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Protein interaction matrix of Papaya ringspot virus type P based on a yeast two-hybrid system

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Summary. – Comprehensive analysis of the interactions between 10 mature proteins of Papaya ringspot virus type P (PRSV-P) was carried out based on a yeast two-hybrid system assay (YTHS). We detected 6 interactions between different viral proteins (VPg-P1, VPg-P3, VPg-CI, VPg-CP, NIaPro-CI, and NIb-P3) and 4 self-interactions (HC-Pro, VPg, NIaPro, and CP). These interactions did not show the same directionality as corresponding interactions detected in other potyviruses and consequently, a protein interaction matrix displayed different patterns. This initial map of the protein interactions of PRSV-P allows further study of various viral proteins in order to develop a new strategy to control PRSV-P infection.

Keywords: Potyvirus; protein-protein interaction; Papaya ringspot virus; yeast two-hybrid system

Introduction

Papaya (*Carica papaya* L.), an important fruit crop cultivated throughout tropical and subtropical regions, is well-known for its nutritional and healing values (Chandrika *et al*., 2003). However, papaya is severely affected by various diseases. A viral disease caused by PRSV-P (the genus *Potyvirus*, the family *Potyviridae*) occurs in most of papaya plantation areas and is considered to be as the most destructive (Bateson *et al*., 1994; Gonsalves, 1998). PRSV is grouped into two types, P and W. Type P infects papaya and cucurbits and type W infects only cucurbits (Gonsalves and Ishii, 1980; Roy *et al*., 1999). In papaya, PRSV-P causes severe mosaic and malformation of leaves, ringspots and streaking on fruits, water-soaked appearance on stems and petioles. The diseased plants become stunted and produce less fruits (Purcifull *et al*., 1984). PRSV is transmitted in a non-persistent manner by a number of aphid species (Purcifull *et al*., 1984; Gonsalves, 1998). PRSV is a positive single-stranded RNA virus and the genomes of different PRSV isolates have been completely sequenced and characterized (Yeh *et al*., 1992; Mangrauthia *et al*., 2008). Recently, we reported the complete genomic sequence of PRSV-P isolate occurring in Hainan Island, China. We also presented the evidence that this virus represented a unique and novel PRSV-P isolate (Lu *et al*., 2008). The viral genome of PRSV-P consists of 10,323 nucleotides (nt) and contains an ORF encoding a large polyprotein processed by virus-encoded proteases into 10 mature functional proteins, namely protein P1, helper component proteinase (HC-Pro), protein P3, 6 K protein 1 (6K1), cylindrical inclusion protein (CI), 6 K protein 2 (6K2), viral genome-linked protein (VPg), nuclear inclusion a proteinase (NIaPro), nuclear inclusion b protein (NIb), and coat protein (CP) (Urcuqui-Inchima *et al*., 2001; Lu *et al*., 2008). The proteins VPg and CP are found in virion, whereas remaining proteins are detected in the infected plants (Dougherty and Carrington, 1988). Most potyviral proteins considered as multifunctional are involved in virus replication, movement, assembly, synergism, aphid transmission, symptom expression, and RNA silencing (Urcuqui-Inchima *et al*., 2001). Some interactions between potyviral proteins play important roles in the viral infec-

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Abbreviations: AD = transcriptional activation domain; BD = DNA-binding domain; ClYVV = Clover yellow vein virus; PVA = Potato virus A; PRSV-P = Papaya ringspot virus type P; PSbMV = Pea seed-borne mosaic virus; SMV = Soybean mosaic virus; SYSV = Shallot yellow stripe virus; YTHS = yeast two-hybrid system

tion and host defense (Blanc *et al.*, 1997; Blanc *et al.*, 1998; Roudet-Tavert *et al.*, 2002; Whitham *et al.*, 2006).

In recent years, viral research methods have made a great progress. YTHS is the most common method for a high-throughput identification of potential protein-protein interactions (Suter *et al.*, 2008). As the number of virus-encoded proteins is limited, it is possible to detect all protein-protein interactions all the way through the genome (Uetz *et al.*, 2004). To date, some comprehensive analyses of protein interactions from a single virus using YTHS have been successfully completed in several potyviruses including Potato virus A (PVA), Pea seed-borne mosaic virus (PSbMV), Soybean mosaic virus (SMV) and Shallot yellow stripe virus (SYSV) (Guo *et al.*, 2001; Kang *et al.*, 2004; Lin *et al.*, 2009). These results indicate that the protein-protein interaction matrix or map of each potyvirus investigated by YTHS is different, although some interactions appear to be common among potyviruses. Namely, each potyvirus may have unique protein–protein interaction patterns (Yambao *et al.*, 2003). Knowledge of potyviral protein interaction maps would contribute to the understanding of initially intricate relationships among proteins and to gain a potential insight into the molecular mechanisms of potyvirus infection. Previous studies on PRSV-P focused mostly on the molecular characterization of viral genome and transgenic papaya resistance to PRSV-P, but the viral proteome and function of PRSV-P remained largely unknown (Guo *et al.*, 2001; Tecson Mendoza *et al.*, 2008; Tripathi *et al.*, 2008).

To investigate the modes of action and function of viral proteins from PRSV-P and to contribute insights into the molecular mechanism of PRSV-P infection, we adopted a GAL4 transcription activator-based YTHS for a protein interaction matrix involving ten PRSV-P proteins. We compared some viral protein interactions of PRSV-P with corresponding interactions reported previously for other potyviruses.

Materials and Methods

Production of PRSV cDNA. PRSV-P (Acc. No. EF183499) was collected in our laboratory (Lu *et al.*, 2008). Total RNA was extracted using TRIzol (Invitrogen) and RNA PCR Kit (AMV) Ver 3.0 (TaKaRa) was used for PRSV cDNA synthesis.

Plasmid construction. GAL4 two-hybrid phagemid vector system (Stratagene) was used to examine the interactions between all ten PRSV-encoded proteins (P1, HCPro, P3, 6K1, CI, 6K2, VPg, NIaPro, Nib, and CP) according to the instruction manual. Each of the 10 full-length genes encoding viral proteins was amplified by PCR from viral cDNAs of PRSV-P with high fidelity enzyme mix (Fermentas). All the primers (Table 1) used for amplification were designed by inserting of the appropriate restriction sites (*Bam*HI, *Eco*RI, *Nhe*I, *Pst*I, *Sal*I, and *Xho*I) that could be used for subcloning of the fragments from pMD18-T vector (Takara) into pBD-GAL4 Cam (*TRP1*, *Cam*^{r}) and pAD-GAL4-2.1(*LEU2, Amp*^{r}) vector (Stratagene). The accuracy of all fusion junction sequences and reading frames in the YTHS vectors were identified by DNA sequencing.

Extraction of yeast proteins and Western blot analysis. YTHS plasmids were transformed into YRG-2 yeast cells individually by lithium acetate method and then plated on SD/-Trp or SD/-Leu medium and grown at 30°C according to the manufacturer's protocol (Stratagene).The total proteins of a single positive yeast transformant were extracted (Atkin *et al.*,1995). Aliquots of 20 μg of protein were separated on 10% SDS-PAGE and transferred to a nitrocellulose membrane. The blots were probed with monoclonal antibodies GAL4-DBD or GAL4-TA diluted 1:300 (Santa Cruz Biotechnology) against BD or AD, respectively, and then with HRP-conjugated goat anti-rabbit IgG diluted 1:2,000 (Tiangen Biotechnology).

Yeast two-hybrid assay. The yeast (*Saccharomyces cerevisiae*) host strain YRG-2 (Stratagene) containing a dual selection reporter gene with *lacZ* and *HIS3* was used for a screening of yeast that generated an interaction between expressed proteins. The promoters that govern an expression of these two reporter genes are optimized to reduce a number of false positives. In addition, the YRG-2 strain also carries the auxotrophic markers, *leu2* and *trp1* for the selection of yeast that were transformed with YTHS vectors pAD-GAL4-2.1 and pBD-GAL4 Cam (Stratagene). To determine protein-protein interactions, the pAD-GAL4- 2.1 and pBD-GAL4 Cam fusion derivatives were cotransformed into YRG-2 cell sequentially and the transformants were screened on SD/-Trp/-Leu/-His medium for 4–7 days at 30°C according to the manufacturer's protocols. Yeast cells cotransformed with pLamin C (aa 67–230 of human lamin C) and pAD-WT (aa 32–236, wild-type lambda cI) were used as a negative control, whereas those cotransformed with pBD-WT and pAD-WT were used as a positive control (Stratagene). The β-galactosidase activity of the putative positive transformants was checked by filter assay (Guo *et al.*, 2001; Yambao *et al.*, 2003). All experiments were repeated at least three times.

Results

Confirmation of viral fusion protein expression in the yeast

To confirm that a negative interaction was not a result of the viral fusion proteins expression absence in YTHS, each of 10 mature protein genes (P1~ 63 K, HC-Pro~52 K, P3~40 K, 6K1~6 K, CI~72 K, 6K2~6 K, VPg~ 21 K, NIaPro~ 27 K, Nib~ 59 K, and CP~ 33 K) from PRSV-P was cloned correctly into both the pAD-GAL4-2.1 and pBD-GAL4 Cam plasmids to be expressed as fusion proteins with either the GAL4 AD (15 K) or the GAL4 BD (17.5 K). Western blot analysis showed that all AD and BD fusion proteins from PRSV-P could be expressed individually in YRG-2 yeast cells at a detectable level (Fig. 1). This finding provided a support to establish interactions that may have not been detected due to the absence of viral fusion proteins expression. Thus, all recombinant plasmids could be used in the YTHS assay.

Verification of recombinant plasmid suitability for YTHS

To eliminate possible false positive interactions that may occur in the pAD-GAL4-2.1 fusion that tend to interact with the GAL4 BD in the absence of interaction partner, the yeast

Table 1. Primers used for amplification of various genes encoding proteins of PRSV-P

Viral gene (size)	Primer (pBD-GAL4 Cam)*	Primer (pAD-GAL4-2.1)*
P1 (1641 nt)	5'-GC*GAATTC*ATGTCTTCATTGTACCAATTGCAAC-3' 5'-GC*GTCGAC*TCAGTATTGCTCCATATGCATGCGAAT-3'	5'-GC*GGATCC*ATGTCTTCATTGTACCAATTGCAAC-3' 5'-GC*GCTAGC*TCAGTATTGCTCCATATGCATGCGAAT-3'
HC-Pro (1371 nt)	5'-GC*GAATTC*AACGATGTCGCTGAGAAATTC-3' 5'-TA*GTCGAC*TCAACCAACAATGTAGTGCTTCATT-3'	5'-GC*GAATTC*AACGATGTCGCTGAGAAATTC-3' 5'-TA*CTCGAG*TCAACCAACAATGTAGTGCTTCATT-3'
P3 (1035 nt)	5'-TA*GTCGAC*TCGGAGAATTCGATCCAACTAC-3' 5'-GC*CTGCAG*TTATTGATGAATTACTGGTATGTAAAT-3'	5'-TA*GCTAGC*GGAGAATTCGATCCAACTAC-3' 5'-GC*CTCGAG*TTATTGATGAATTACTGGTATGTAAAT-3'
6K1 (156 nt)	5'-CG*GAATTC*GCAAAATCGGACAATGAAAAG-3' 5'-CG*GTCGAC*TTATTGATGATAAACACTTGTATTG-3'	5'-CG*GAATTC*GCAAAATCGGACAATGAAAAG-3' 5'-CG*CTCGAG*TTATTGATGATAAACACTTGTATTG-3'
CI (1905 nt)	5'-CA*GTCGAC*TCTCTTTGGATGACATTCGGG-3' 5'-TA*CTGCAG*CTATTGATGATACACAGCCCTC-3'	5'-CA*GCTAGCT*CTTTGGATGACATTCGGG-3' 5'-CA*CTCGA*GCTATTGATGATACACAGCCCTC-3'
6K2 (171 nt)	5'-TA*GAATTC*AGCGTTGATGGGGTTAAGCAC-3' 5'-GC*GTCGAC*CTATTGATGGAACACGTGAACATTTG-3'	5'-TA*GAATTC*AGCGTTGATGGGGTTAAGCAC-3' 5'-GC*CTCGAG*CTATTGATGGAACACGTGAACATTTG-3'
VPg (567 nt)	5'-TA*GAATTC*GGTTTCTCCGCGCGAC-3' 5'-GC*GTCGAC*CTATTCATGGTGAACAGATTTTG-3'	5'-TA*GAATTC*GGTTTCTCCGCGCGAC-3' 5'-GC*CTCGAG*CTATTCATGGTGAACAGATTTTG-3'
NIaPro (714 nt)	5'-GC*GAATTC*GGAAAGAGTCTTTGCCAAGG-3' 5'-GC*GTCGAC*TCACTGCTCAAAAACATTTAATTGATT-3'	5'-GC*GAATTC*GGAAAGAGTCTTTGCCAAGG-3' 5'-GC*CTCGAG*TCACTGCTCAAAAACATTTAATTGATT-3'
NIb (1611 nt)	5'-GC*GAATTC*AGTGGAAGTCGATGGCTTT-3' 5'-GC*GTCGAC*TTACTGGTGAAATACATGTGCAT-3'	5'-GC*GAATTC*AGTGGAAGTCGATGGCTTT-3' 5'-CA*CTCGAG*TTACTGGTGAAATACATGTGCAT-3'
CP (861 nt)	5'-TA*GTCGAC*TCAATTACGCATACCCAGGAGAGAG-3' 5'-GC*GAATTC*TCCAAAAATGAAGCTGTGGATGC-3'	5'-GC*GCTAGC*TCCAAAAATGAAGCTGTGGATGC-3' 5'-TA*CTCGAG*TCAATTACGCATACCCAGGAGAGAG-3'

*Restriction sites are in italics.

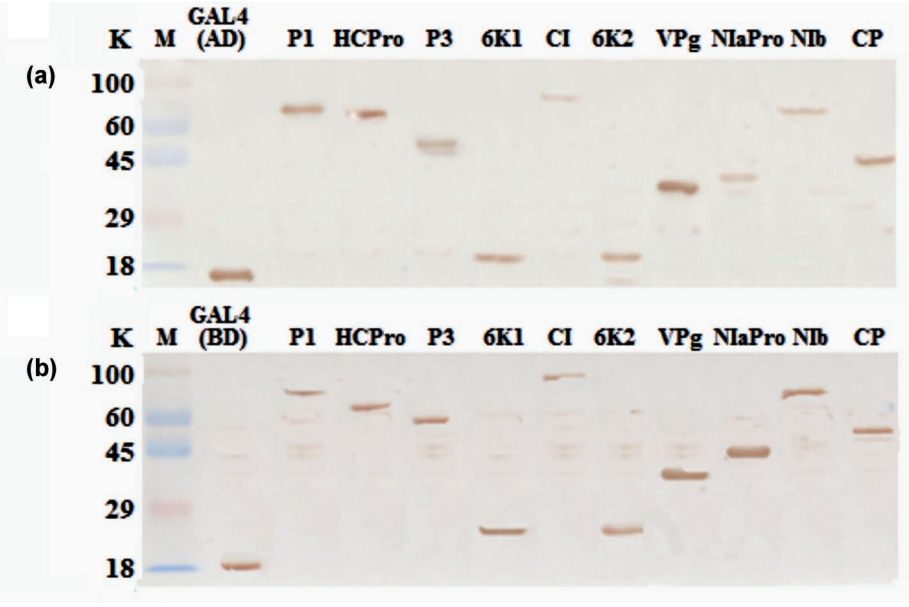

Fig. 1

Western blot analysis of AD (a) and BD (b) fusion proteins of PRSV-P expressed in yeast cell YRG-2

Protein size marker (lane M).

cells transformed with recombinant pAD-GAL4-2.1 DNAs carrying viral genes and the empty pBD-GAL4 Cam vector were first detected on SD/-Trp/-Leu/-His medium at 30°C. Similar to the negative control, no colonies were observed on the synthetic medium for up to 5 days of incubation. The same result was obtained, when the reciprocal combination

Table 2. Protein-protein interaction matrix of PRSV-P based on YTHS

Binding domain	Activation domain									
	P1	HC-Pro	P3	6K1	CI	6K2	VPg	NIaPro	NIb	CP
P1	–	–	–	–	–	–	++	–	–	–
HC-Pro	–	++	–	–	–	–	–	–	–	–
P3	–	–	–	–	–	–	+	–	+	–
6K1	–	–	–	–	–	–	–	–	–	–
CI	–	–	–	–	–	–	+	+	–	–
6K2	–	–	–	–	–	–	–	–	–	–
VPg	++	–	+	–	+	–	+++	–	–	++
NIaPro	–	–	–	–	+	–	–	+	–	–
NIb	–	–	+	–	–	–	–	–	–	–
CP	–	–	–	–	–	–	++	–	–	+++

Colonies turned blue in 2 hrs (+++), 2–6 hrs (++) and 6–12 hrs (+). No colonies appeared on the selection medium after 5 days or colonies from transformant selection medium did not turn blue after 12 hrs (–).

of the empty pAD-GAL4-2.1 vector and the recombinant pBD-GAL4 Cam DNAs carrying viral genes was tested. The results suggested that all recombinant constructs were suitable for the detection of protein-protein interactions in YTHS.

Identification of protein-protein interactions of PRSV-P based on YTHS

A total of 100 individual interaction combinations between all 10 viral proteins of PRSV-P were evaluated in a pairwise matrix based on YTHS. As a result, 16 combinations (Table 2) were detected by screening of the yeast cotransformants on SD/-Trp/-Leu/-His medium for up to 5 days at 30°C. Six pairs of interactions (VPg-P1, VPg-P3, VPg-CI, VPg-CP, NIaPro-CI, and NIb-P3) were detected, when these proteins were expressed as fused not only with BD and AD, but also with AD and BD, respectively. These results implied that the detected interactions could occur in both directions. In addition, 4 self-interactions for HC-Pro, VPg, NIaPro, and CP were observed. In the filter assay, each positive clone harboring interacting partner developed a visible blue color in different time period depending on the activation of reporter gene *lacZ* (Table 2). The color development correlated with β-galactosidase activity and therefore the intensity of color could represent the relative strengths of different interactions.

Discussion

In this study, all detected protein-protein interactions were symmetrical and dissimilar to the same interactions detected in other potyviruses such as PVA, PSbMV, SMV, SYSV, and Clover yellow vein virus (ClYVV) (Guo *et al.*, 2001; Yambao *et al.*, 2003; Lin *et al.*, 2009). For example, the interaction between P3 and NIb of SMV was detected only when both proteins were expressed in fusion with AD and BD, but the same interaction in SYSV was observed in the opposite direction (Lin *et al.*, 2009). The different directionality between some viral protein interactions suggested that the fused protein might have different protein folding or exposure of binding sites in both directions. However, it was suggested to consider such interaction examples with some caution (Guo *et al.*, 2001; Lin *et al.*, 2009).

Four self-interactions (HC-Pro, VPg, NIaPro, and CP) reported previously for PVA, PSbMV, SMV, SYSV, and ClYVV were also detected in this work (Guo *et al.*, 2001; Yambao *et al.*, 2003; Kang *et al.*, 2004; Lin *et al.*, 2009), but a self-interaction of the same viral protein had different level of intensity when compared to various potyviruses. In our hands, the CP-CP and VPg-VPg interactions were found as strong ones, while NIaPro self-interaction represented a weak one. The CP-CP interaction was suggested to have a significant role in the encapsidation of viral RNA (Kang *et al.*, 2004) and VPg-VPg interaction might be involved in viral genomic RNA replication and translation (Urcuqui-Inchima *et al.*, 2001; Guo *et al.*, 2001; Yambao *et al.*, 2003). The C-terminal region of VPg in ClYVV was proven as important for the VPg self-interaction (Yambao *et al.*, 2003). In addition, a moderate self-interaction of HC-Pro was also detected in our experiments. Some previous studies concluded that N- and C-termini of PVA HC-Pro were required for a self-interaction, while N-terminus was important for PVY and Lettuce mosaic virus HC-Pro self-interaction (Guo *et al.*, 1999; Urcuqui-Inchima *et al.*, 1999; Plisson *et al.*, 2003).

Potyvirus VPg is linked to the 5' end of the viral genome (Urcuqui-Inchima *et al.*, 2001) and showed a wide range of

interactions with 4 viral proteins (P1, P3, CI, and CP) besides its self-interaction. These 4 interactions have not been reported previously for any potyvirus except for SYSV very recently (Lin *et al.*, 2009). But in this study the interaction between VPg and P3 or CP did not show the same directionality as the same interaction in SYSV (Lin *et al.*, 2009). Newly, VPg is considered as a multifunctional protein including its function as a virulence factor (Grzela *et al.*, 2008). Natural plant resistance to the potyvirus infection may be due to the inability of VPg to interact with eukaryotic translation initiation factor eIF4E (Wittmann *et al.*, 1997; Grzela *et al.*, 2006; Charron *et al.*, 2008). Previous studies revealed that P1, P3, CI, and CP had various functions in the potyvirus infection (Urcuqui-Inchima *et al.*, 2001). Therefore, VPg implication in multiple interactions with potyviral or host proteins might be essential for viral translation, replication and movement that contribute to the identification of multiple functions of VPg in the potyvirus life cycle.

A newly described interaction between NIaPro and CI proteins from SMV or SYSV was also observed (Lin *et al.*, 2009). NIaPro is a major proteinase responsible for processing of the potyviral polyprotein into functional products (Urcuqui-Inchima *et al.*, 2001). Amino acid Lys27 of NIaPro determines the host-specificity of PRSV-P and PRSV-W types (Roy *et al.*, 1999; Chen *et al.*, 2008). The inability of PRSV-W to infect papaya may be due to its inability to carry out viral replication or movement in the host (Chen *et al.*, 2008). The CI protein forms the cylindrical inclusions typical of potyviral infection and is involved in the genome replication and virus movement (Gómez de Cedrón *et al.*, 2006; Gabrenaite-Verkhovskaya *et al.*, 2008). Hence, identification of the interaction between NIaPro and CI in the potyvirus infection process might be useful for the further research.

To our knowledge, this is the first report about the interactions involving 10 viral proteins of PRSV-P examined by YTHS matrix screen. Although the YTHS approach has some limitations and our findings have to be confirmed by other assays (Uetz *et al.*, 2004; Suter *et al.*, 2008), we believe that the observed protein interactions of PRSV-P can provide a platform for further study of viral protein functions. Transgenic papaya resistance to PRSV-P is generally considered as the best strategy for long-term virus control. However, different specificities of PRSV resistance were presented in transgenic papaya lines expressing different PRSV CP genes against PRSV isolates from different geographic origins. Moreover, the use of transgenic papaya lines is limited in other areas of the world, because of the strain-specific resistance (Chiang *et al.*, 2001; Bau *et al.*, 2003). Thus, our findings can serve as a basis for elucidation of the interaction relationship between virus and host and develop a new effective broad-spectrum strategy to control PRSV-P especially with the availability of papaya genome database that has been recently published (Ming *et al.*, 2008).

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