Investigations Into Molecular Interactions Between Potato Virus Y and *Myzus persicae*

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INTRODUCTION

One way to control the spread of aphid-borne viruses is to control their aphid vector, but this is not always effective as many aphid-borne potato viruses, including the most important ones, are transmitted non-persistently, being acquired within a very short time before agrochemicals can act. Thus in order to devise alternative approaches to control this class of viruses a better understanding of the interaction between the virus, the host plant and the aphid vector is required. In this respect a study was undertaken to identify aphid proteins that may be involved in the virus-vector interaction and some aphid cuticle proteins were identified to interact with potato virus Y helper component (HC-Pro) through screening of an aphid cDNA expression library, and their potential role in virus transmission was discussed. Additionally, virus RNA was detected by a nested RT-PCR assay within aphids that had transmitted PVY but could no longer transmit after sequential transfer to healthy plants. This result challenges the concept of short retention of non-persistent viruses inside their aphid vectors.

CONCLUSIONS

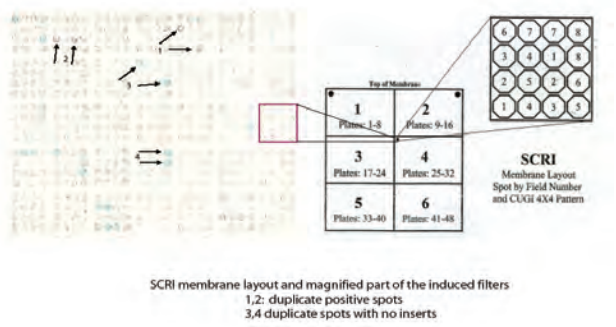
1-Library screening revealed that HC-Pro interacted with eight proteins including aphid cuticle proteins, beta-tubulin, some enzymes, and other genes of unknown function.
2-One of the cuticle proteins, clone P72F, shares a high level of similarity with other aphid CUP proteins and bioinfomatic analysis suggests that these proteins may form a multi-gene family. The amino acid sequence shows that P72F clone is different from other CUP proteins characterized before from *M. persicae*.
3. Overlay blot analysis showed that P72F interacted with HC-Pro preparations from PVY and TEV.
4. PVY was detected within the head and body of aphids that had transmitted PVY to healthy tobacco but were no longer able to transmit virus although aphids were thought to be carrying sufficient virus to initiate an infection.

5-Taken together, the observations suggest:
I. That HC-Pro can bind to several different aphid cuticle proteins.
II. This may point to the existence of more than one type of virus receptor inside aphid vector and that the virus detected was defective for transmission or was not available for transmission (possibly it could not be released because it was not accessible to aphid saliva thought to be important in virus release from stylets).

SUGGESTED FUTURE WORK

Specificity of interaction between HC-Pro and CUPs
To find the range of CUPs to which HC-Pro can bind, experiments should be expanded to other aphid vector and non-vector species.
Localization of virus receptors
The system developed by Uzest et al. (2007) can be employed to localize the interaction between aphid stylets and PVY HC-Pro. A fusion of the green fluorescent protein (GFP) to the protein sequence of the PVY HC-Pro can be constructed. The construct then can be agro-infiltrated into *N. benthamiana* leaves, then functionality of the GFP-HC-Pro fusion may be revealed by feeding aphids on the agroinfiltrated leaves then on PVY purified particles through parafilm membranes. If GFP-HC-Pro is functional for transmission, the GFP-HC-Pro binding site in the aphid stylets can be investigated by fluorescent microscopy.
Aphid feeding studies through membranes
This technique can be used to investigate whether aphids can transmit purified PVY preparations in the attogram range in the presence of active HC-Pro. This will indicate whether the infectivity of virus particles was disabled or virus particles are firmly attached to aphid CUPs. Additionally, blocking of aphid receptors can be tested by preparing antiserum against P72F protein, then blocking of aphid transmission of PVY can be tested by feeding aphids on the antibody then testing transmission efficiency compared to the controls. Another thought would be to fuse the antibody with a fluorescent tag and investigate the position of interaction by looking at the aphid stylet under the confocal microscope.

cDNA LIBRARY SCREENING

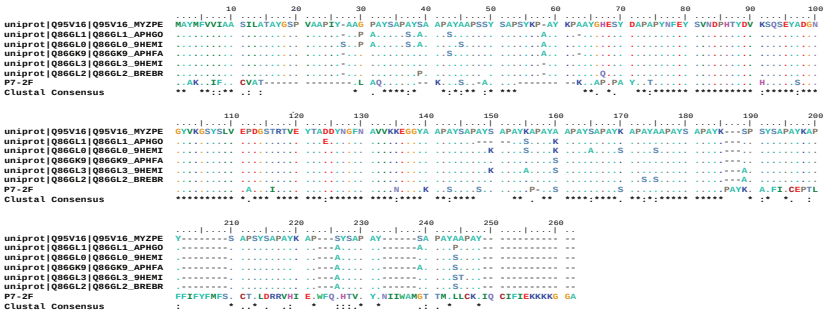


SCRI membrane layout and magnified part of the induced filters
1,2: duplicate positive spots
3,4 duplicate spots with no inserts

Each cluster contains 16 spots, representing eight clones in duplicates. Each large membrane can accommodate up to 48 Genetix 384-well plates, divided into 6 sub-areas each with clusters of clones from 8 plates. Therefore the whole library of 7680 clones can be screened on one large filter. Dark purple colour shows a reaction; blue colour shows that the clone did not contain an insert

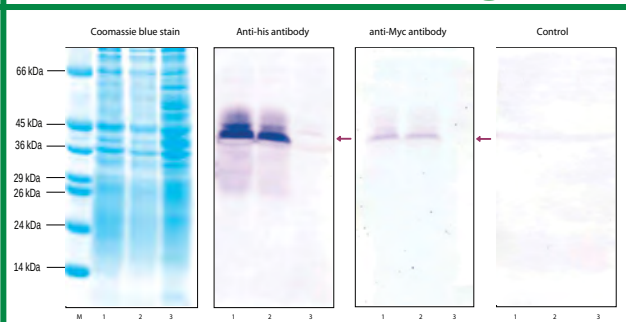
Selected clone	EST search (Nucleotide sequence)		Protein sequence search		
	EST match / CDNA accession number	EST identity	EMBL protein match/ Swiss prot accession number/Referance	Organism	identity
P72F	*M.Persicae* ES451638.1	96%	RR2 cuticle protein/ Q95V16_MYZPE (MPCP1, Dobrovsky *et al.* 2003)	*M. persicae*	91%
	M. Persicae ES450274.1	96%	RR2 cuticle protein/ Q45V97_MYZPE (MPCP3, Dombrovsky *et al.* 2007)	*M. persicae*	52%
P817P	*M. persicae* ES450783	97%	RR1 cuticle protein/ Q45V94_MYZPE (MPCP5, Dombrovsky *et al.* 2007a)	*M. persicae*	99%
	M. Persicae ES450857.1	96%			
P820P	*M. Persicae* ES450462.1	99%	Exoskeleton protein Nousiainen *et al.* (1997)	*Homarus americanus*	66%
			Full=Cuticular protein 111, RR-3 family Nousiainen *et al.* (1997)	Anopheles gambiae	72%

Using this system, eight clones were identified to interact with N-HC-Pro, and inserts were sequenced. The above table presents BLAST result searches of the NCBI database . P72F and P817P gave high matches with *M. persicae* cuticular proteins that were previously characterized and reported to interact with HC-Pro of ZYMV (Dombrovsky et al., 2007). P820P gave match with exoskeleton protein belong to other insects



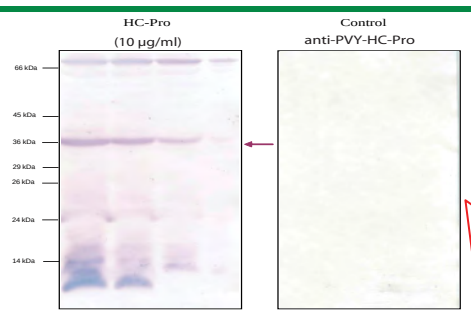
The sequence data of clone P72F were subjected to BLAST search via the NCBI website, BLASTX programme was used. The closest protein matches with clone P72F obtained from sequence database were aligned together using the ClustalW multiple alignment programme through the BioEdit program or on the EMBL website. The result indicates that there is a high level of identity among different sequences and the P72F clone on the N-terminus. However, clone P72F is different sequence at the C-terminus.

CONFIRMING INTERACTION WITH HC-PRO OF PVY AND TEV



M: Molecular size markers., 1, 2, 3 : Whole cell extracts prepared from three separate clones of P72F.

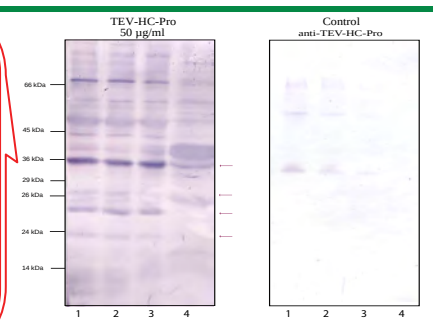
P72F clone gene was sub-cloned into the pQE-30 vector, so that on expression it could be fused to the epitope tags 6-His and c-Myc. Recombinant protein was expressed and whole cell extracts prepared. However, the molecular mass of induced proteins separated by SDS-PAGE was greater than expected from the sequence.
The detected bands were between 36 and 45 kDa. This discrepancy between the migration of proteins in SDS gels and their predicted size from amino acid sequence is a characteristic of cuticular proteins,which typically migrate 20-40% more slowly than expected (Cox and Willis, 1987).



Positions of molecular size markers were shown on the left.1, 2, 3 : inclusion bodies prepared from three separate clones of P72F. Lane 4 represents empty pQE-30 vector.

HC-Pro expressed from PVX in *N. benthamiana* (Sasaya et al., 2000) interacted with the inclusion body preparations from the induced P72F clones. Interaction was with a protein of about 36 kDa in molecular mass,which is inconsistent with the result obtained with the α-His, and α-Myc antisera. In addition, HC-Pro interacted with protein products of smaller mass (14-26 kDa), which may represent degraded protein products.

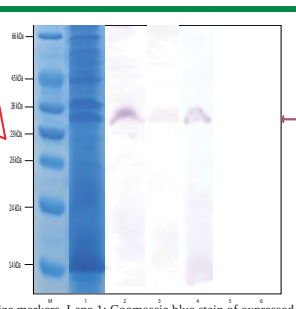
A band of about 36 kDa molecular mass interacts with HC-Pro belong to PVY or TEV. The deduced molecular mass of the cloned product of the P72F clone is approximately 20 kDa. Due to the cloning strategy additional sequence of 20 amino acids from the pSPORT vector, as well as the 6-histidine and the c-Myc tags given an estimated mass of 23.2 kDa.



Positions of molecular size markers were shown on the left.1, 2, 3 : inclusion bodies prepared from three separate clones of P72F. Lane 4 represents empty pQE-30 vector.

It was decided to use His-tagged TEV HC-Pro(Blanc et al., 1999) because of the high HC-Pro yield produced from this engineered clone. TEV HC-Pro was incubated with electroblotted aphid proteins prepared from the inclusion bodies of P72F sub-cloned in pQE-30. the result suggests that TEV HC-Pro interacts with P72F IB protein preparations at the same position as did PVY HC-Pro derived from the PVX vector. There also appears to be a reaction with smaller mass products which may be degradation products, as seen before in the interaction with PVY HC-Pro

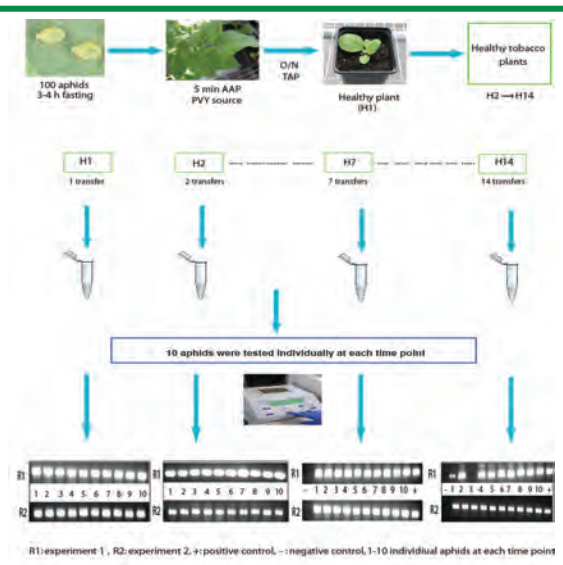
The results confirm that the band of mass of 36 kDa does interact with HC-Pro



M: Molecular size markers. Lane 1: Coomassie blue stain of expressed P72F inclusion body protein, lane 2: protein was incubated with α-His antibody, lane 3: protein was incubated with α-Myc antibody, lane 4: protein was incubated with TEV HC-Pro, lane 5: protein was incubated with α-mouse-AP only, lane 6: protein was incubated with α-TEV HC-Pro and α-rabbit-AP.

Since different bands reacted with TEV HC-Pro, a further experiment was done to confirm that P72F protein preparations react at the same position as with PVY HC-Pro. A portion of the gel was stained with Coomassie blue and directly compared with sections of the same gel electroblotted and incubated with α-His, α-Myc or TEV HC-Pro preparation followed by α-TEV HC-Pro antiserum.

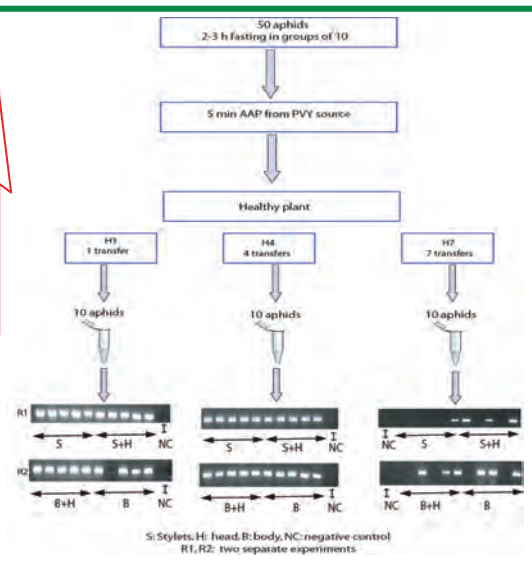
RETENTION OF PVY INSIDE APHIDS



R1: experiment 1 , R2: experiment 2, +: positive control, - : negative control, 1-10 individual aphids at each time point

The results show that virus was detectable in all 10 aphids after 7 successive transfers to healthy plants (7days). After 14 transfers (14 days), which is the longest period tested, virus was detectable in 9/10(R1) and 10/10 (R2) aphids. Using ELISA, only the first tobacco plant (H1) tested virus positive, and all the other plants (H2-H14) were found to be virus-free.

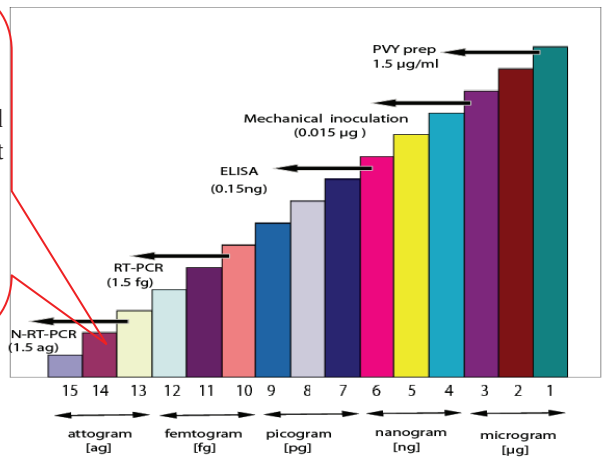
PVY was detected within the head and body of aphids that had transmitted PVY to healthy tobacco but were no longer able to transmit virus. Possibly it could not be released because it was not accessible to aphid saliva which is important in virus release from stylets.



S: Stylets, H: head, B: body, NC: negative control
R1, R2: two separate experiments

The results show that virus is retained in all parts of the insect body (stylet, head, and body), but was detected in fewer aphids after 7 transfers. This finding is contrary to the currently accepted view of non-persistent viruses being only stylet-borne. However, this result may suggest that more than one type of receptor exists in the aphid's body, but only the one located at the tip of the stylets carries transmissible virus particles.

N-RT-PCR detection limit was in the attogram range which is in agreement with the threshold reported by Moury et al. (2007) that approx. 1–3 virus particles (attogram range) were enough for an aphid to initiate aninfection with PVY



1-15 : 10 folds dilutions , start at 1.5µg and end with 0.015 ag

A 10-fold dilution series of PVY preparation (1.5 µg /µl) was tested by N-RT-PCR, RT-PCR and ELISA. And ten- fold dilutions were tested by inoculation to healthy tobacco plants. The results from two experiments revealed that the detection limit of the mechanical inoculation method is 0.015 µg /µl compared with 0.00015 µg /µl using ELISA. This means that ELISA is approx. 100 times more sensitive than mechanical inoculation. The sensitivity of detection was increased by a further factor of 10^4 times by using RT-PCR alone. The lowest detection limit was 0.015 ag/µl by using nested RT-PCR

MATERIALS AND METHODS/ LIBRARY SCREENING

A portion of the cDNA expression library described by Ramsey et al. (2007), 7680 clones constructed by Dr. B. Fenton, and colleagues (SCRI), was screened using the Q-Bot for colony printing in a completely automated process. Antisera used in this work were: Anti-6-histidine (Sigma), anti-Myc (SCRI), anti-PVY-HC-Pro (SCRI), and anti-TEV-HC-Pro (J.J. Lopez-Moya, Spain).

MATERIALS AND METHODS/ VIRUS RETENTION

For N-RT-PCR, the primers used were Singh S0, Singh AS480 (Singh et al., 1998) for the first round and Malloch F, Malloch R (Malloch, unpublished, SCRI) for the second round. For RT-PCR, Singh et al.(1996) primers were used.

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