

The central and C-terminal domains of VPg of *Clover yellow vein virus* are important for VPg–HCPPro and VPg–VPg interactions

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Interactions between the major proteins of *Clover yellow vein virus* (CIYVV) were investigated using a GAL4 transcription activator-based yeast two-hybrid system (YTHS). Self-interactions manifested by VPg and HCPPro and an interaction between NIb and NIaPro were observed in CIYVV. In addition, a strong HCPPro–VPg interaction was detected by both YTHS and by *in vitro* far-Western blot analysis in CIYVV. A potyvirus HCPPro–VPg interaction has not been reported previously. Using YTHS, domains in CIYVV for the VPg self-interaction and the HCPPro–VPg interaction were mapped. The VPg C-terminal region (38 amino acids) was important for the VPg–VPg interaction and the central 19 amino acids were needed for the HCPPro–VPg interaction.

INTRODUCTION

Clover yellow vein virus (CIYVV) is a member of the genus *Potyvirus* and has a single-stranded, positive-sense RNA genome of about 10 kb (Takahashi *et al.*, 1997). Mature functional proteins (P1, HCPPro, P3, 6K1, CI, 6K2, VPg, NIaPro, NIb and CP) are generated by viral polyprotein processing by three virus-encoded proteases (P1, HCPPro and NIa) (Riechmann *et al.*, 1992). Although substantial information has been reported on the similarity of the interactions between potyviral proteins, differences are also observed within members of this genus.

Most potyviral proteins are multifunctional. For example, HCPPro is involved in aphid transmission (Atreya *et al.*, 1990), genome replication (Klein *et al.*, 1994; Kasschau *et al.*, 1997), long-distance movement (Cronin *et al.*, 1995; Kasschau *et al.*, 1997; Saenz *et al.*, 2002) and autoproteolysis (Carrington *et al.*, 1989). In addition, it has been demonstrated that HCPPro has plasmodesmatal gating (Rojas *et al.*, 1997) and nucleic acid binding properties (Maia & Bernardi, 1996). Recently HCPPro has been identified as a suppressor of post-transcriptional gene silencing (reviewed by Marathe *et al.*, 2000; Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau & Carrington, 1998).

VPg is also multifunctional and needed for virus replication (Shahabuddin *et al.*, 1988; Murphy *et al.*, 1990, 1991, 1996). It is also needed for virus cell-to-cell and long-distance movement (Nicolas *et al.*, 1997; Schaad *et al.*, 1997; Keller *et al.*, 1998; Rajamäki & Valkonen, 2002). It contains a

nuclear localization signal that is important for virus replication (Schaad *et al.*, 1996) and a sequence-non-specific RNA-binding domain (Merits *et al.*, 1998). Furthermore, the plant cap-binding proteins eIF4E and eIF(iso)4E have been found to bind the VPg of potyviruses (Wittmann *et al.*, 1997; Leonard *et al.*, 2000; Schaad *et al.*, 2000). This interaction was found to be important for the replication of the *Turnip mosaic virus* (TuMV), *Lettuce mosaic virus* (LMV), *Potato virus Y* (PVY) and *Tobacco etch virus* (TEV) in *Arabidopsis thaliana* and pepper (Duprat *et al.*, 2002; Lellis *et al.*, 2002; Ruffel *et al.*, 2002).

Protein–protein interactions play important roles in the virus infection cycle. Several interactions between potyviral proteins have been studied using the yeast two-hybrid system (YTHS) (Guo *et al.*, 2001). However, the reported interactions between major proteins are not necessarily common to all potyviruses, and there are many inconsistent observations among the potyviruses that have been analysed by YTHS (Hong *et al.*, 1995; Li *et al.*, 1997; Merits *et al.*, 1999; Urcuqui-Inchima *et al.*, 1999; Choi *et al.*, 2000; Guo *et al.*, 2001; Lopez *et al.*, 2001). In this report, we describe a novel protein–protein interaction between HCPPro and VPg in CIYVV that has not been reported in any other potyvirus, suggesting that the HCPPro–VPg interaction participates in some function unique to CIYVV. Since the reported results for protein–protein interactions of other potyviruses are not always applicable to CIYVV, the present work will serve as a molecular basis for further characterization of CIYVV proteins.

METHODS

Viruses and plants. CIYVV no. 30 is maintained as a clone, pCIYVV, in our laboratory (Takahashi *et al.*, 1997). It was originally isolated from French bean (*Phaseolus vulgaris*) (Uyeda *et al.*, 1975) and induces lethal necrosis on broad bean (*Vicia faba*). The infected tissues used in this paper were all prepared from infected broad beans.

Yeast two-hybrid system and plasmid construction. Yeast two-hybrid assays were carried out according to the MATCHMAKER Two-Hybrid System 3 protocol (Clontech). The yeast (*Saccharomyces cerevisiae*) strain AH109 was used to determine protein–protein interactions. The DNA binding domain (BD) vector pAS2-1 and the activation domain (AD) vector pACT2 were used throughout. HCPro cDNA was inserted in-frame into the BD between the *Sma*I and *Sal*I sites of pAS2-1 and in-frame into the AD between the *Bam*HI and *Sac*I sites of pACT2. Wild-type VPg cDNA and its deletion clones were inserted in-frame into the BD between the *Eco*RI and *Bam*HI sites of pAS2-1, or into the *Bam*HI and *Eco*RI sites of pACT2. PCR primers were designed to add the required restriction endonuclease recognition sequences so that the PCR products could be cloned in-frame into the AD or BD gene. Recombinant plasmids were transformed into *E. coli* strain JM109. Plasmid DNA was isolated by Quantum Prep Plasmid Miniprep Kit (Bio-Rad). Competent cells of *S. cerevisiae* strain AH109 were transformed simultaneously with pACT2 and pAS2-1 recombinant DNAs by the lithium acetate method (Gietz *et al.*, 1995). Yeast cells were plated on a high-stringency selective medium without tryptophan, leucine, histidine and adenine, and their β -galactosidase activities were tested at 30 °C on the same selective media containing X- α -Gal. Yeast cells co-transformed with pAS2-1 and pACT2 without inserts were used as a negative control, whereas those co-transformed with pTAD1-1 [AD simian virus 40 (SV40) large T antigen] and pVA3-1 (BD mouse p53) were used as a positive control.

Anti-VPg polyclonal antibody production. Due to the insolubility of the full-length VPg expressed in *E. coli*, the 5' (aa 1–100) and 3' (aa 153–191) halves of the VPg gene were separately cloned into the pGEX vector (Amersham) to produce glutathione S-transferase (GST)–VPg fusion proteins in *E. coli* JM109. The former protein was designated VPgN and the latter VPgC. Each purified fusion protein was used to immunize BALB/c mice.

Anti-HCPro monoclonal antibody production. The entire HCPro gene was cloned into pMAL-c2 (New England Biolabs) to express the maltose-binding protein (MBP)–HCPro fusion protein in *E. coli* JM109. The fusion protein was purified with an MBP affinity column and used to produce a monoclonal antibody against HCPro, essentially as described by Inoue *et al.* (1984).

Western blot analysis. Total proteins were prepared from either the leaves or stem tissues of infected plants. Tissues were homogenized in denaturing buffer containing 2% SDS and 5% β -mercaptoethanol (Laemmli, 1970) or in PBS. The extracts were boiled for 5 min and centrifuged to collect the supernatants. Equal amounts of protein (~10 μ g) were separated by SDS-PAGE. Proteins were then transferred to nitrocellulose membranes (Amersham Pharmacia Biotech) and the blots were probed with anti-HCPro monoclonal antibody. Proteins were visualized using anti-mouse secondary antibodies conjugated to alkaline phosphatase, followed by treatment with NBT/BCIP.

Extraction of yeast proteins. Yeast cells were grown in appropriate selective synthetic medium SD (Difco) to reach the mid-exponential phase (OD_{600} = 0.6–0.8). Total proteins from yeast were prepared as described in the *Yeast Protocols Handbook* (Clontech). Yeast cells were lysed with glass beads (425–600 μ m) in cracking buffer (8 M urea, 5% SDS, 40 mM Tris/HCl, pH 8.8, 0.1 mM EDTA and 0.4 mg bromophenol blue ml^{-1}) supplemented with β -mercaptoethanol and

protease inhibitors. Protein samples (10–20 μ g) were fractionated by SDS-PAGE through a 12.5% gel, transferred to nitrocellulose membranes and subjected to immunoblot analysis. The membranes were probed with antibodies against GAL4 AD or BD (1:200 dilution; Santa Cruz Biotechnology) and then with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:250 dilution; Bio-Rad).

Far-Western blot analysis. The VPg–HCPro interaction was further examined *in vitro* by far-Western blotting. Two different probes, GST–VPg (VPgN + VPgC) and MBP–HCPro, were used. Both probes were produced in *E. coli* JM109. Bacterially expressed proteins and total protein extracts from CIYVV-infected or healthy stem tissues were separated by SDS-PAGE and the fractionated proteins were then transferred on to nitrocellulose membranes. Alternatively, bacterially expressed proteins were directly dot-blotted on to membranes. The blots were first incubated in binding buffer (0.2 M PBS) containing *E. coli*-produced probe proteins (GST–VPg or GST only and MBP–HCPro or MBP only). After being probed with these proteins, the membranes were extensively washed in PBS and treated with specific antibodies (anti-GST–VPg polyclonal antibodies or anti-HCPro monoclonal antibody). All the membranes were treated with alkaline phosphatase-conjugated anti-mouse IgG (Bio-Rad) and finally immersed in the substrate solution containing BCIP/NBT as described above.

RESULTS

Interactions between CIYVV proteins

Interactions between CIYVV-encoded proteins (P1, HCPro, P3, CI, VPg, NIaPro, NIB and CP) were examined using YTHS. Yeast strain AH109 has three reporter genes, *HIS3*, *ADE2* and *lacZ*, under the control of the promoter sequences from the *GAL1*, *GAL2* and *MEL1* genes, respectively. The use of three reporters eliminates false positives since artificial interactions are not able to activate all of the promoter sequences. To acquire an initial understanding of the interrelationships among CIYVV-encoded proteins, we cloned each of the eight ORFs into both the pACT2 and pAS2-1 plasmids so that each protein could be expressed as a fusion protein with either the GAL4 AD or the DNA BD. AH109 yeast cells transformed with the recombinant pAS2-1 DNAs carrying viral genes and the empty pACT2 vector were first tested for self-activation on the selective medium lacking tryptophan, leucine, histidine and adenine. Yeast cells did not grow on the synthetic medium in any of the control experiments, which were observed for up to 5 days. The same result was obtained when the reciprocal combination of the empty pAS2-1 vector and the recombinant pACT2 DNAs carrying viral genes was tested. Among the different genes evaluated for protein–protein interactions, strong positive interactions were observed between HCPro and HCPro, VPg and Vpg, NIaPro and NIB, and VPg and HCPro (Table 1). While the first three interactions have been found previously in potyviruses (Thornbury *et al.*, 1985; Li *et al.*, 1997; Wang & Pirone, 1999; Guo *et al.*, 1999, 2001; Oruetxebarria *et al.*, 2001), the VPg–HCPro interaction has not previously been described in a potyvirus. Dimerization of HCPro was further indicated by Western blot analysis (Fig. 1) using a monoclonal antibody against HCPro. In addition to the 50 kDa monomer band, a distinct 100 kDa

Table 1. Protein–protein interactions between CIYVV proteins in the yeast two-hybrid system

Binding domain (BD)	Activation domain (AD)					
	HCPro	VPg	NIaPro	NIb	SV40 large T antigen	pACT2
HCPro	++	+	–	–	NT	–
VPg	++	++	–	–	NT	–
NIaPro	–	–	–	–	NT	–
NIb	–	–	++	–	NT	–
pAS2-1	–	–	–	–	NT	–
Murine p53	NT	NT	NT	NT	++	NT

++ , Colonies turned blue within 2 h; + , colonies turned blue between 2 and 6 h; – , no colonies appeared on growth selection medium; NT, not tested.

band was detected, suggesting the existence of strong HCPro dimerization. However, in the present study we cannot exclude the possibility that HCPro could form a heterodimer with either a viral or host protein.

Identification of the domain of VPg involved in the VPg–HCPro interaction

To define the protein domains involved in this interaction, a series of deletions in VPg were made. All truncated forms

of VPg, in either BD or AD, were again evaluated for autoactivation of the marker genes in yeast. When transformed with a deletion mutant and an empty vector in reciprocal combinations, no growth was observed in the selective medium. The truncated VPgs, as well as the full-length HCPro expressed from either the BD or AD, were all tested by Western blot analysis to confirm stable expression of those proteins in yeast (Fig. 2). The nature of the deletion mutant constructs is shown in Fig. 3. Fifty and eighty amino acids from the N terminus of VPg were deleted, creating BD 51–191 and BD 81–191, respectively. The constructs containing the N-terminal domain (BD 1–99) and the central domain (BD 77–153) were also prepared. AH109 yeast cells transformed with these plasmids and a plasmid containing the full-length HCPro (either in BD or AD) turned blue when transferred on to a medium containing X- α -Gal, whereas cells transformed with BD 1–80 and AD HCPro did not grow. These results indicate that a short peptide between positions 80 and 99 (19 amino acids) is important for VPg–HCPro interaction (Fig. 3).

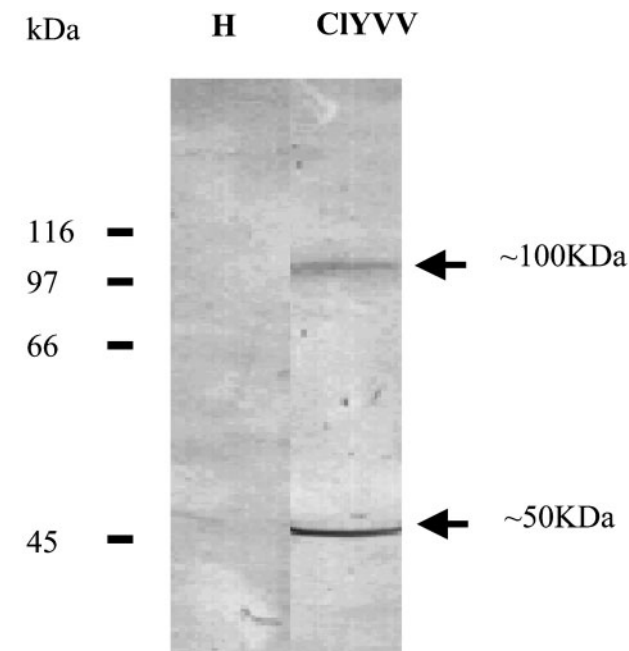


Fig. 1. Detection of HCPro isolated from infected tissues using an anti-HCPro monoclonal antibody. Proteins were extracted in PBS and separated by SDS-PAGE. Two bands of 50 and 100 kDa were detected in CIYVV-infected leaves of broad beans after extraction in PBS. H, healthy leaves; CIYVV, upper systemic leaves infected with CIYVV. The positions of molecular mass markers are indicated on the left.

In vitro interaction of VPg and HCPro

As described above, the CIYVV VPg was shown to interact with HCPro using the YTHS, and the VPg domain important for the VPg–HCPro interaction was also mapped by the same method. The interaction was further examined *in vitro* by far-Western blots. As shown in Fig. 4(A), the MBP–HCPro fusion protein corresponding to 92 kDa (left membrane, uppermost arrow) was clearly detected, but MBP alone was not detectable (left membrane), indicating specific positive interactions between MBP–HCPro and GST–VPg (VPgN + VPgC). The lower faint bands may be degradation products of MBP–HCPro. In the same membrane, total protein extracts from CIYVV-infected tissues containing VPg and HCPro were also analysed. Two bands corresponding to the molecular masses of HCPro (50 kDa, upper arrow) and VPg (22 kDa) were visualized. No bands were found from total proteins from the healthy tissues.

The reverse direction for the far-Western analysis was also

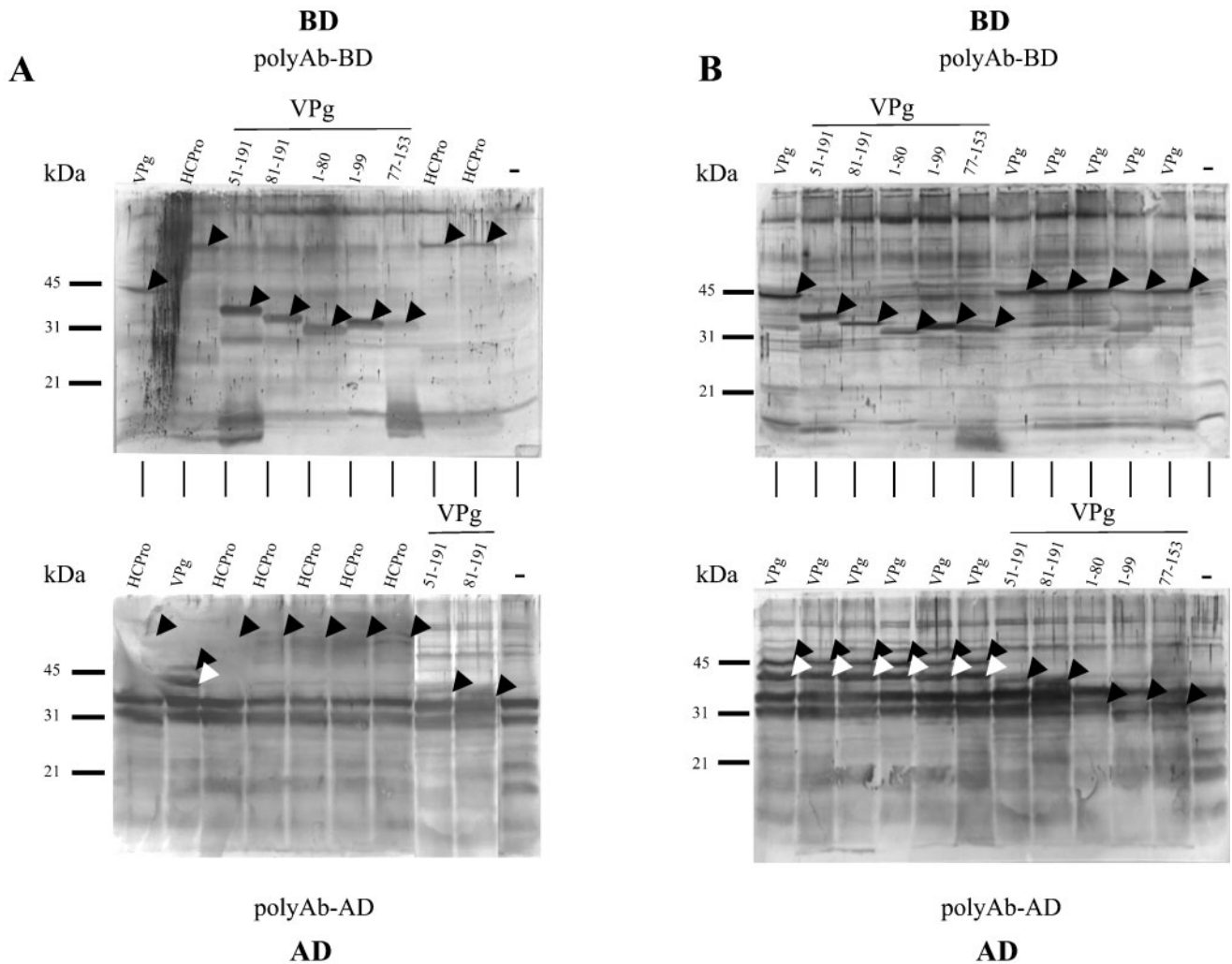


Fig. 2. Western blot analysis of CIYVV protein expression in yeast cells. Total protein was prepared from yeast transformed with the plasmids indicated above the lanes and from non-transformed yeast (-). (A) Proteins extracted from VPg-HCPro interactions as shown in Fig. 3. (B) Representative proteins extracted from VPg-VPg interactions as shown in Fig. 4. After SDS-PAGE and transfer to a nitrocellulose membrane, the fusion proteins were detected by immunoreaction with polyclonal antibodies against the BD (upper panels) and AD (lower panels). The positions of the proteins expected from the respective plasmids are indicated by arrows. The sizes are shown on the left. A white arrow indicates a possible processed protein from the AD VPg.

determined, using the *E. coli*-expressed MBP-HCPro as a probe (Fig. 4B). When GST-VPg (VPgN+VPgC) was separated by SDS-PAGE, anti-GST-VPg antibodies reacted very weakly to GST-VPg, although they had originally been used to raise the antibodies. Instead, using a dot-blot far-Western assay, it was shown that GST-VPg could interact with anti-GST-VPg antibodies (Fig. 4B, right membrane). The blotted GST-VPgN reacted to the overlaid MBP-HCPro (left membrane, arrow), whereas there was no signal on the blotted GST-VPgC or GST only. No positive signal was found with MBP (middle membrane) suggesting that MBP did not bind to either GST or VPg. These observations are consistent with the domain identified by the YTHS, indicating that the domain of aa 80-99 of VPg (also within GST-VPgN) is important for the VPg-HCPro interaction.

However, our *in vitro* far-Western blot experiments do not exclude the possibility that the C-terminal half may also contribute to the protein-protein interaction *in planta*.

Identification of the domain of VPg involved in its self-interaction

We also analysed the VPg domain(s) involved in its self-interaction. A series of VPg deletion mutants from both the BD and AD vectors were made (Fig. 5). When the C terminus of VPg was deleted, no growth was observed on the selective medium. To eliminate the possibility that the lack of interaction was due to instability of the proteins expressed in the yeast cells, Western blot analysis was performed (Fig. 2). Comparison of the results obtained

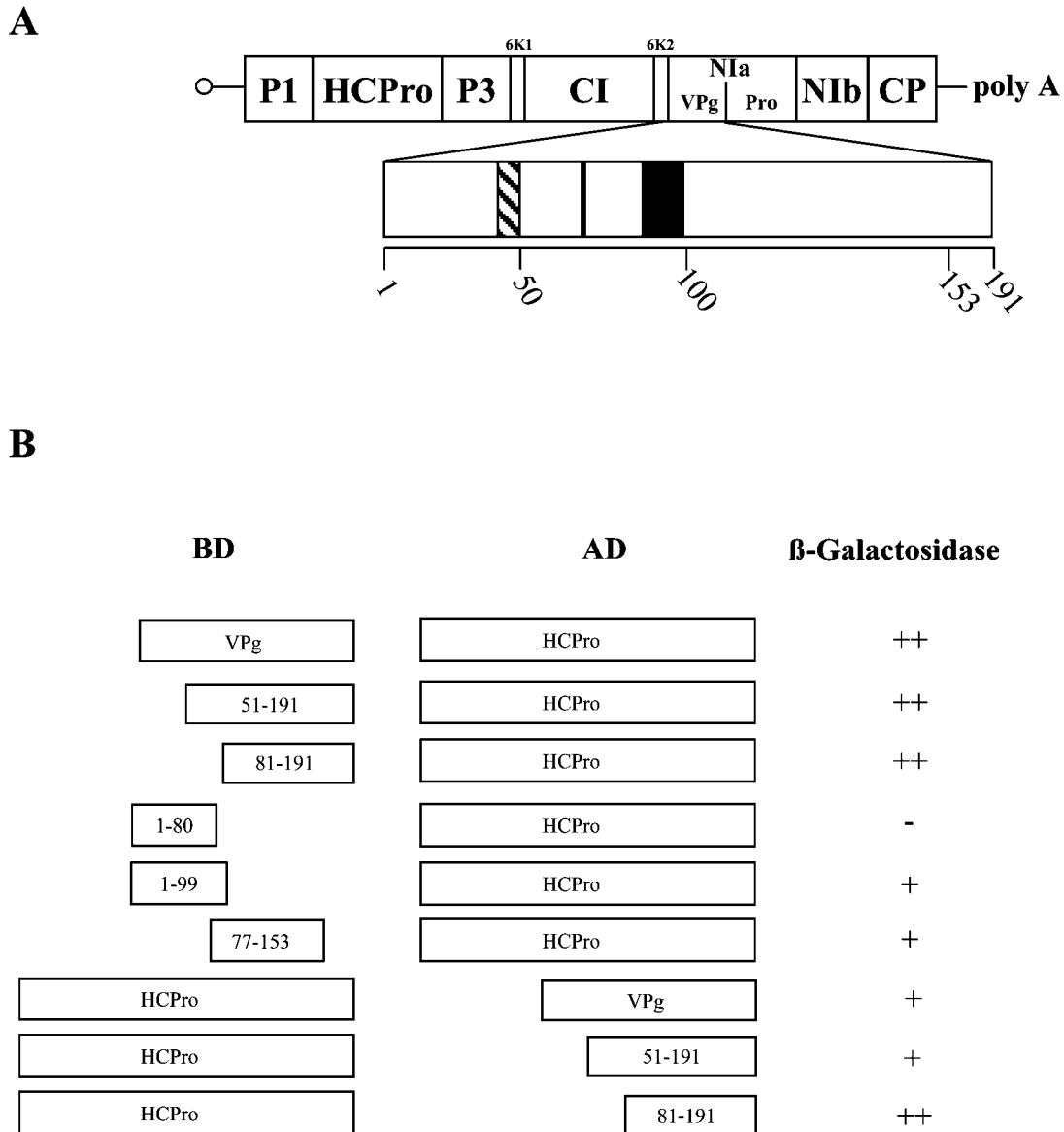


Fig. 3. Estimation of the domain sequence responsible for the VPg–HCPro interaction using the yeast two-hybrid system. (A) Genome organization of CIYVW. Some functional domains of VPg are indicated based on TEV and PVA research results (Schaad *et al.*, 1997; Rajamäki & Valkonen, 2002). The nuclear localization signal and genome amplification are shown by a hatched box; the tyrosine residue bound to the viral 5' end is shown by a vertical line; and the region involved in long-distance movement is shown by a black box. (B) Protein–protein interactions between VPg deletion constructs and HCPro. ++, Colonies turned blue within 2 h; +, colonies turned blue between 2 and 6 h; –, no colonies appeared on the selective medium. Numbers inside the boxes are the amino acid positions relative to the intact VPg.

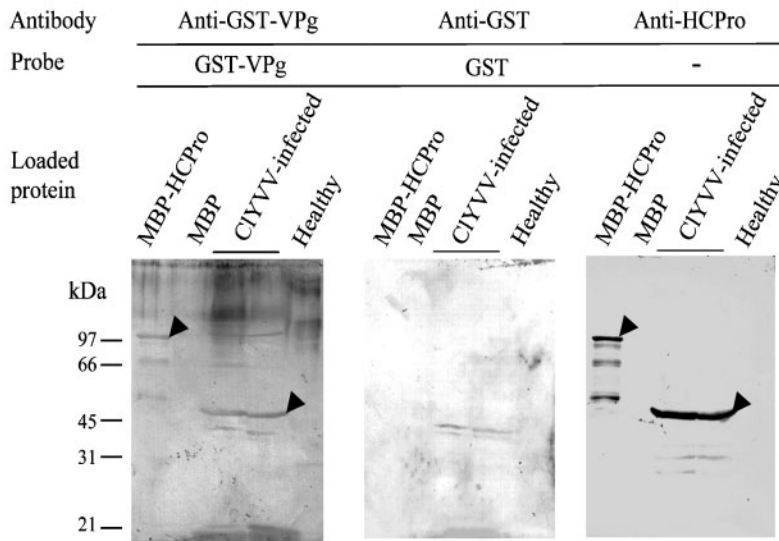
from BD 51–191 and BD 81–191 with BD 77–153 and BD 50–153 suggested that the 38 C-terminal amino acids (residues 153–191) are important for VPg self-interaction (Fig. 5). Furthermore, the combination of BD 81–191 and AD 81–191 showed a positive interaction (Fig. 5). The interaction was stronger in both directions when the combination of the region from residues 51 to 191 and the full-length VPg was used. The structural requirement for

VPg self-interaction probably favours the entire VPg, although the C-terminal region is indispensable.

DISCUSSION

Protein–protein interactions between different genes from the genera *Potyvirus* and *Tritimovirus*, members of the *Potyviridae* family, is well documented (Hong *et al.*, 1995; Li

A



B

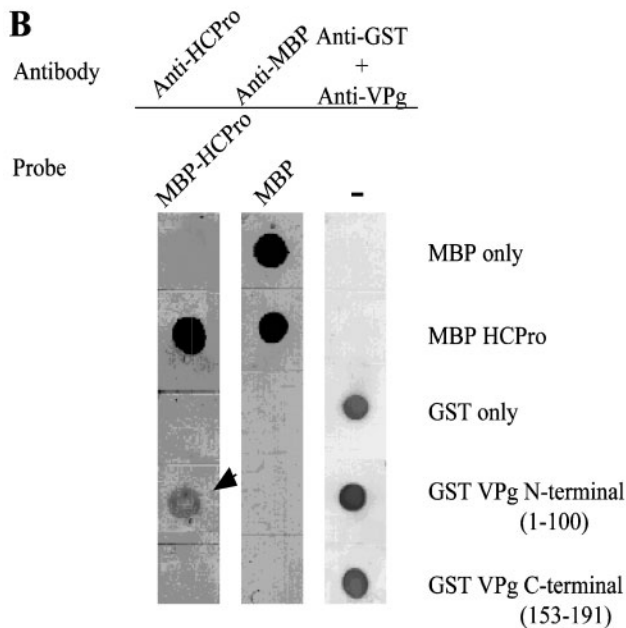


Fig. 4. Far-Western blot analysis of the VPg–HCPro interaction. (A) Detection of HCPro using GST–VPg (GST–VPgN+GST–VPgC) as probes. Samples were first separated by SDS-PAGE and transferred to nitrocellulose membranes. Three duplicate membranes were prepared. The left membrane was first overlaid with GST–VPgN+GST–VPgC, then incubated with anti-GST–VPg antibodies. The middle membrane was first overlaid with MBP and then incubated with anti-MBP antibodies. The right membrane was directly incubated with an anti-HCPro monoclonal antibody. The positions of molecular mass markers are indicated on the left. The molecular masses are: MBP–HCPro, 92 kDa; HCPro, 50 kDa; VPg, 22 kDa. (B) Detection of GST–VPg using MBP–HCPro as a probe. *E. coli*-produced proteins (indicated on the right) were first dot-blotted on to nitrocellulose membranes. The left membrane was probed with MBP–HCPro, the middle with MBP and right was directly incubated with anti-GST–VPg antibodies. The arrow indicates an interaction between VPg and HCPro.

et al., 1997; Guo *et al.*, 1999; Merits *et al.*, 1999; Urcuqui-Inchima *et al.*, 1999; Choi *et al.*, 2000; Lopez *et al.*, 2001; Oruetebarria *et al.*, 2001). However, there has been little information on the virus-encoded proteins of the potyvirus CIYVV. We are currently characterizing the functions of each protein of CIYVV. Although similarities in the sequences within the *Potyvirus* genus are not necessarily high, some conserved sequences between members of this group have been reported. The conserved proteins should have similar functions in common within this genus. In this study, we found interactions between HCPro and HCPro, VPg and VPg, and NIaPro and Nib. The HCPro–HCPro interaction was initially reported in TEV by Thornbury *et al.*

(1985) using biochemical methods. Using YTHS, HCPro self-interactions have also been reported in *Potato virus A* (PVA), *Pea seed-borne mosaic virus* (PSbMV) and LMV (Guo *et al.*, 1999, 2001; Urcuqui-Inchima *et al.*, 1999). Two different domains were found to be important for self-interaction. For PVY and LMV, the N terminus is important, while both the N and C termini are important for PVA (Guo *et al.*, 1999; Urcuqui-Inchima *et al.*, 1999). VPg self-interaction was first shown in PVA (Oruetebarria *et al.*, 2001) and then in PSbMV (Guo *et al.*, 2001). The NIB protein has been reported to interact with VPg in *Tobacco vein mottling virus* (TVMV) (Hong *et al.*, 1995). In contrast, it interacts with NIaPro, but not with VPg, in TEV and

BD	AD	β -Galactosidase
VPg	VPg	++
51-191	VPg	++
81-191	VPg	+
1-80	VPg	-
1-99	VPg	-
77-153	VPg	-
50-153	VPg	-
VPg	51-191	++
VPg	81-191	+
VPg	1-80	-
VPg	1-99	-
VPg	77-153	-
VPg	50-153	-
1-80	1-80	-
1-80	81-191	-
81-191	81-191	+
81-191	1-80	-

Fig. 5. Estimation of the domain sequence responsible for VPg self-interaction using the yeast two-hybrid system by protein–protein interaction between VPg deletion constructs. ++, Colonies turned blue within 2 h; +, colonies turned blue between 2 and 6 h; –, no colonies appeared on the selective medium. The C-terminal region 154–191 was found to be important for self-interaction of VPg. Numbers inside the boxes indicate the amino acid positions relative to the intact VPg.

PSbMV (Li *et al.*, 1997; Guo *et al.*, 2001). Taken together, these results indicate that such interactions are not necessarily common among potyviruses but may be differential, i.e. each potyvirus may have unique protein–protein interaction patterns.

The interaction found between VPg and HCPro in CIYVV is a novel feature, so far not shown for any other *Potyvirus*. Whether this interaction is required for CIYVV replication, protein synthesis or movement is unclear. Potyviruses encode several proteins with common functions in their infection cycle. At least seven proteins (P1, HCPro, P3, CI, 6K2, VPg and Nib) have been found to be involved in replication (reviewed by Revers *et al.*, 1999), and five (HCPro, CI, 6K2, VPg and CP) are involved in virus movement (Dolja *et al.*, 1994, 1995; Klein *et al.*, 1994; Cronin *et al.*, 1995; Kasschau *et al.*, 1997; Schaad *et al.*, 1997; Andersen & Johansen, 1998; Carrington *et al.*, 1998; Lopez-Moya & Pirone, 1998; Rajamäki & Valkonen, 1999).

Although HCPro and VPg have common functions, especially in replication and virus movement, it is conceivable that VPg and HCPro act only cooperatively and not independently in replication and movement.

The involvement of VPg in potyviral movement has been reported in TEV, TMVMV and PVA (Schaad *et al.*, 1997; Nicolas *et al.*, 1997; Rajamäki & Valkonen, 1999, 2002). In these systems, these authors indicated that VPg formed a ‘movement complex’ with another viral protein and/or a host factor to function in virus movement. Furthermore, in a separate study VPg was found in an early stage of infection of PVA in the companion cells of infected *Solanum commersonii*, emphasizing its role in the movement of potyviruses (Rajamäki & Valkonen, 2003). Since our observations indicate that the central domain of VPg is important for its interaction with HCPro and the same domain was reported to be important for viral movement in other potyviruses (Rajamäki & Valkonen, 2002), we speculate that CIYVV VPg and HCPro may play an important role in the virus movement process. In view of the fact that VPg is attached to the 5′ end of viral RNA and that HCPro has been identified as a movement protein that increases the size exclusion limit of plasmodesmata (Rojas *et al.*, 1997), the association of VPg and HCPro might facilitate the movement process in a host plant.

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