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Interaction between potyvirus helper component-proteinase and capsid protein in infected plants

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Monoclonal antibodies were raised against helper component-proteinase (HcPro) purified from plants infected with the potyvirus *Lettuce mosaic virus* (LMV). These antibodies were used in a two-site triple antibody sandwich ELISA assay together with polyclonal antibodies directed against purified virions. An interaction between HcPro and the viral coat protein (CP) was demonstrated in extracts of LMV-infected leaves, as well as for two other potyviruses, *Plum pox virus* and *Potato virus Y*. The CP–HcPro interaction was not abolished in LMV derivatives with an HcPro GFP N-terminal fusion, or with a deletion from the CP of the amino acids involved in aphid transmission. Electron microscopy indicated that HcPro probably does not interact with the CP in the form of assembled virions or virus-like particles. Together, these results suggest that the interaction detected between CP and HcPro might be involved in a process of the potyvirus cycle different from aphid transmission.

Potyviruses are the most numerous and economically devastating plant viruses (Shukla *et al.*, 1994). Their single-stranded genomic RNA, encapsidated in flexuous virions 800 nm long, contains about 10 000 nucleotides and encodes a polyprotein processed by three proteinases to yield the mature viral proteins (for a review see Revers *et al.*, 1997). The involvement of all potyviral proteins has been shown at one stage or another of the virus cycle (Klein *et al.*, 1994). One of them, helper component-proteinase (HcPro), is especially important in this respect because it is a multifunctional protein involved in a number of processes of the virus cycle (Maia *et al.*, 1996; Revers *et al.*, 1999).

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In addition to the proteinase activity responsible for its own C-terminal processing (Carrington *et al.*, 1989), HcPro is involved in genome amplification (Klein *et al.*, 1994), short- and long-distance movement (Kasschau *et al.*, 1997; Rojas *et al.*, 1997; Kasschau & Carrington, 2001) and aphid transmission (Huet *et al.*, 1994). In addition, HcPro has been described as a pathogenicity determinant (Revers *et al.*, 1999; Redondo *et al.*, 2001), and also responsible for the synergism observed in mixed infections involving a potyvirus (Vance *et al.*, 1995). Some of these properties at least could be related to the ability of HcPro to suppress plant defence responses based on gene silencing (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau & Carrington, 1998, 2001).

HcPro acts coordinately with the capsid protein (CP) for several of these functions: aphid transmission (Blanc *et al.*, 1997) and also, less expectedly, virus movement (Rojas *et al.*, 1997). This opens the possibility that these proteins are engaged in a direct interaction. Indeed, such an interaction, demonstrated by *in vitro* overlay assay (Blanc *et al.*, 1997; Peng *et al.*, 1998; Manoussopoulos *et al.*, 2000), was correlated with aphid transmission (Blanc *et al.*, 1997). Biological analysis of mutants of *Potato virus A* also produced indirect evidence for an interaction (Andrejeva *et al.*, 1999). However, the CP–HcPro interaction has never been demonstrated *in vivo*, even using the yeast two-hybrid system (Guo *et al.*, 2001; J. Walter, personal communication). Here we report the detection of an interaction *in planta* between HcPro and CP of *Lettuce mosaic virus* (LMV) and of two other potyviruses, and discuss its implication for biological processes in which HcPro and CP are engaged.

The fusion of a polyhistidine tag to the N terminus of LMV-E HcPro was engineered in an infectious cDNA (Yang *et al.*, 1998). His-tagged HcPro from lettuce plants infected with this cDNA was purified to near-homogeneity by affinity chromatography on nickel-containing resins (Blanc *et al.*, 1997), and used to produce anti-HcPro mouse monoclonal antibodies. Two monoclonal antibodies, 3C5-10 and 2G10-13, were selected and tested further by Western blot assays for their specificity and polyvalence (data not shown). For all LMV isolates analysed, both antibodies reacted with a single protein of about 50 kDa, in agreement with the calculated molecular

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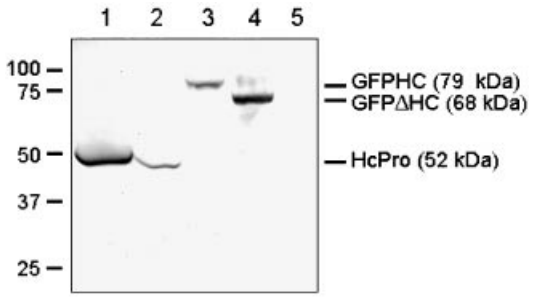

Fig. 1. Western blot detection of various natural or recombinant LMV isolates in infected lettuce leaf extracts using anti-HcPro monoclonal antibody 3C5-10. 1, LMV-0; 2, LMV-0-GFPclvHC; 3, LMV-0-GFPHC; 4, LMV-0-GFPΔHC; 5, non-inoculated plant extract. The positions of protein molecular mass markers (Benchmark, Bio-Rad) are indicated on the left, and those of every form of HcPro on the right, with their predicted molecular mass given in parentheses. See text for a description of the structure of the recombinant LMV isolates used.

mass of LMV HcPro; no reaction with any other protein, including the 31 kDa CP, could be detected (data not shown). Both monoclonal antibodies also reacted with the HcPro of *Potato virus Y* (PVY). In addition, 3C5-10 reacted with lower molecular mass material specifically present in extracts of plants infected with *Plum pox virus* (PPV) and presumably representing degraded forms of PPV HcPro. Neither of the monoclonal antibodies reacted with HcPro of *Pea seed-borne mosaic virus* (data not shown). 3C5-10 was used for further experiments unless otherwise stated.

Three recombinant forms of LMV-0, with various alterations engineered at the N terminus of their HcPro (German-Retana *et al.*, 2000; S. German-Retana, unpublished), were tested by Western blot using 3C5-10 (Fig. 1). Two of these recombinant viruses carried a GFP marker gene fused to the N terminus of HcPro either with (LMV-0-GFPclvHC) or without (LMV-0-GFPHC) an engineered NIa cleavage site added between the two proteins (S. German-Retana, unpublished). The third LMV-0 derivative had GFP fused to a truncated form of HcPro lacking the first 102 amino acids of its N terminus (LMV-0-GFPΔHC). In each extract, 3C5-10 reacted with a single protein with a size as expected for the respective engineered forms of HcPro (Fig. 1), confirming its specificity; in particular, no cross-reaction was detected with the 31 kDa CP. These results also indicate that 3C5-10 recognition does not target a site within the first 102 amino acids of HcPro, and also that it is not abolished by the presence of GFP fused to the N terminus of intact or truncated HcPro. Antibody 3C5-10 was also used to successfully detect HcPro in immunoprints (Fakhfakh *et al.*, 2001; Krause-Sakate *et al.*, 2002) or double-antibody-sandwich (DAS) ELISA (Clark & Adams, 1977) prepared with infected lettuce, as well as in Western blots after heterologous expression in *Escherichia coli*, showing that recognition was not sensitive to eukaryotic post-translational modifications (data not shown).

A two-site triple-antibody-sandwich (2S-TAS) ELISA using 3C5-10 and polyclonal rabbit antibodies raised against LMV virions (anti-CP), provided by Hervé Lot (INRA-Avignon, France), was performed on extracts of lettuce systemically infected with LMV-0. Briefly, infected leaves were homogenized in a mortar in the presence of 3 vols per weight unit of sample buffer (PBS containing 0·05 % Tween and 2 % PVP), and the extract was clarified by low speed centrifugation before being placed in the antibody-coated microtitre plates. Either 3C5-10 (2 μg/ml; similar results were obtained with 1 μg/ml) or anti-CP (1 μg/ml) were used as the primary or as the secondary antibody and revealed by adding goat anti-mouse or anti-rabbit antibodies conjugated with alkaline phosphatase (Jackson ImmunoResearch). The chromogenic reaction was performed at room temperature with *p*-nitrophenyl phosphate (Sigma) as the substrate. All other experimental conditions were performed as described for DAS-ELISA (Clark & Adams, 1977). DAS-ELISA was routinely performed on the same extracts using anti-CP antibodies and conjugate in order to evaluate the relative virus concentrations.

Extracts of infected plants produced a positive signal with either anti-CP DAS-ELISA, or a combination of either polarity of anti-CP and 3C5-10 or 2G10-13 in the 2S-TAS-ELISA assay (Table 1 and data not shown). A signal was always obtained but its intensity varied depending on particular plants and experiments (Table 1A vs Table 1B for instance). No significant signal was observed if any one of the steps of the assay was omitted (Table 1B), or if extracts of non-infected plants were used (Table 1A). No signal was detected when monoclonal antibodies raised against another LMV non-structural protein, VPg, or if polyclonal antibodies against virions of another potyvirus, PPV, or against an unrelated virus, *Grapevine chrome mosaic virus* (genus *Nepovirus*), were used in place of 3C5-10 (data not shown). 2S-TAS-ELISA gave a positive signal in plants at least 6 days and up to 60 days after inoculation (data not shown). Altogether, our results suggest the presence, in infected plant extracts, of a complex involving CP and HcPro.

In order to evaluate the general value of this observation, two other isolates of LMV (LMV-E and LMV-AF199) were similarly assayed and produced similar results (data not shown). Similar results were also obtained when two other potyviruses, PPV (strains PPV-M, PPV-D and PPV-NAT) and PVY (strain PVY-Fr), were assayed using specific antibodies prepared against their virions and the LMV HcPro-specific monoclonal antibody 3C5-10 (Table 1C). However, in contrast to the results obtained with LMV, a signal was detected only when 3C5-10 was used as the primary antibody. The basis for this difference in behaviour is unclear at the moment.

Two-site TAS-ELISA thus proved a reliable assay to reveal an *in planta* interaction between HcPro and CP in the case of the three potyviruses tested. The assay was then used in an attempt to further characterize the CP–HcPro complex, using various forms of genetically engineered LMV. Specifically, experiments were carried out using LMV-0-GFPHC and another LMV-0 derivative lacking the DAG triplet in the N-

Table 1. Interaction between CP and HcPro assessed by 2S-TAS-ELISA using the 3C5-10 anti-HcPro monoclonal antibody and anti-CP polyclonal antibodies

Absorbance readings (A_{405}) in three different ELISA experiments are given. Ab1, primary antibody; Ab2, secondary antibody; n.i., not inoculated. (A) 2S-TAS-ELISA A_{405} values obtained using extracts of lettuce leaves infected with LMV-0. Healthy plants were assayed as controls, and DAS-ELISA was performed in parallel using anti-CP. The average A_{405} value for four independent plants tested in this particular experiment is given, as well as the standard deviation (SD). (B) 2S-TAS-ELISA A_{405} values obtained using LMV-0 infected leaf extracts, omitting separate steps in the 2S-TAS-ELISAS procedure. Five independent plants were assayed in this particular experiment, and the mean and standard deviation of the A_{405} values are shown. (C) 2S-TAS-ELISA A_{405} values obtained using extracts of plants infected with *Plum pox virus* [PPV; in *Nicotiana benthamiana* or in peach (*Prunus persica*)] or *Potatoe virus Y* [PVY; in pepper (*Capsicum annuum*)]. PPV-D and PPV-M isolates were tested (Kerlan & Dunez, 1979) as well as the non-aphid transmitted PPV-Nat (Maiss *et al.*, 1989). The antibodies used were homologous anti-CP and 3C5-10, specific for LMV HcPro. DAS-ELISA was used for the anti-CP/anti-CP combination.

(A)

	(2S-TAS-ELISA)				(DAS-ELISA)	
Ab1	anti-CP		3C5-10		anti-CP	
Ab2	3C5-10		anti-CP		anti-CP	
Inoculum	n.i.	LMV-0	n.i.	LMV-0	n.i.	LMV-0
Plant 1	0.083	0.917	0.056	0.464	0.037	1.386
Plant 2	0.074	2.876	0.033	1.252	0.018	1.954
Plant 3	0.050	1.900	0.068	1.339	0.057	1.654
Plant 4	0.045	2.256	0.053	0.675	0.042	0.874
Mean	0.063	1.987	0.053	0.933	0.039	1.467
SD	0.018	0.820	0.015	0.429	0.016	0.458

(B)

		Ab1	–	–	+	+	+	+
		LMV-0	–	+	–	+	+	+
		Ab2	–	+	+	–	+	+
Ab1	Ab2	Conjugate	–	+	+	+	–	+
anti-CP	3C5-10	Plant 1	0.011	0.161	0.090	0.065	0.004	0.929
		Plant 2	0.016	0.088	0.081	0.042	0.004	1.152
		Plant 3	–0.016	0.062	0.082	0.032	–0.007	0.714
		Plant 4	–0.005	0.047	0.069	0.032	0.000	0.494
		Plant 5	–0.008	0.065	0.042	0.018	0.015	1.121
		Mean	0.000	0.085	0.073	0.038	0.004	0.882
		SD	0.013	0.045	0.019	0.017	0.008	0.279
3C5-10	anti-CP	Plant 1	0.002	0.164	0.109	0.134	–0.002	0.547
		Plant 2	0.000	0.070	0.077	0.044	–0.003	0.662
		Plant 3	0.001	0.091	0.100	0.064	–0.004	0.278
		Plant 4	0.000	0.071	0.085	0.044	–0.004	0.162
		Plant 5	–0.001	0.086	0.079	0.064	–0.006	0.193
		Mean	0.000	0.096	0.090	0.070	–0.004	0.368
		SD	0.001	0.039	0.014	0.037	0.001	0.223

(C)

		Nicotiana benthamiana		*Prunus persica*			*Capsicum annuum*	
Ab1	Ab2	n.i.	PPV-D	n.i.	PPV-NAT	PPV-M	n.i.	PVY-Fr
anti-CP	anti-CP	0.012	0.923	–0.008	0.624	0.704	0.005	2.422
anti-CP	3C5-10	0.036	0.037	0.044	0.037	0.041	0.003	–0.006
3C5-10	anti-CP	0.065	1.433	0.091	1.172	1.673	–0.135	1.264

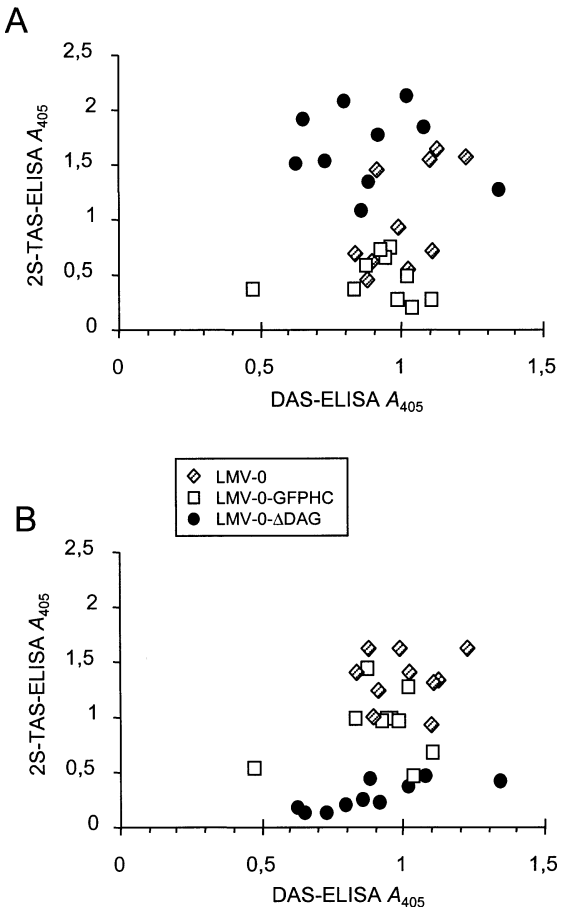

Fig. 2. CP–HcPro interaction in wild-type or mutant forms of LMV-0. Extracts of ten independent plants (two separate experiments involving five plants each) inoculated with various derivatives of LMV-0 were subjected to 2S-TAS-ELISA and DAS-ELISA. Individual 2S-TAS-ELISA A_{405} values (*y*-ordinate), illustrating the interaction strength, were plotted against those obtained in anti-CP DAS-ELISA (*x*-ordinate), illustrating the virus concentration in each individual extract. A, 3C5-10 was used as the primary antibody and anti-CP as the secondary antibody; B, anti-CP was used as the primary antibody and 3C5-10 as the secondary antibody. The extracts analysed were from plants inoculated with LMV-0 (diamonds), LMV-0-GFPHC (squares) or LMV-0ΔDAG (circles). Non-inoculated plants (not illustrated) yielded A_{405} values ranging from −0·013 to 0·019 in DAS-ELISA (average −0·001), from 0·016 to 0·088 (average 0·059) in 2S-TAS-ELISA using 3C5-10 as the primary antibody and from −0·009 to 0·022 (average 0·008) in 2S-TAS-ELISA using anti-CP as the primary antibody; in no case did these ranges overlap with those from inoculated plants.

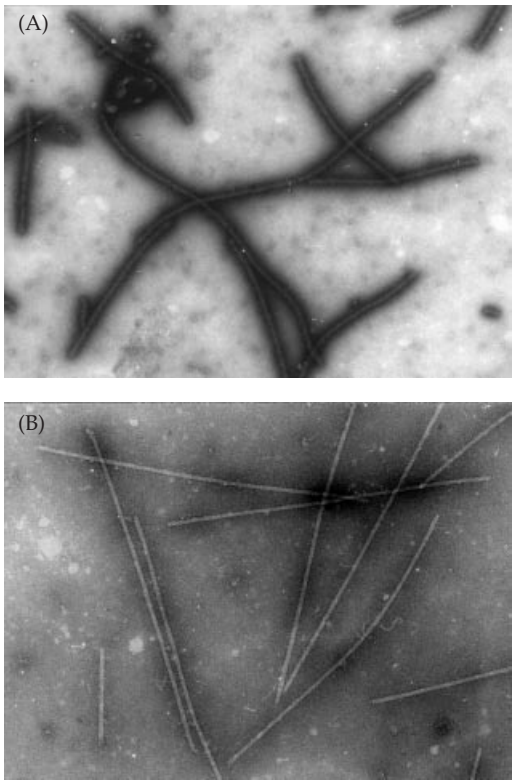

Fig. 3. Electron microscope observation of LMV-0 particles trapped with anti-CP. (A) anti-CP as the secondary antibody; (B) 3C5-10 as the secondary antibody.

terminal region of the CP (LMV-0ΔDAG; S. German-Retana, unpublished). The effect of these modifications on virus accumulation was undetectable in a anti-CP-based DAS-ELISA (Fig. 2).

When 3C5-10 was used as the primary antibody, the HcPro interaction was not significantly different in intensity for LMV-0ΔDAG and wild-type LMV-0 (Fig. 2A). However, the use of anti-CP as the primary antibody resulted in a weaker but still significant signal for LMV-0ΔDAG (Fig. 2B). Although we have no explanation for the observation that this difference in signal intensity was only observed for this particular construct, it suggests an involvement of the DAG triplet in the interaction, as described in an *in vitro* approach for another potyvirus (Blanc *et al.*, 1997). However, this requirement does not seem to be absolute because the interaction signal was not completely abolished.

In contrast, the CP–HcPro interaction was only slightly attenuated in LMV-0-GFPHC (Fig. 2), suggesting that the presence of exogenous sequences at the N terminus of HcPro only marginally affects the ability of HcPro to interact with CP. Interaction was also observed in the case of a similar construct obtained from the LMV-E isolate, LMV-E-GFPHC (data not shown).

The 2S-TAS-ELISA scheme was transferred to an immunosorbent electron microscopy (ISEM) format in an attempt to visualize any structure that could be related to the CP–HcPro interaction. The same extraction buffer and protocol as for 2S-TAS-ELISA were used, and a control 2S-TAS-ELISA assay was performed in parallel to the ISEM experiments. Anti-CP very efficiently trapped LMV virions, which could be further decorated using the same antibody (Fig. 3A). However, no decoration of the virus particles was apparent when 3C5-10 was used as the secondary antibody (Fig. 3B). Conversely, when 3C5-10 was used as the primary antibody, the density of virus particles per surface unit was not distinguishable from the

background obtained when omitting the deposition of a primary antibody (data not shown). Although it is clearly possible that the structure leading to the 2S-TAS-ELISA signal is unstable at the surface of electron microscopy grids, these results suggest that the CP–HcPro interaction does not take place in virus-like structures. The absence of decoration of virions by 3C5-10 would be compatible with a scattered or localized repartition of HcPro subunits at the surface of virions, but then virus particles should have been trapped by 3C5-10 when it was used as the primary antibody. Thus, the CP–HcPro interaction is probably materialized in another structure. The data currently available do not allow us to establish whether this alternative structure consists of monomers, oligomers, or polymers of CP and HcPro.

In conclusion, the results presented here provide evidence for a direct physical interaction between CP and HcPro in extracts of plants infected with LMV, but also with two other potyviruses tested. Thus, this phenomenon probably reflects a general property of potyviruses, rather than a specificity of the LMV–lettuce system. 2S-TAS-ELISA is simple, reliable and at least partially quantitative, and thus provides a useful tool to study this interaction directly in plant extracts, especially when coupled with the use of genetically engineered viruses.

The CP–HcPro interaction we report could be involved in aphid transmission, like the one shown *in vitro* (Blanc *et al.*, 1997). Alternatively, but not exclusively, this interaction could be related to other biological functions of HcPro and CP. The residual level of interaction after deletion of the DAG triplet in the CP N terminus argues for the second hypothesis because this triplet is necessary for aphid transmission in several potyviruses (Atreya *et al.*, 1991; Blanc *et al.*, 1997). This is also supported by the detection of an interaction in LMV-E-GFPHC, a mutant in which GFP is fused to the N terminus of the HcPro, which was previously shown to have lost aphid transmissibility (German-Retana *et al.*, 2000). Lastly, we also detected an interaction in plants infected with the aphid non-transmissible PPV-NAT, which has a natural deletion of 15 amino acids, including the DAG triplet in the N terminus of the CP (Maiss *et al.*, 1989) (Table 1C). Altogether, these data are compatible with a relationship between the CP–HcPro interaction and a biological trait other than aphid transmission, such as short- and/or long-distance virus movement in the plant. More investigations are needed, however, in order to understand how CP and HcPro interact with each other, and the biological role of this interaction in the potyvirus cycle.

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