Interactions among *Dendrolimus punctatus* cypovirus proteins and identification of the genomic segment encoding its A-spike

Chuangang Cheng, Yunpeng Shao, Lan Su, Yin Zhou and Xiulian Sun

Revealing the interactions among cypovirus proteins would facilitate our understanding of the replication and assembly of this virus. In the present study, interactions among proteins encoded by the 10 segments of *Dendrolimus punctatus* cypovirus (DpCPV) were identified using yeast two-hybrid (Y2H) and far-Western blotting assays. In total, 24 pairs of interactions were detected. Twelve pairs of one-direction interactions, four pairs of binary interactions and four pairs of self-associations were identified in the Y2H assays. Another four pairs of interactions were identified by far-Western blotting. The interactions between the methyltransferase domain of the turret protein (VP3) and VP4 as well as between polyhedrin and VP4 were further confirmed by far-Western blotting and pull-down assays, respectively. In addition, immunogold labelling showed that the A-spike of DpCPV is formed by VP4. In conclusion, we obtained a protein–protein interaction network of DpCPV and showed that its A-spike is formed by VP4 encoded by genomic segment 6.

INTRODUCTION

Cypovirus (CPV) belongs to the genus *Cypovirus* within the family *Reoviridae*. Unlike the multishelled viruses in the family *Reoviridae*, CPV virions are single shelled and embedded in polyhedral inclusion bodies. This structure is stable and is fully capable of carrying out functions such as cell entry and endogenous RNA transcription (Zhou et al., 2003). The CPV genome is typically composed of 10 dsRNA segments (S1–S10). Based on the electrophoretic migration patterns of the dsRNA segments in agarose or acrylamide gels, CPVs have been classified into 16 different types (King et al., 2011). *Dendrolimus punctatus* cypovirus (DpCPV; type 1) is an important pathogen of *Dendrolimus punctatus*, 2003). The CPV genome is typically composed of 10 segments of *D. punctatus* (Kunimi, 2007), and its complete nucleotide sequence has been determined (Zhao et al., 2003a, b).

The fine structures of the three major capsid proteins (VP1, VP3 and VP5) of *Bombyx mori* cypovirus (BmCPV) have been elucidated by cryo-electron microscopy (cryo-EM) (Cheng et al., 2011; Yu et al., 2011). Cryo-EM studies have demonstrated that the capsid shell of CPV is approximately 660 Å in diameter (excluding the fivefold turret). There are 120 copies of the capsid shell proteins (CSPs), which make up VP1; 12 B-spikes (also known as turret proteins, TPs), which are composed of 60 copies of VP3 and function as guanylyltransferases (GTases) and methyltransferases (MTases), and 120 copies of the large protrusion proteins (LPP), making up VP5, which functions as a clamp protein on the capsid shell (Xia et al., 2003; Yu et al., 2011; Zhou et al., 2003). In addition, VP5 displays RNA chaperone-like activity, which destabilizes RNA helices and accelerates strand annealing (Yang et al., 2013). The RNA-dependent RNA polymerase (RdRp), which is a main component of transcriptase enzyme complexes, attaches to the inner surface of the capsid shell at the icosahedral fivefold vertices (Xia et al., 2003; Zhang et al., 1999). VP2 is associated with the CSPs as an inner capsid to maintain the stability of the capsid shell (Chakrabarti et al., 2010). VP4 binds to viral RNA and helps in replication and transcription through ATP binding and hydrolysis (Chavali et al., 2008), but its localization on the particle remains unknown. To date, the genomic segment that encodes the A-spike of CPV has not been identified. The coordinate spike structure of rotavirus (RV) (Ludert et al., 1996) and σ1 of mammalian orthoreovirus (MRV) (Furlong et al., 1988) has been identified as a viral attachment protein. CPV NSP1 (p101) is remarkably similar to foot-and-mouth disease virus 2A protease (Hagiwara et al., 2001). CPV NSP3 (p36) binds to ssRNA and dsRNA (Chen et al., 2007), and its N-terminal transmembrane domain has an important function in the virus life cycle (Chen et al., 2006).

Revealing the protein–protein interactions of CPV would facilitate our understanding of the replication and assembly...
of this virus. Some protein–protein interactions of viruses in the family *Reoviridae* have been identified. NS80 of grass carp reovirus (GCRV) has been identified as interacting with the proteins NS38, VP4 and VP6; in addition, NS80 and NS38 interact with themselves (Cai et al., 2011; Shao et al., 2013). The non-structural protein μNS of MRV forms virogenic stroma (Broering et al., 2005), which also are associated with the viral core proteins μ2, λ1, λ2, λ3 and σ2, as well as the RNA-binding non-structural protein σNS (Miller et al., 2010). In CPVs, interactions among VP1, VP3 and VP5 have been observed by cryo-EM (Cheng et al., 2011; Yu et al., 2011).

In the present study, molecular biology methods, such as yeast two-hydrid (Y2H) assays, far-Western blotting, pull-down and fluorescence co-localization assays were used to identify interactions among the 10 proteins encoded by the DpCPV genomic segments. Furthermore, the genomic segment encoding the A-spike was determined.

**RESULTS**

**Y2H and far-Western blotting assays reveal 24 pairs of protein–protein interactions**

The Y2H assays showed that 10 proteins encoded by the DpCPV genomic segments were involved in one or more interactions. In total, 20 pairs of interactions were detected (Table 1 and Fig. 1a), comprising 12 pairs (derived from 19 pairs of intact and truncated proteins) of one-direction interactions, four pairs of binary interactions (VP4–VP5, VP4–polyhedrin, VP5–polyhedrin and polyhedrin–NSP2) and four pairs of self-associations (VP4–VP4, VP5–VP5, polyhedrin–polyhedrin and NSP3–NSP3) (Fig. 1a).

In detail, four interactions among the non-structural proteins were identified from the Y2H assays: polyhedrin–NSP1, polyhedrin–NSP2, polyhedrin–polyhedrin and NSP3–NSP3 (Fig. 1a), whilst NSP3 did not interact with any other proteins. Six interactions between the structural and non-structural proteins were also identified: VP1–polyhedrin, RdRp–polyhedrin, VP2–polyhedrin, VP4–NSP2, VP4–polyhedrin and VP5–polyhedrin (Fig. 1a). Ten interactions among the structural proteins were detected: VP5 interacted with all five of the other structural proteins; VP1, VP3 and VP4 interacted with each other; and both VP4 and VP5 self-associated (Fig. 1a).

Four interactions among the structural proteins were not obtained by Y2H but were identified using far-Western blotting (Fig. 1b). These included VP1–VP1 (Fig. S1b, available in the online Supplementary Material), VP2–VP1 (Fig. S1c), VP2–RdRp (Fig. S2b) and GTase–RdRp (Fig. S2c).

In total, 24 interactions among the DpCPV proteins were identified through Y2H and far-Western blotting assays (Fig. 1).

**A fluorescence co-localization assay confirms the VP5–polyhedrin interaction**

A fluorescence co-localization assay was performed to further verify the VP5–polyhedrin interaction. When the plasmid PIB-DsRed-S10 and the bacmid vBac-S7-EGFP were co-transfected into Sf9 cells, VP5 and polyhedrin were found to be co-localized in the cytoplasm at 48 h post-transfection (Fig. 2).

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<th>Prey (protein)</th>
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A far-Western blotting assay verifies the MTase–VP4 interaction

A far-Western blotting assay was performed to verify the MTase–VP4 interaction (Fig. 3). It was found that maltose binding protein (MBP)-MTase specifically bound to His–VP4 (Fig. 3b, lane 1) and did not bind to His–VP5 or His–polyhedrin (Fig. 3b, lanes 2 and 3). These results were consistent with the Y2H assay results (Table 1) and further demonstrated that the interaction between MTase and VP4 was direct and did not require any other viral proteins.

A His pull-down assay verifies the VP4–polyhedrin interaction

His–polyhedrin as the bait protein was detected by the anti-His mAb at approximately 30 kDa (Fig. 4, lanes 1 and 2). MBP–VP4 and MBP as the prey proteins were incubated with His-polyhedrin; after pull-down, Western blotting was performed with the anti-MBP mAb. MBP–VP4 was detected at approximately 114 kDa (Fig. 4, lane 2), whilst MBP, as the negative control, was not detected (Fig. 4, lane 1). The prokaryotically expressed MBP protein was used in the Western blot (Fig. 4, lane 3) to compare with lane 1.

Anti-VP4-conjugated immunogold particles aggregate around the projecting part of the virions

To determine whether VP4 formed the A-spike of DpCPV, virions were loaded onto carbon-coated grids (Fig. 5a) and incubated with immunogold-conjugated anti-VP4 antibody, raised from the prokaryotic expression of genomic segment 6. As shown by transmission electron microscopy (TEM) analysis, the anti-VP4-conjugated immunogold particles aggregated around the projecting part of the virions (Fig. 5b). The gold particles were dispersed

Fig. 1. Summary of DpCPV protein interactions from Y2H and far-Western blotting assays. (a) Summary of protein interactions from Y2H assays. Solid lines represent binary interactions and self-associations and dashed lines represent one-direction interactions. Rectangles represent structural proteins and ovals represent non-structural proteins. (b) Protein interactions from far-Western blotting assays.

Fig. 2. Interaction of VP5 and polyhedrin, confirmed by a fluorescence co-localization assay. Sf9 cells were co-transfected with 2 μg plasmid PIB-DsRed-S10 and bacmid vBac-S7-EGFP using 8 μl Cellfectin according to the manufacturer’s instructions. (a) Expression of polyhedrin visualized by DsRed. (b) Expression of VP5 visualized by EGFP. (c) DAPI staining of Sf9 cell nuclei. (d) Merged image of (a), (b) and (c). Bar, 5 μm.

Fig. 3. Interaction of MTase domain (aa 407–1057) and VP4 verified by a far-Western blotting assay. (a) The proteins were separated by SDS-PAGE and stained with Coomassie blue. (b) Interactions were detected using a far-Western blotting assay. Samples were separated by SDS-PAGE, transferred to a PVDF membrane, denatured and renatured, and then incubated with maltose binding protein (MBP)–MTase, and detected by anti-MBP mAb as the primary antibody and HRP-conjugated goat anti-mouse as the secondary antibody. Lanes: M, protein molecular mass marker; 1, His–VP4; 2, His–VP5; 3, His–polyhedrin.
randomly on the grids when rabbit pre-immune serum was used as a negative control (data not shown).

DISCUSSION

The interactions of the major capsid proteins of CPV have been elucidated previously by cryo-EM (Cheng et al., 2011; Yu et al., 2011), but little has been known regarding the interactions among the non-structural proteins as well as those between the non-structural and structural proteins of CPVs. In the current study, we identified interactions between non-structural and structural proteins, e.g. NSP2 and VP4, polyhedrin and VP4, and between non-structural proteins, e.g. NSP1 and polyhedrin, NSP2 and polyhedrin, which provided information on the methods of virus replication and assembly.

There were two conformers for both VP1 (CSP-A and CSP-B) and VP5 (LPP-3 and LPP-5). CSPs interact with LPPs, and both CSPs and LPPs self-associate (Cheng et al., 2011; Yu et al., 2011). In this study, the self-association of VP1 was detected by far-Western blotting (Fig. S1b), whilst the interaction between VP1 and VP5 and the self-association of VP5 were detected by Y2H assays (Fig. 1). These results were consistent with previous cryo-EM studies (Cheng et al., 2011; Yu et al., 2011). Moreover, our results revealed that VP2 interacted with VP1 (Fig. S1c), which is consistent with the report that VP2 maintains the stability of the capsid shell (Chakrabarti et al., 2010).

It has been reported that the N terminus of VP3 could bring heterologous proteins into the polyhedrin (Ijiri et al., 2009; Ikeda et al., 2001, 2006), indicating that VP3 interacts with polyhedrin. However, recent studies have indicated that the B-spike is too far away to interact with polyhedrin, as the A-spike is located in the middle of the B-spike and polyhedrin (Chen et al., 2011; Yu et al., 2011). Our Y2H assay (Fig. 1) and far-Western blotting assay (Fig. 3) results also showed that there was no interaction between VP3 and polyhedrin, whilst VP4 interacted with both the MTase domain 1 (aa 407–726) of VP3 and polyhedrin (Table 1, Figs 3 and 4).

The A-spike between the B-spike and polyhedrin has been observed by electron tomography (Chen et al., 2011), but the segment encoding the A-spike has not been identified. In this study, immunogold labelling indicated that the A-spike is formed by VP4, encoded by genomic segment 6. It has been reported that both s1 of MRV and VP4 of RV form spikes on the surface of the virions that function as the viral attachment protein (Furlong et al., 1988; Ludert et al., 1996). As the A-spike is the most projecting portion of CPV particles, we believe that the A-spike may also play the attachment role in CPV infection.
The RdRp is located below the TP and forms the transcriptase complex (Xia et al., 2003; Zhang et al., 1999), indicating that RdRp could interact with the GTase of TP. Although we did not detect an interaction between RdRp and GTase by Y2H, RdRp (aa 1–546) interacted with GTase, according to the far-Western blotting results (Fig. S2c). This interaction facilitates our understanding of the highly effective formation of the CPV mRNA. It has been reported that positive strands of the dsRNA segments are unwound, passed through the TPs, and followed by a capping reaction and methylation reaction by the GTase and MTase domains of VP3 (Cheng et al., 2011; Yu et al., 2011).

The NS80 of GCRV interacts with NS38, VP4 and VP6, and also self-interacts (Cai et al., 2011), whilst the μNS of MRV interacts with μ2, λ1, λ2, λ3, n2 and σNS (Miller et al., 2010). As a pair of homologous proteins, μNS and NS80 are related to the formation of virogenic stroma in GCRV and MRV replication, and act in recruiting viral proteins and assembling them into integrated virions (Broering et al., 2005; Shao et al., 2013). Similarly, according to the Y2H results, the polyhedrin of DpCPV interacted with most of the structural and non-structural proteins, i.e. VP1, VP2, VP4, VP5, RdRp, NSP1, NSP2 and itself. These multiple associations imply that polyhedrin might act as the virogenic stroma during CPV assembly.

In conclusion, we obtained a protein–protein interaction network of DpCPV and showed that its A-spike is formed by VP4. To the best of our knowledge, this report is the first to study the interaction network of CPVs using molecular biology methods. Further research should focus on the role of the A-spike in attachment and the functions of polyhedrin in virus assembly.

METHODS

Viruses and cell lines. DpCPV was initially isolated from D. punctatus larvae during a natural outbreak in Macheng, Hubei, China, and propagated by infecting fifth-instar Spodoptera exigua larvae. S. exigua larvae were obtained from the Experimental Animal Center of Wuhan Institute of Virology, Chinese Academy of Sciences. Sf9 cells (Invitrogen) were cultured at 27

70 min, 4

C) to remove the sucrose. The sample purity was confirmed by SDS-PAGE and electron microscopy.

Construction of clones expressing intact or truncated fragments of DpCPV for Y2H screening. Eighteen cDNA segments (including the truncated ones) of the 10 DpCPV dsRNA genomic segments were cloned into vectors for the Y2H screening assay using the primers listed in Table S1. Based on the structures of VP1 and VP3 (Cheng et al., 2011; Yu et al., 2011) and motif analysis of RdRp (Zhao et al., 2003a), the following were constructed: five truncated fragments of VP1: VP1 (aa 1–144), VP1 (aa 145–404), VP1 (aa 405–827), VP1 (aa 828–1069) and VP1 (aa 1070–1333); truncation of the RdRp protein to RdRp (aa 1–546), RdRp (aa 547–681) and RdRp (aa 682–1225); and truncation of VP3 to GTase (aa 1–406), MTase 1 (aa 407–726) and MTase 2 (aa 829–1057). There were 11 truncated fragments representing three ORFs, plus seven other intact ORFs, making a total of 18 construct pairs. All 18 fragments were cloned into both pGBKTK7 and pGADT7 vectors and confirmed by sequence analysis.

Y2H assays. Y2H assays were performed according to the manufacturer’s protocol (Matchmaker Gold Yeast Two- Hybrid System; Clontech). The bait plasmids were transformed into the yeast strain Y2HGold. The yeasts grew on Trp-synthetically defined (SD/−Trp) plates to test auto-activation and toxicity. All five truncations of the VP1 bait plasmids, pGBKTK7-RdRp (aa 547–681), pGBKTK7-VP2, pGBKTK7-NSP1 and pGBKTK7-MTase 1 had auto-activation. Except for these plasmids, the combinations of the bait and prey plasmids were transformed into the yeast strain Y2HGold to identify the protein–protein interactions; the negative control (pGBKTK7-53 and pGADT7-T) and negative control (pGBKTK7-1am and pGADT7-T) were executed at the same time. Briefly, for each transformation, 100 ng bait recombinant plasmid and 100 ng prey recombinant plasmid were mixed with 5 μl denatured Yeastmaker Carrier DNA (Yeastmaker Yeast Transformation System 2; Clontech) and transformed into the Y2HGold yeast according to the user manual. The transformants were then grown at 30°C on synthetically defined (SD) medium in double-dropout plates of SD/−Leu−Trp/X-gal/AbA, and the blue colonies that grew on this medium were picked and placed onto higher stringency quadruple-dropout plates of SD/−Ade/−His/−Leu/−Trp. Positive results were repeated twice, and negative results were repeated once.

Fluorescence co-localization assay. The plasmid PIB-DS-red-S10 was constructed to express polyhedrin fused with red fluorescence protein at the N terminus. The bacmid vBac-S7-EGFP, which expresses VP5 fused with EGFP, was constructed as described previously by Jin et al. (2013). Sf9 cells were seeded on the day before transfection at a density of 1.0 × 10^6 cells ml^−1 in six-well plates containing glass coverslips. Transfections were performed with 2 μg PIB-DS-red-S10, 2 μg vBac-S7-EGFP DNA and 8 μl Cellfectin (Invitrogen) per well according to the manufacturer’s directions. Cells were fixed with 4% paraformaldehyde at 48 h post-transfection and stained with DAPI. Samples were examined on a TCS SP2 confocal laser scanning microscope (Leica).

Far-Western blotting assay. The far-Western blotting method was performed as described previously (Wu et al., 2007), with appropriate modifications. The prokaryotic expression proteins (His−VP4, His−VP5 and His−polyhedrin) were purified, separated by SDS-PAGE (12% acrylamide), transferred onto PVDF membranes (Millipore), denatured and renatured by AC buffer [100 mM NaCl, 20 mM Tris/ HCl (pH 7.6), 0.5 mM EDTA, 10% glycerol, 0.1% Tween 20, 2% BSA, 1 mM DTT] containing 6 M, 3 M and 1 M guanidine/HCl at room temperature, and 0.1 M guanidine/HCl at 4°C for 30 min, successively. The membrane was immersed in AC buffer without guanidine/HCl overnight at 4°C and then blocked with 5% BSA for 1 h at room temperature. Subsequently, the membrane was incubated with the prokaryotic expression protein MBP-MTase (aa 407–1057) or MBP (as the negative control) for 2 h at room temperature,
followed by incubation with anti-MBP mAb (New England BioLabs) for 90 min at room temperature. After three washes with PBST buffer [0.1% Tween 20 (pH 7.4)], the samples were incubated with HRP-conjugated goat anti-mouse IgG (Boster) for 90 min. The results were detected using Enhanced Chemiluminescence Reagent Plus (Thermo Scientific).

**His pull-down assay.** Samples (5 mg) of prokaryotic expression protein MBP (as the negative control) or MBP–VP4 were incubated with His–polyhedrin at 4 °C for 30 min, and each mixture was added to 1 ml Ni-nitrilotriacetic acid (NTA) resin (Invitrogen) and incubated at 4 °C for 30 min, respectively. After washing with 4 ml washing buffer [60 mM imidazole, 0.5 M NaCl, 20 mM Tris/HisCl (pH 7.9)], the protein was eluted from the resin with elution buffer [1 M imidazole, 0.5 M NaCl, 20 mM Tris/HisCl (pH 7.9)]. Samples were separated by SDS-PAGE (12% acrylamide), followed by Western blotting with an anti-MBP mAb (New England BioLabs) and anti-His mAb (Beyotime) mixture as the primary antibody; HRP-conjugated goat anti-mouse IgG (Boster) was used as the secondary antibody.

**TEM analysis of virus particles incubated with immunogold-conjugated anti-VP4.** Segment 6 of DpCPV was cloned into vector pET28a (Invitrogen) and transformed into *Escherichia coli* BL21 (Invitrogen), followed by induction with 1 mM IPTG. The expressed VP4 protein was purified by a Ni-NTA agarose column (Invitrogen). A rabbit was immunized with VP4 protein to produce the polyclonal anti-VP4 antibody. Immunogold staining was performed according to a method described previously (Chakrabarti et al., 2010). Briefly, after absorption of virus particles onto carbon-coated grids, blocking was carried out using 1% BSA in 20 mM PBS. After washing with 20 mM PBS, affinity-purified anti-VP4 antibody was added at a dilution of 1:100, and the grids were incubated for 30 min. The grids were then washed again with 20 mM PBS, and gold-tagged anti-VP4 antibody was added at a dilution of 1:100. The grids were then washed three times with double-distilled water, and the samples were stained with 2% aqueous uranyl acetate. After overnight drying, the samples were examined by TEM (Hitachi H-800; Hitachi Co.) at 100 kV.

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