Studies on interaction of cucurbit aphid-borne yellow virus proteins using yeast two-hybrid system and bimolecular fluorescence complementation

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Summary. – In this article, yeast two-hybrid system (YTHS) and bimolecular fluorescence complementation (BiFC) were used to analyze the interactions of cucurbit aphid-borne yellows virus (CABYV)-encoded proteins. P0, P1, P1-2, P3, P4, and P5 were tested by YTHS in all possible pairwise combinations, and only P3/P3 interaction was detected. Results obtained by BiFC further confirmed the self-interaction of P3, and the subcellular localization of reconstituted YFP fluorescence was observed mainly in nuclei of Nicotiana benthamiana leaf epidermal cells. Domains involved in P3/P3 self-interaction were analyzed by YTHS and BiFC using deletion mutants. The results showed that R domain (residues 1–61) in the N-terminus could self-interact, and it also interacted with the S domain (residues 62–199) in the C-terminus of P3. The present work would serve as a molecular basis for further characterization of CABYV proteins, and the regions involved in P3/P3 self-interaction could provide the clue for understanding the capsid assembly pathway of CABYV.

Keywords: Polerovirus; protein-protein interaction; subcellular localization; interaction domain

Introduction

CABYV is a member of the genus Polerovirus in the family Luteoviridae. Its icosahedral virion is approximately 25 nm in diameter, containing a single-stranded RNA of 5.6 Kb in length (Guilley et al., 1994). The virus was first reported to infect cultivated cucurbits in France, causing a severe disease (Lecoq et al., 1992). CABYV now infects cucurbits (almost all Cucurbitaceae) widely throughout the world with economic importance. In China, our previous works showed that CABYV occurred widely in Mainland (Xiang et al., 2008; Shang et al., 2009) and the complete RNA genome of the CABYV Chinese isolate (CABYV-CHN) was determined (Xiang et al., 2008).

CABYV genome comprises six major open reading frames (ORFs 0–5) encoding proteins P0, P1, P1-2, P3, P4, and P5 (Guilley et al., 1994; Xiang et al., 2008). In the genus Polerovirus, P0 encoded by the first open reading frame (ORF0) is a potent suppressor of gene silencing (Pazhouhandeh et al., 2006), and it is indispensable for virus accumulation (Sadowy et al., 2001). P1 and P1-2 (expressed as an ORF1-ORF2 fusion protein by translational frameshift) are necessary for virus replication (Nickel et al., 2008). P3 (the major capsid protein), encoded by the ORF3, controls the virion formation, and it is essential in the aphid transmission process (Brault et al., 2003). P4 is thought to be the movement protein based on its biochemical properties and subcellular localization (Tacke et al., 1993; Schmitz et al., 1997), but the requirement of the putative movement protein might be host dependent (Lee et al., 2002). P5, known as readthrough protein, is a minor coat protein required for aphid transmission (Brault et al., 1995) and is also involved in...

Abbreviations: BiFC = bimolecular fluorescence complementation; CABYV = cucurbit aphid-borne yellows virus; CP = coat protein; DAPI = 4′, 6-diamidino-2-phenylindole; YTHS = yeast two-hybrid system

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virus movement and accumulation in plants (Mutteret al. et al., 1999; Peter et al., 2008).

Poleroviruses are strictly phloem-limited and dependent on aphids for transmission. Infectious cDNA clones served to investigate biological roles of viral proteins (Prüfer et al., 1995; Lee et al., 2002; Braun et al., 2003; Peter et al., 2008), however, the functions of poleroviral proteins remain largely unclear. The assessment of protein interactions is an important way to better understand functions of viral proteins and the interplay between virus and host. Many examples of viral protein interactions have been reported (Guo et al., 2001; Lin et al., 2009; Stewart et al., 2009; Shen et al., 2010), however, very limited information is available for the family Luteoviridae, except that potato leafroll virus P4 could form homodimers (Tacke et al., 1993).

In this article, we now report the protein interaction properties of CabyV using YTHS and BiFC. Our results could provide novel information about functions of proteins and capsid assembly pathway of CabyV.

Materials and methods

Plant materials and growth conditions. N. benthamiana plants were grown in 10 cm pots filled with a mixture of 60% vermiculite and 40% meadow soil and cultivated in growth chambers (16 hrs light/8 hrs dark at 25–26°C).

Construction of plasmids. Full-length cDNA of CabyV-CHN (GenBank accession no. EU0000535) was kept in our laboratory. The plasmids used in BiFC assay, pSPYNE-35S and pSPYCE-35S (for split YFP N-terminal/C-terminal fragment expression), were kind gifts from Dr Jörg Kudla (Walter et al., 2004). To construct plasmids for YTHS, the coding sequences of P0, P1, P2, P3, P4, P5, and N-terminal (amino acid residues 1–61) and C-terminal (residues 62–199) fragments of CabyV P3 were amplified separately using LaTaq polymerase (Takara) with primer pairs P1(5′-CGCGAATTCATGCCAGACATTCGGGTTTTGGACC-3′), F9/R10(5′-TATGGATCCATGAATACGGCCGTGGGTTTGG-3′), F9(5′-TATGGATCCATGAATACGGCCGTGGC-3′)/R10(5′-AGTCCTCGAGTTTCGGGTTTTGGACC-3′), F9(5′-TATGGATCCATGAATACGGCCGTGGC-3′)/R10(5′-AGTCCTCGAGTTTCGGGTTTTGGACC-3′), F9/R10(5′-TATGGATCCATGAATACGGCCGTGGGTTTGG-3′), F9(5′-TATGGATCCATGAATACGGCCGTGGC-3′)/R10(5′-AGTCCTCGAGTTTCGGGTTTTGGACC-3′), and F10(5′-CGCGGATCCAGGAAACTCGATTTTTC-3′)/R10(5′-CGCGGATCCAGGAAACTCGATTTTTC-3′), respectively. The PCR fragments were digested with BamHI/Xhol and cloned into the vectors pSPYCE-35S and pSPYNE-35S to produce recombinant plasmids pP0-YFP, pP0-YFPN, pP4-YFP, pP4-YFPN, pP3-YFP, pP3-YFPN, pP3(1-61)-YFP, pP3(1-61)-YFPN, pP3(62-199)-YFP, and pP3(62-199)-YFPN, respectively.

BiFC assay and confocal laser scanning microscopy. N. benthamiana leaves were used for agroinfiltration. Agrobacterium tumefaciens strain EHA105 carrying pP0-YFP, pP4-YFP, pP3-YFP, pP3(1-61)-YFP, pP3(62-199)-YFP, pP3(1-61)-YFPN, pP3(62-199)-YFPN, or pP3(62-199)-YFPN was separately cultured in a shaker overnight at 28°C in LB medium containing streptomycin (100 mg/ml) and kanamycin (50 mg/ml), and the cells were resuspended to an OD60 of 0.6 with MMB buffer (10 mmol/L MES/NaOH, pH 5.6, 10 mmol/L MgCl2, 200 mmol/L acetosyringone). For coinfiltration, equal volumes of combinations were mixed prior to infiltration. Yb protein encoded by barley stripe mosaic virus could self-interact (Bragg and Jackson, 2004). Therefore the combination of pyb-YFP/pyb-YFPN (Kept in State Key Laboratory for Agrobiotechnology, China Agricultural University) was used as a positive control, and the pair of pSPYNE-35S/pSPYCE-35S served as the negative control. Observation of leaf epidermal cells for fluorescence was performed at 48–72 hrs after infiltration. To locate nuclei, the leaf tissues were infiltrated with PBS containing 10 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) for about 5 mins prior to observation.
Confocal microscopy was performed on a confocal laser scanning microscope (Nikon C1). A 488 nm argon laser with an emission band of 550–590 nm and a 408 nm argon laser with an emission band of 515–530 nm were used for YFP and DAPI staining detections, respectively.

Results and Discussion

P3/P3 self-interaction in yeast cells

CABYV-encoded proteins, P0, P1, P1-2, P3, P4, and P5 were assessed in all possible pairwise combinations (6 activation domain fusions × 6 binding domain fusions = 36 pairwise combinations) for interactions in a YTHS. It was concluded that six CABYV proteins could not interact with each other, because transformants of protein pairs (P0/P1, P0/P1-2, P0/P3, P0/P4, P1/P1-2, P1/P3, P1/P4, P1/P5, P1-2/P3, P1-2/P4, P1-2/P5, P3/P4, P3/P5, and P4/P5) could not grow on agar plates of SD/-Leu/-Trp/-Ade/-His media. Limitations of YTHS may account for the failure to detect some interactions, but it is possible that proteins of CABYV might not interact directly, or host factors are necessary to detect interactions. Further studies on protein-protein interaction in CABYV-infected plants are needed to test these possibilities.

Analysis of interactions of P0, P3, and P4 of CABYV by BiFC

Panels 1–11, images of *N. benthamiana* leaf epidermis transformed with constructs pP0-YFPN/pP0-YFPC, pP0-YFPN/pP3-YFPC, pP0-YFPN/pP4-YFPC, pP0-YFPC/pP3-YFPN, pP0-YFPC/pP4-YFPN, pP3-YFPC/pP4-YFPC, pSPYNE-35S/pSPYCE-35S (negative control), pγb-YFPC/pγb-YFPN (positive control), and pP3-YFPN/pP3-YFPC, respectively. YFP: YFP fluorescence image (green); Light: bright-field image; DAPI: DNA-selective dye DAPI image indicating the positions of nuclei in cells; Merged: YFP, DAPI, and bright-field overlay.
We also tested for self-interaction properties of P0, P1, P1-2, P3, P4, and P5, and only transformants of pGBK-P3/pGAD-P3 could grow on the high-stringency selection medium, which suggested P3 could self-interact. Virus capsid assembly requires repeated interaction of CP (coat protein) subunits. Self-interaction of CAYBV P3 in yeast is consistent with observations in other viruses (Guo et al., 2001; Hallan and Gafni, 2001; Lin et al., 2009).

**Confirmation of P3/P3 interaction in living plant cells by BiFC**

Based on the results obtained by YTHS, we further analyzed interaction properties of P3 by BiFC, and also chose P0 and P4 for further demonstration. No or negligible fluorescence was displayed in leaves that were co-infiltrated with combinations of pP0-YFPN/pP0-YFPC, pP0-YFPN/pP3-YFPC, pP0-YFPN/pP4-YFPC, pP0-YFPN/pP4-YFPN, pP0-YFPY/pP3-YFPY, pP0-YFPY/pP4-YFPY, pP3-YFPN/pP4-YFPN, pP3-YFPN/pP4-YFPY, pP3-YFPY/pP4-YFPN, or pP4-YFPN/pP4-YFPY (Fig. 1, panels 1–8), indicating that P0, P3, and P4 could not interact with each other, and P0, P4 were not able to self-interact in plant cells. However, the reconstitution of YFP fluorescence was observed in N. benthamiana leaf epidermis coinfiltrated with pP3(1-61)-YFPN/pP3(1-61)-YFPN (Fig. 1, panel 11), which confirmed the P3 self-interaction.

We did not observe the P4/P4 interaction neither by YTHS nor by BiFC. Each member in the same genus may have unique protein-protein interaction patterns (Urcuqui-Inchima et al., 1999), and as for P4, sequence identity between potato leafroll virus and CAYBV is relatively low (about 40%), so homotypic interaction of P4 may not be a general phenomenon for all Polerovirus members. Another possibility is that the limitations of YTHS and BiFC, such as nuclear entry of fusion proteins in YTHS and insufficient flexibility to allow reconstitution of the split YFP fragments in BiFC assay, might account for the failure to detect the interaction.

Self-interaction of intact CAYBV P3 protein was observed mainly in nucleus, and weak fluorescence was detected in the cytoplasm (Fig. 2, panel 11). Predicted nuclear localization signals (NLSs) in arginine-rich region of CAYBV P3 analyzed with PSORT II prediction (http://psort.hgc.jp/form2. html) (data not shown here) might account for the nuclear targeting. We currently do not know why CAYBV CP enters the nucleus, however, our results are quite similar to those reported for other Luteoviridae members (Nass et al., 1995; Haupt et al., 2005).

**R domain interacted with itself and with S domain in P3**

Polerovirus capsids are thought to be assembled from approximately 180 CP subunits according to \( T = 3 \) symmetry (Lee et al., 2005). The CP of icosahedral viruses generally contains two domains, the N-terminal arginine-rich domain (R) and the shell domain (S) (Terradot et al., 2001), and both domains are critical for viral capsid formation (Lokesh et al., 2002; Brault et al., 2003; Kaplan et al., 2007). Therefore, two deletion mutants for each YTHS and BiFC assay were created to define regions required for the P3 self-interaction. R domain (residues 1–61) in the N-terminus of P3 could self-interact, for positive and reproducible interaction was detected when yeast cells were co-transformed with combination of pGBK-p3(1-61)/pGAD-p3(1-61) (Fig. 2a). R domain also interacted with S domain (residues 62–199) in the C-terminus of P3, but it showed directionality, because interaction was observed only when the pair of pGBK-p3(62-199)/pGAD-p3(1-61) was co-transformed (Fig. 2a).

Fluorescent signal observed in N. benthamiana leaf epidermis coinfiltrated with pP3(1-61)-YFPN/pP3(1-61)-YFPY pair further confirmed the R/R self-interaction (Fig. 2b, panel 5). The interaction between R and S domains was also detected in plant cells and also showed directionality, because reconstituted YFP fluorescence was only observed in leaves coinfiltrated with the pair of pP3(1-61)-YFPN/pP3(62-199)-YFPY (Fig. 2b, panel 6). The interaction between the N-terminus of one CP and the C-terminus of the other has been reported to be needed for dimer formation in \( T = 1 \) icosahedral particles (Choi and Loesch-Fries, 1999; Hallan and Gafini, 2001). Based on our results we suggest that R/R and R/S interactions are probably required to assemble three CAYBV capsid subunits to form a trimer.

We observed that both R/R and R/S interactions were exclusively presented in nuclei (Fig. 2b, panel 5 and 6), suggesting the capability for directing nuclear transport might be enhanced when R domain was expressed alone.

The phenomenon of directional interaction between R and S domains in yeast may be due to the fact that protein fusions in one direction might have a more favorable protein folding or exposure of binding site than those in the other direction (Guo et al., 2001). On the other hand, in BiFC assay, the phenomenon may be caused by the orientation of the interacting protein pair relative to the split YFP domain that results in the insufficient flexibility to allow reconstitution of the split YFP fragments (Bracha-Drori et al., 2004).

Taken together, it was demonstrated that CAYBV P3 protein could interact with itself in yeast and plant cells. Our results also showed that the R domain interacting with itself and with S domain in P3 was responsible for self-interaction of P3. There is no crystallographic data available for Luteovirus coat protein, thus the domains involved in self-interaction of P3 might provide the clue to the understanding of polerovirus capsid assembly.
Fig. 2

Domains involved in P3/P3 self-interaction

(a) Detection of domains required for P3/P3 interaction by YTHS. 1, pGBK7/pGADT7 (negative control); 2, pGBK7-53/pGADT7-RecT (positive control); 3, pGBK-P3(1-61)/pGADT7(negative control); 4, pGBK-P3(1-61)/pGAD-P3(1-61); 5, pGBK7/pGAD-P3(1-61) (negative control); 6, pGBK-P3(62-199)/pGAD-P3(62-199); 7, pGBK-P3(62-199)/pGADT7(negative control); 8, pGBK7/pGAD-P3(62-199)(negative control); 9, pGBK-P3(62-199)/pGAD-P3(62-199); 10, pGBK-P3(1-61)/pGAD-P3(62-199). (b) Detection of domains involved in self-interaction of P3 by BiFC. Panels 1–6, images of N. benthamiana leaf epidermis transformed with constructs of pP3(62-199)-YFP N/pP3(62-199)-YFP C, pP3(1-61)-YFP C/pP3(62-199)-YFP N, pSPYNE-35S/pSPYCE-35S (negative control), pγb-YFP C/pγb-YFP N (positive control), pP3(1-61)-YFP N/pP3(1-61)-YFP C, and pP3(1-61)-YFP N/pP3(62-199)-YFP C. YFP: YFP fluorescence image (green); Light: bright-field image; DAPI: DAPI image; Merged: YFP, DAPI, and bright-field overlay.
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References


