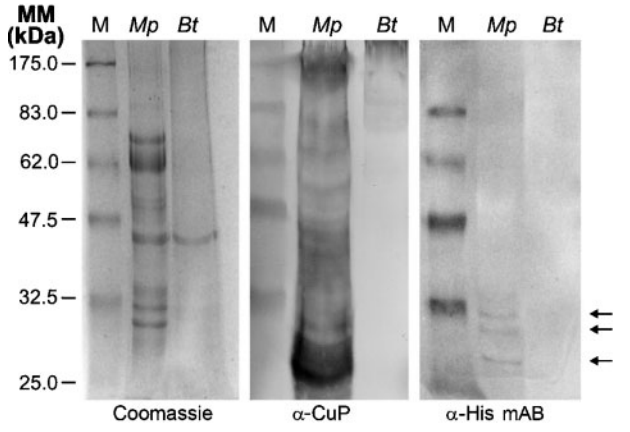

Fig. 3. Comparison of the profile of protein extracts obtained with 8 M urea from *M. persicae* (*Mp*) and *B. tabaci* (*Bt*). The proteins were separated by SDS-PAGE (12 % gels). Left panel, Coomassie blue staining; centre panel, reaction of proteins electroblotted onto nitrocellulose membranes and overlaid with polyclonal antibodies against the CuPs of *M. persicae* (1 : 1500); right panel, protein extracts from *M. persicae* electroblotted onto nitrocellulose membranes and overlaid with purified His-tagged ZYMV-KLSC HC–Pro. Bound helper protein was identified with mAbs to histidine residues (1 : 4000), then AP-conjugated α-mouse antibodies (1 : 30 000) were added. MM, Molecular mass marker.

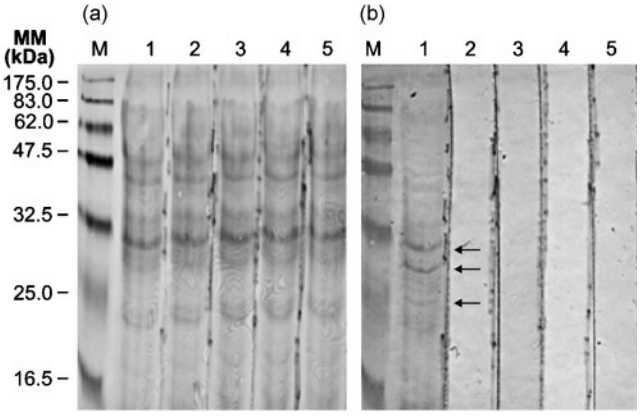

Fig. 4. Determination of the virions binding to protein extracts obtained by 8 M urea from *M. persicae*. (a) Profile of protein extract obtained by 8 M urea from *M. persicae*, separated by SDS-PAGE (12 % gels) and stained with Ponceau (lanes 1–3). (b) Electroblotted proteins overlaid with a mixture of ZYMV-KLSC HC–Pro and ZYMV virions (lane 1), ZYMV-KLSC HC–Pro without virions (lane 2) or virions of ZYMV without HC–Pro (lane 3). Lane 4, crude extract of virus-free squash; lane 5, PBS buffer. The presence of ZYMV was identified by far-Western blot analysis using polyclonal antibodies against ZYMV (1 : 2000). MM, Molecular mass markers. Purified His-tagged HC–Pro was obtained by binding to Ni–NTA resin.

2-D separations of putative CuPs in extracts of *M. persicae*

Identifying the proteins in MpPE reacting with HC–Pro required amino acid sequencing. The resolution of 1-D separation of the proteins by PAGE was not sufficient to enable the excision of single, well-separated protein bands. Therefore, MpPE was separated by 2-D electrophoresis. At first, five separations were carried out and the protein spots were stained with Coomassie blue. A representative of the distribution of proteins in the 2-D gel is shown in Fig. 5(a). The mean ± SD of the number of proteins in the five separations was 140.2 ± 20.2, 80 % of which were present between MMs of 20 and 60 kDa and between pH 4 and 8. The MpCuPAb enabled us to identify about 50 % of the protein spots shown in Fig. 5(a) as CuPs (Fig. 5b). Most of these proteins were found between 20 and 80 kDa and pH 4 and 8. It is apparent that more proteins reacted to MpCuPAb in the pH range 4–6 than in the pH range 7–9 (Fig. 5b).

Binding of 2-D-separated protein spots to the HC–Pro of ZYMV-KLSC

Four 2-D gels were used to identify the proteins in MpPE that interacted with HC–Pro. After electroblotting, one membrane was overlaid with purified ZYMV-ELSC HC–Pro (defective in transmission) and three membranes were overlaid with purified ZYMV-KLSC HC–Pro (active in transmission). No reaction was observed for proteins overlaid with the defective HC–Pro (Fig. 5d). The other three membranes – overlaid with ZYMV-KLSC HC–Pro – produced 20 (Fig. 5c), 14 and 12 proteins reacting with HC–Pro (membranes not shown). Examination of the three gels revealed that eight to ten of the reacting proteins were grouped within the ranges of 20–32.5 kDa and pH 4–7, and four to ten proteins were within the ranges of 18–40 kDa and pH 7–9.

The membrane shown in Fig. 5(c) presented the highest number of reacting proteins. Therefore, this membrane was also selected for excision of protein spots and amino acid sequencing. Not all proteins were excised; only those that were separated from other protein spots and visible were selected [see nine circled and numbered proteins on the membrane in Fig. 5(c)] and excised from the respective Coomassie blue-stained gel (Fig. 5a). The excised proteins were microsequenced by the LC-MS/MS method (Table 1). The sequence of the proteins in three other spots (1, 6 and 8) did not show a measurable similarity to peptides in GenBank. However, spots 6 and 8 did react with MpCuPAb; therefore, they should be regarded as putative CuPs. The results of the reactions of the 2-D-separated proteins to HC–Pro and their amino acid sequences are presented in Table 1. Proteins 3, 4, 5 and 9 represent peptides that were present in CuPs of aphids that were deposited in GenBank. Peptides in these four proteins included a conserved amino acid sequence found in the R&R consensus of CuPs (bold sequence in Table 1). In

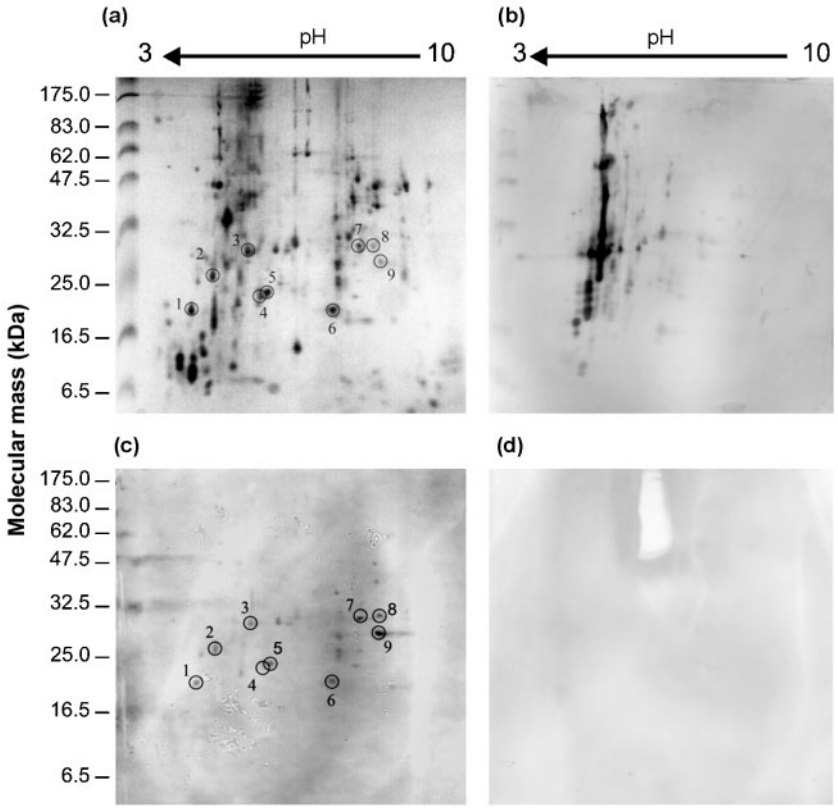

Fig. 5. 2-D separation of protein extracts obtained by 8 M urea from *M. persicae* and determination of protein spots that bind HC–Pros. pH values are shown at the top. (a) Colloidal Coomassie blue staining. (b) Protein spots reacting to antibodies against the CuPs of *M. persicae* (MpCuPAb) (1 : 1500). (c) Overlay assay with His-tagged ZYMV-KLSC HC–Pro. Protein spots were selected (marked by circles) for protein sequencing by the LC-MS/MS system. (d) Overlay assay with His-tagged ZYMV-ELSC HC–Pro (His-tagged HC–Pros were purified from infected plant tissue by binding to Ni–NTA resin). Bound HC–Pro was visualized by α-His mAb (1 : 4000).

Table 1. Identification of putative CuPs from *M. persicae* reacting with overlaid HC–Pro from ZYMV-KLSC

Spot no.*	Protein identity	GenBank accession no.	No. peptides	Peptide sequence†	Coverage (%)	MM (kDa)		pI	
						Observed	Predicted	Observed	Predicted
2	GroEL *Buchnera aphidicola*	AAR21862	1	ANDAAGDGTTTATLLAQSIVNEGLK	4.7	24.2	55.40	4.56	5.17
3	CuP *Aphis gossypii*	AAO63549	4	APAYSAPAYK SQSEYADGNGYVK APYSAPSYSAPAYK **TVEYTADDYNGFNAVVK**	30.7	30.0	23.02	5.37	6.08
4	CuP *Myzus persicae*	AAL29466	4	SQSEYADGNGYVK **TVEYTADDYNGFNAVVK** APAYAAPAYSAPAYK APAYSAPAYK	37.1	22.2	23.40	5.75	6.07
5	CuP *Myzus persicae*	AAZ20451	5	GYYPGAPAVYPAVGAVTPAPIIAPVPVVPK VVSPVYKPVDNKLPAIIR NAGSENAAQVIEGSYSYVGDDGAPVEVK **YYADETGYHAVGNVV**PTIPSEIAK SLELIASQPQKPEDSKK	47.0	22.3	26.50	5.88	6.587
7	Hypothetical protein *Apis mellifera*	XP_397538	1	AVPVSVPHPVPVTVDRPYPVDVPR	14.3	31.0	17.80	7.60	9.28
9	CuP *Myzus persicae*	AAZ20447	8	LVAPAVPVAK VVQVVPAQFQPDPSYTFAYQVQDQITGDSK YSLIEPDGTR **RTVDYTADPTNGFNAYVQK** SDVQQAVFVPSVSTDDVETIK VDTIEVEQAR YAPSGSKPLKNTLAVPETK KNTLAVPETKT	52.0	28.5	24.30	8.12	6.58

*Protein spot number refers to number circled in Fig. 5, representing far-Western reaction of CuPs to the ZYMV-KLSC HC–Pro.

†Amino acid sequence of proteins in each protein spot was determined by LC-MS/MS. Peptide sequences in bold represent homology to the R&R consensus in the CuPs (chitin-binding domain).

these proteins, the rate of coverage ranged from 30.7 % for the CuP of *Aphis gossypii* to 52 % for that of *M. persicae*. Two other proteins interacted with HC–Pro and did not resemble known CuPs (spots 2 and 7). Although these two proteins showed little resemblance (4.7 and 14.3 % coverage, respectively) to other proteins in GenBank, it is difficult to speculate on their significance. The differences between the MMs and pI values of the CuPs observed in MpPE and those predicted from GenBank could indicate that the CuPs in the extracts were not identical to those reported previously, except for protein 4, which shows similar values (Table 1).

DISCUSSION

Retention of potyviruses in aphids' stylets has been shown, by both biological tests and electron microscopy, to depend on the presence of an active HC–Pro. Ammar *et al.* (1994), Wang *et al.* (1996) and Blanc *et al.* (1998) showed that the majority of virions were retained near the proximal ends of the stylets. Molecular studies since the 1990s have provided valuable information on viral domains involved in aphid transmission: the roles of the Asp–Ala–Gly domain in the coat protein (Atreya *et al.*, 1990; Gal-On *et al.*, 1992) and of the Lys–Ile–Thr–Cys and Pro–Thr–Lys domains in HC–Pro (Thornbury *et al.*, 1990; Huet *et al.*, 1994). Harris & Harris (2001) proposed an aphid cuticular receptor for non-persistently transmitted viruses. However, prior to the present study, little was done to identify the domains in the aphid cuticle that are associated with the viral proteins involved in transmission by aphids. This is because of lack of information on aphid proteins in general and on CuPs in particular. For persistently transmitted viruses, interesting findings were reported for putative aphid receptors among the proteins in the midgut of *M. persicae*, for both luteoviruses (Gray & Gildow, 2003) and polerovirus (Seddas *et al.*, 2004). In the past few years, we attempted to isolate and characterize CuPs from aphids (Dombrovsky *et al.*, 2003, 2007). In the present paper, we report an *in vitro* association of CuPs extracted from *M. persicae* with the HC–Pro of ZYMV. The reaction of HC–Pro with blotted CuPs was recorded for the active HC–Pro with a wild-type KLSC motif, but not for the inactive HC–Pro with the ELSC motif. Also, protein extracts from *B. tabaci*, a non-vector for potyviruses, did not bind to ZYMV HC–Pro. Amino acid sequencing of protein spots of MpPE that reacted with the HC–Pro of ZYMV showed the conserved amino acid sequence that is present in the R&R consensus (chitin-binding domain). This consensus is typical of most insect CuPs, including those of aphids (Dombrovsky *et al.*, 2007). The rate of coverage ranged from 30 to 52 % when analysed against aphid CuPs deposited in GenBank.

Identification of peptides in protein spots reacting to HC–Pro as CuPs suggests that these putative proteins are potentially involved in HC–Pro binding. Also, most proteins detected as CuPs were in the MM range 22–30 kDa (Table 1). It is of interest to note that bands of a similar size (MMs of 27 and 30 kDa) bound HC–Pro and reacted to MpCuPAb in the 1-D gels. None of the CuPs in the MpPE were identical to the CuPs deposited in GenBank. Differences in MM and pI among CuPs are to be expected, even though they share conserved parts of the proteins. It is well established that insects are composed of many different CuPs that differ in their chitin-binding domains (RR-1 and RR-2) and terminal repeats (Dombrovsky *et al.*, 2007).

These differences may arise from the variable amino acid repeats in the regions flanking the chitin-binding domain (Dombrovsky *et al.*, 2003, 2007). The finding that the CuPs obtained from the cDNA library of *M. persicae* did not react with HC–Pro may imply that Mpcp2, Mpcp3, Mpcp4 and Mpcp5 (Dombrovsky *et al.*, 2007) are not present in the stylets or that CuPs expressed in bacteria may differ in structure or composition from CuPs expressed in aphids. One may question the validity of testing whole-body extracts of aphids when the site of attachment lies in the stylets. Unfortunately, however, the size and protein content of the stylets did not allow extraction of a discernible quantity of CuPs. On the other hand, research on the cuticles of other insects revealed that the CuPs are mobilized in the body and that those found in one organ are not necessarily synthesized there (Csikos *et al.*, 1999). Therefore, knowledge about CuPs from the whole body may well also apply to those found in the stylets. Antibodies to ZYMV revealed the binding of virions to MpPE bands when applied in a mixture with active HC–Pro.

The finding that virions did not bind when applied without HC–Pro suggests that their binding was indirect (HC–Pro bound to the CuPs and virions bound to the HC–Pro). This finding supports the 'bridge hypothesis' (Govier & Kassanis, 1974; Pirone & Blanc, 1996). However, additional tests are needed to verify the indirect binding to CuPs.

In summary, the present study provides evidence for an *in vitro* association between a component of the aphid cuticle and the potyviral HC–Pro and, therefore, may suggest a potential role as a putative aphid cuticular receptor, as proposed by Harris & Harris (2001). However, in order to understand the significance of the *in vitro* binding between the HC–Pro and CuPs, there is a need for extensive biological and electron microscopic studies. Elucidation of a role for CuPs in the transmission of non-persistently transmitted viruses may shed additional light on the transmission process.

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