Acta virologica **55**: 235 – 241, 2011 doi:10.4149/av_2011_03_235

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

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Received January 21, 2011; accepted August 22, 2011

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

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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

virus movement and accumulation in plants (Mutterer *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; Brault *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 cultured 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. EU000535) 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, P1-2, 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 F1(5′-CGCGAATTCATGCAAATTGAGTCTG-3′)/R1(5′-ACTGGATCCTCAGCGTTGTAAGATCTTC-3′), F2(5′-CGACATATGATGGAAGCGAAACACTTTTC-3′)/R2(5′-TCACATATGTCAGTTCAGCTTCCGC-3′), F2/R3(5′-GACCATATGTTATATCTTTTGTGGCTGC-3′), F3(5′-ACTGAATTCATGAATACGGCCGTGGCTAG-3′)/R4(5′-CTAGGATCCCTATTTCGGGTTTTGGAC-3′), F4(5′-AATGAATTCATGCAGGGAGGCGGAGG-3′)/R5(5′-GATGGATCCCTACCTATTTCGGGTTTTGG-3′), F5(5′-GCACATATGATGAATACGGCCGTGGCTAG-3′)/R6(5′-GCAGGATTCTTATGAGGTTTTATCAGCTAG-3′), F3/R7(5′-TATGGATCCGCCTGGACTCCTTCC-3′), and F6(5′-CCGGAATTCGAAACATTCGTATTTTC-3′)/R4, respectively. The PCR fragments were digested with *Eco*RI/*Bam*HI (for P0, P4, P3, and P3 deletion mutants), *Nde*I (for P1 and P1-2), or *Nde*I/*Bam*HI (for P5), and then cloned into vectors pGBKT7 and pGADT7 (Clontech laboratories, inc.), to generate the recombinant plasmids pGBK-P0, pGAD-P0, pGBK-P1, pGAD-P1, pGBK-P1-2, pGAD-P1-2, pGBK-P3, pGAD-P3, pGBK-P4, pGAD-P4, pGBK-P5, pGAD-P5, pGBK-P3(1-61), pGAD-P3(1-61), pGBK-P3(62-199) and pGAD-P3(62-199), respectively.

For BiFC, the full-length coding sequences of P0, P4, P3, N- and C-terminal fragments of P3 were amplified separately with the primer pairs F7(5′-CGAGGATCCATGCAAATTGAGTCTGTTC-3′)/R8(5′-AGTCTCGAGGCGTTGTAAGATCTTCTG-3′), F8(5′-TATGGATCCATGCAGGGAGGCGGAGG-3′)/R9(5′-CAGCTCGAGCCTATTTCGGGTTTTGG-3′), F9(5′-TATGGATCCATGAATACGGCCGTGGC-3′)/R10(5′-ACGCTCGAGTTTCGGGTTTTGGACC-3′), F9/R11(5′-GATCTCGAGGCCTGGGCTCCTTCCTC-3′), and F10(5′-CGCGGATCCATGGAAACATTCGTATTTTC-3′)/R10, respectively. The PCR fragments were digested with *BamHI*/*Xho*I and cloned into the vectors pSPYCE-35S and pSPYNE-35S to produce recombinant plasmids pP0-YFP^{C}, pP0-YFP^{N}, pP4-YFP^{C}, pP4-YFP^{N}, pP3-YFP^{C}, pP3-YFP^{N}, pP3(1-61)-YFP^{C}, pP3(1-61)-YFP^{N}, pP3(62-199)-YFP^{C}, and pP3(62-199)-YFP^{N}, respectively.

YTHS. YTHS tests were performed using the BD Matchmaker Library Construction and Screening kits (Clontech). Small-scale lithium acetat-mediated transformation method was used to transform pairs of constructs simultaneously into *Saccharomyces cerevisiae* strain AH109 according to the manufacturer′s protocols. Transformed yeast cells were plated on synthetic dropout (SD) medium lacking leucine, tryptophan, adenine and histidine (SD/-Leu/-Trp/-Ade/-His) (high-stringency selection medium). Protein interaction was determined by colony growth on the SD/-Leu/-Trp/-Ade/-His medium. Plasmid combination of pGBKT7-53/pGADT7-RecT supplied with the kits served as a positive control. All experiments were repeated at least three times, and identical results were obtained.

BiFC assay and confocal laser scanning microscopy. *N. benthamiana* leaves were used for agroinfiltration. Agrobacterium tumefaciens strain *EHA*105 carrying pP0-YFP^{C}, pP4-YFP^{C}, pP3-YFP^{C}, pP3(1-61)-YFP^{C}, pP3(62-199)-YFP^{C}, pP0-YFP^{N}, pP4-YFP^{N}, pP3-YFP^{N}, pP3(1-61)-YFP^{N}, or pP3(62-199)-YFP^{N} 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 OD_{600} of 0.6 with MMA buffer (10 mmol/l MES/NaOH, pH 5.6, 10 mmol/l $MgCl_2$, 200 mmol/l acetosyringone). For coinfiltration, equal volumes of combinations were mixed prior to infiltration. γb protein encoded by barley stripe mosaic virus could self-interact (Bragg and Jackson, 2004). Therefore the combination of pγb-YFP^{C}/pγb-YFP^{N} (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, P0/P5, 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.

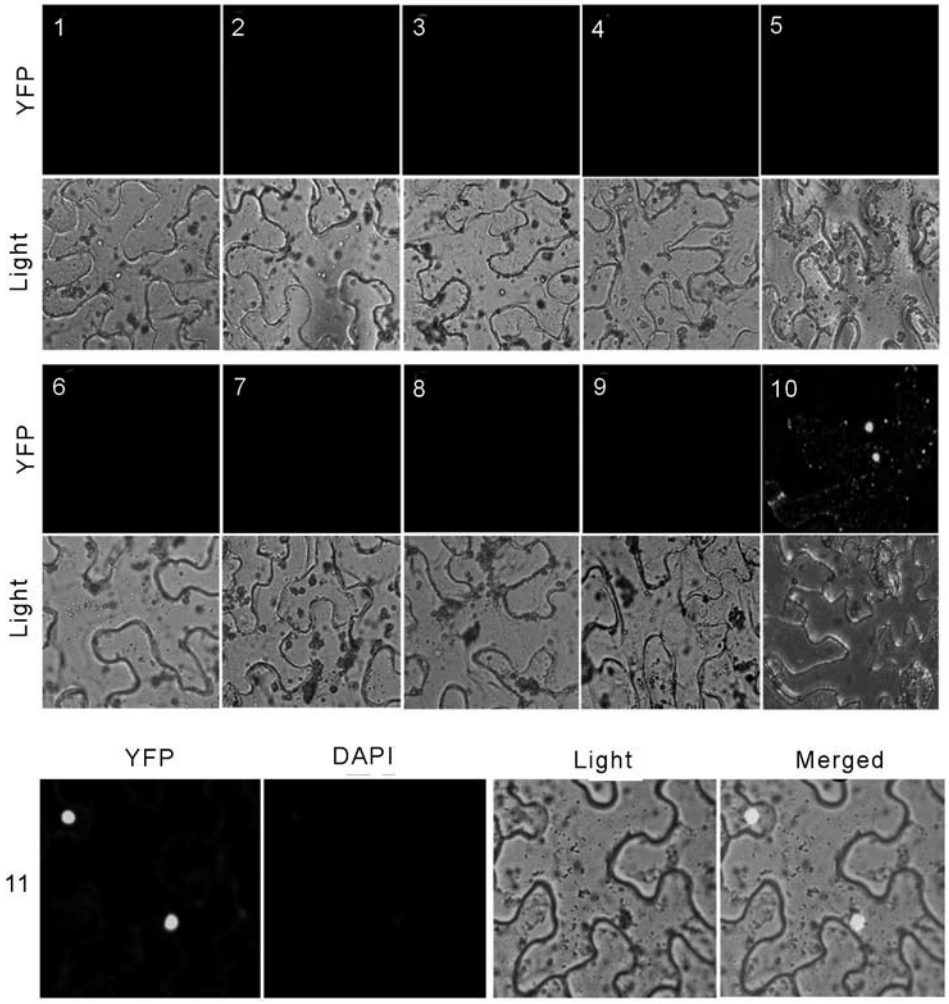

Fig. 1

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-YFP^N/pP0-YFP^C, pP0-YFP^N/pP3-YFP^C, pP0-YFP^N/pP4-YFP^C, pP0-YFP^C/pP3-YFP^N, pP0-YFP^C/pP4-YFP^N, pP3-YFP^N/pP4-YFP^C, pP3-YFP^C/pP4-YFP^N, pP4-YFP^N/pP4-YFP^C, pSPYNE-35S/pSPYCE-35S (negative control), pγb-YFP^C/pγb-YFP^N (positive control), and pP3-YFP^N/pP3-YFP^C, 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 CABYV 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-YFP^N/pP0-YFP^C, pP0-YFP^N/pP3-YFP^C, pP0-YFP^N/pP4-YFP^C, pP0-YFP^C/pP3-YFP^N, pP0-YFP^C/pP4-YFP^N, pP3-YFP^N/pP4-YFP^C, pP3-YFP^C/pP4-YFP^N, or pP4-YFP^N/pP4-YFP^C (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 the pair of pP3-YFP^N/pP3-YFP^C (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 CABYV 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 CABYV 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 CABYV P3 analyzed with PSORT II predition (http://psort.hgc.jp/form2.html) (data not shown here) might account for the nuclear targeting. We currently do not know why CABYV 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)-YFP^N/pP3(1-61)-YFP^C 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)-YFP^N/pP3(62-199)-YFP^C (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 CABYV 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 CABYV 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 *Luteovirid* coat protein, thus the domains involved in self-interaction of P3 might provide the clue to the understanding of polerovirus capsid assembly.

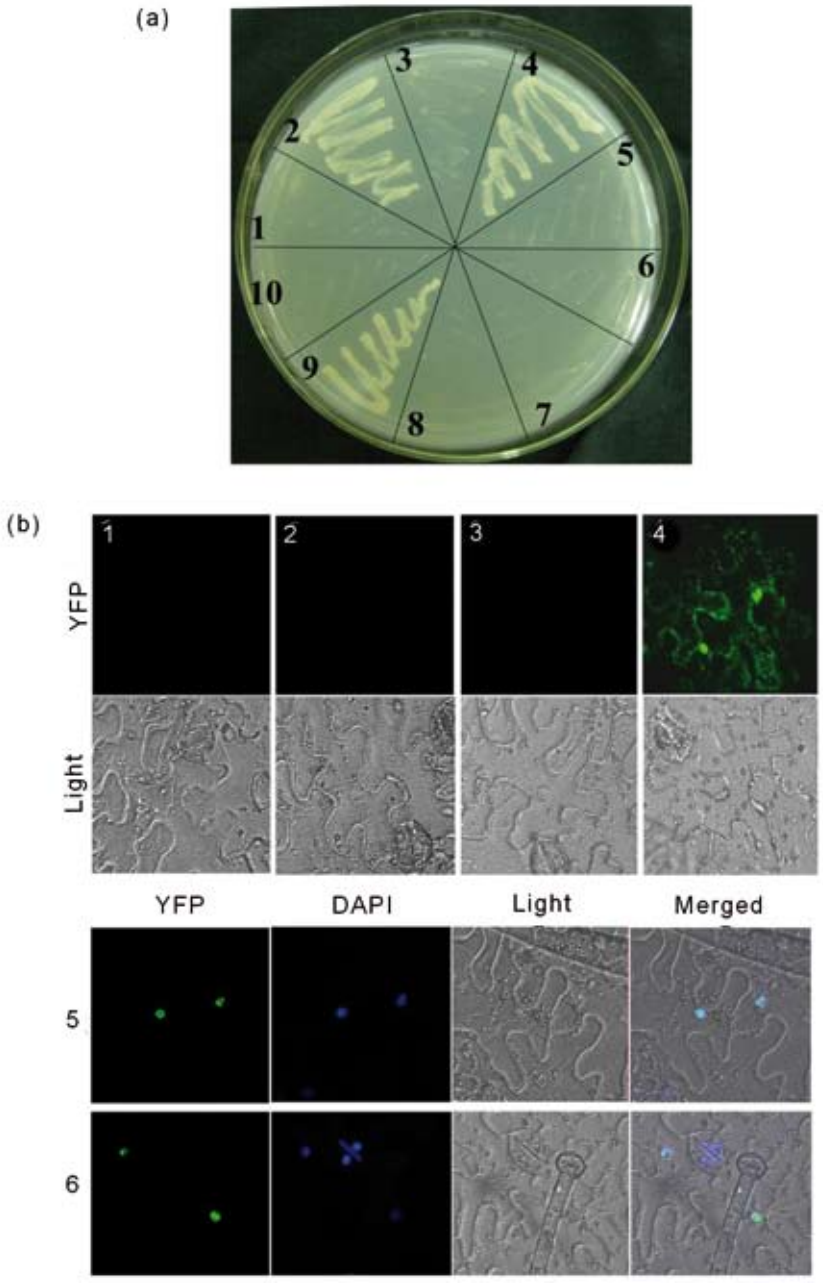

Fig.2

Domains involved in P3/P3 self-interaction

(a) Detection of domains required for P3/P3 interaction by YTHS. 1, pGBKT7/pGADT7 (negative control), 2, pGBKT7-53/pGADT7-RecT (positive control); 3, pGBK-P3(1-61)/pGADT7(negative control); 4, pGBK-P3(1-61)/pGAD-P3(1-61); 5, pGBKT7/pGAD-P3(1-61)(negative control); 6, pGBK-P3(62-199)/pGAD-P3(62-199); 7, pGBK-P3(62-199)/pGADT7(negative control); 8, pGBKT7/pGAD-P3(62-199)(negative control); 9, pGBK-p3(62-199)/pGAD-p3(1-61); 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.

Acknowledgements. This work was partially supported by the National Natural Science Foundation of China (31071663, 30971896), and the Natural Science Foundation of Beijing, P.R. China (6082006).

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