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A set of host proteins interacting with papaya ringspot virus NIa-Pro protein identified in a yeast two-hybrid system

L. GAO[1, 2#], W. T. SHEN[2*#], P. YAN[2], D. C. TUO[2], X. Y. LI[2], P. ZHOU[2*]

[1]College of Agriculture, Hainan University, Haikou 570228, Hainan, P.R. China; [2]Institute of Tropical Bioscience and Biotechnology & Analysis and Testing Center of Chinese Academy of Tropical Agricultural Science, Key Laboratory of Biology and Genetic Resources of Tropical Crops, Ministry of Agriculture, Haikou 571101, Hainan, P.R. China

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Summary. – The protein-protein interactions between viral and host proteins play an essential role in plant virus infection and host defense. The potyviral nuclear inclusion protein a protease (NIa-Pro) is involved in various steps of viral infection. In this study, the host proteins interacting with papaya ringspot virus (PRSV) NIa-Pro were screened in a *Carica papaya* L. plant cDNA library using a Sos recruitment two-hybrid system (SRS). We confirmed that the full-length EIF3G, FBPA1, FK506BP, GTPBP, MSRB1, and MTL from papaya can interact specifically with PRSV NIa-Pro in yeast, respectively. These proteins fufill important functions in plant protein translation, biotic and abiotic stress, energy metabolism and signal transduction. In this paper, we discuss possible functions of interactions between these host proteins and NIa-Pro in PRSV infection and their role in host defense.

Keywords: Sos recruitment two-hybrid system; papaya ringspot virus; NIa-Pro; protein-protein interaction

Introduction

Papaya ringspot virus (PRSV, the genus *Potyvirus*, the family *Potyviridae*) is considered to be the most destructive virus that occurs in almost all papaya plantations of the world (Gonsalves, 1998). PRSV genome has a positive-sense single-stranded RNA of 10,323–10,326 nt in length and encodes a 381-kDa polyprotein, which is processed into one structural and nine non-structural proteins via three virus-encoded proteinases named P1 protein (P1), helper component proteinase (HC-Pro) and NIa-Pro (Yeh and Gonsalves, 1985; Yeh *et al*., 1992). As a multifunctional proteinase, potyviral NIa-Pro has been intensively studied. NIa-Pro is a trypsin-like cysteine protease that cleaves the virus polyprotein at seven distinct locations and has independent nuclear localization signals that allow it to accumulate in the nucleus (Kang *et al*., 2001; Hajimorad *et al*., 1996). NIa-Pro also has RNA binding and nonspecific DNase activity, suggesting its involvement in virus replication and host cell DNA degradation in later stages of infection cycle (Daros and Carrington, 1997; Anindya and Savithri, 2004). Furthermore, it is reported that PVY NIa-Pro acts as an elicitor by its structure binding to or cleavage of host-encoded protein (s) to elicit the Ry-mediated disease resistance in potato (Mestre *et al*., 2000, 2003). There are two types of PRSV: type P, which infects papaya and cucurbits, and type W, which infects cucurbits but not papaya (Roy *et al*., 1999). The amino acid Lys[27] of NIa-Pro determines host specificity of PRSV for papaya infection (Chen *et al*., 2008). Thus, NIa-Pro as a multifunctional protein would be expected to involve multiple interactions with different host factors.

*Corresponding authors. E-mail: dnaswt@hotmail.com, zhp6301@126.com; phone: +86-898-66890687. #These authors contributed equally to this work.

Abbreviations: eIF = eukaryotic translation initiation factor; FBPA1 = fructose 1, 6 bisphosphate aldolase class 1 protein; FK506BP = fk506-binding protein; GTPBP = GTP-binding family protein; Hc-Pro = helper component proteinase; MSRB1 = methionine sulfoxide reductase B1 protein; MTL = metallothionein-like protein; NIa-Pro = nuclear inclusion protein a protease; PRSV = papaya ringspot virus; SRS = Sos recruitment two-hybrid system; EIF3G = eukaryotic translation initiation factor 3G protein

Host proteins play important roles in viral infection cycle and can interact with potyviral proteins to allow or overcome viral infection. The discovery of interacting plant and viral proteins can help to elucidate molecular mechanisms of viral infection and host defense. Previously, the yeast two-hybrid system has been used to identify host proteins that interact with potyviral proteins such as P1 (Shi *et al.*, 2007), HC-Pro (Jin *et al.*, 2007; Cheng *et al.*, 2008; Shen *et al.*, 2010; Ala-Poikela *et al.*, 2011), viral genome-linked protein (VPg) (Léonard *et al.*, 2000; Léonard *et al.*, 2004; Khan *et al.*, 2006; Michon *et al.*, 2006), cylindrical inclusion body (Jiménez *et al.*, 2006), nuclear inclusion protein b (Dufresne *et al.*, 2008) or coat protein (McClintock *et al.*, 1998; Feki *et al.*, 2005; Hofius *et al.*, 2007). However, little is known about the host proteins that interact with the NIa-Pro protein. In this study, PRSV NIa-Pro protein was used as a bait to screen a cDNA library of papaya plants using SRS in yeast cytoplasm (Broder *et al.*, 1998). Finally, we confirmed that six different host encoded proteins from the papaya cDNA library, with high sequence identity to the eukaryotic translation initiation factor 3G protein (EIF3G), fructose 1, 6 bisphosphate aldolase class 1 protein (FBPA1), fk506-binding protein (FK506BP), GTP-binding family protein (GTPBP), methionine sulfoxide reductase B1 protein (MSRB1) and metallothionein-like protein (MTL), can interact specifically with PRSV NIa-Pro in yeast. Furthermore, we discuss the possible function of these host proteins in PRSV infection and host defense.

Materials and Methods

Virus isolates and plant materials. PRSV-P isolate HN (Lu *et al.*, 2008) was isolated in our laboratory. *C. papaya* L. seedlings, with 7–8 true leaves, were kept at 28°C in a greenhouse under a 16 hrs photoperiod and 8 hrs darkness cycle.

NIa-Pro bait plasmid construction. The complete nucleotide sequence encoding the NIa-Pro protein was amplified by PCR from full-length cDNA of PRSV-P isolate HN (GenBank Acc. No. EF1834997) using primers NP-F and NP-R (Table 1) (Lu *et al.*, 2008). The PCR product was digested and subcloned into the *Bam*HI/*Sac*I site of the bait vector pSos (Leu^+) (Stratagene, USA), to be fused with the hSos sequence, resulting in pSos-NP construct. The accuracy of the NIa-Pro fusion junction sequence and the reading frames in the bait vector were identified by DNA sequencing.

Papaya cDNA library construction. Total RNA was extracted from three whole healthy *C. papaya* L. plants at the 7–8 leaf stage using TRIzol (Invitrogen, USA). Poly $(A)^+$-RNA (5.0 μg), isolated with an Oligotex mRNA kit (Qiagen, Germany), was used for cDNA synthesis. cDNAs larger than 500 bp as *Eco*RI-*Xho*I fragments were inserted into the pMyr vector (Ura^+) (Stratagene, USA) to be expressed with a myristoylation signal, which anchors the proteins in the membrane. The pMyr-cDNAs were transformed into XL10-Gold Kan super-competent *Escherichia coli* cells (Stratagene, USA), as described in the CytoTrap™ XR Library Construction Kit instruction manual (Stratagene, USA). The cDNA library plasmids were isolated using a Plasmid Maxi kit (Qiagen, Germany).

Screening of the papaya cDNA library using SRS. The yeast cdc25H strains co-transformed with pMyr-cDNA library plasmids (40 μg) and pSos-NP plasmids (40 μg) were initially selected by plating onto SD/glucose (–UL) at room temperature (22–25°C) for 48 hrs, as detailed in the manufacturer's instructions (Stratagene, USA); the transformants were then replica-plated onto SD/galactose (–UL) plates using velvet pads to induce the expression of the Sos-NIa-Pro bait at 37°C. After 6 days, the growing colonies (candidate for protein interaction) were picked up and resuspended in sterile H_2O. Resuspended co-transformants were patched (dotted) onto SD/glucose (–UL) plates using a pin multi-blot replicator and incubated for 2 days at 25°C. Putative positives were then identified among the candidates by two rounds of patching cell tests for growth on SD/galactose (–UL) or SD/glucose (–UL) plates for 6 days at either 25°C or 37°C. Meanwhile, cdc25H yeast cells co-transformed with either pSos-MAFB /pMyr-MAFB (Stratagene, USA) or pSos-MAFB/ pMyr-Lamin C (Stratagene, USA) were used as standard positive and negative controls, respectively, in all two-hybrid assays. Finally, the pMyr plasmids were isolated from yeast positive protein interaction candidates and amplified in *E. coli* for DNA sequence analysis; further retransformation of the plasmids with the pSos-NP bait construct into yeast cells was conducted as a false positive test.

Positive clone analysis and acquisition of the full-length cDNAs. After sequencing, the positive clones were analyzed and classified by using a BLAST search of the GenBank database. To obtain the full-length cDNAs of PaEIF3G, PaFBPA1, PaFK506BP, PaGTPBP, PaMSRB1, and PaMTL, RT-PCR were carried out using the primers PaEIF3G-F/PaEIF3G-R, PaFBPA1-F/PaFBPA1-R, PaFK506BP-F/PaFK506BP-R, PaGTPBP-F/PaGTPBP-R, PaMSRB1-F/PaMSRB1-R, and PaMTL-F/PaMTL-R, respectively (Table 1). The specific primers were designed according to the sequence alignment with whole-genome shotgun reads (wgs) of *C. papaya* L. in GenBank. The full-length cDNAs were cloned into pMD18-T (Takara, Japan) and verified by sequencing. The amino acid sequences were analyzed using the PROSITE database.

SRS/CytoTrap two-hybrid assay. The full-length cDNAs of PaEIF3G, PaFBPA1, PaFK506BP, PaGTPBP, PaMSRB1, and PaMTL, were cloned into pMyr via the *Eco*RI/*Sal*I sites to form pMyr-PaEIF3G, pMyr-PaFBPA1, pMyr -PaFK506BP, pMyr-PaGTPBP, pMyr-PaMTL, and pMyr-PaMSRB1, respectively. *Saccharomyces cerevisiae* strain cdc25H was co-transformed with the pMyr-PaEIF3G/pSos-NP, pMyr-PaFBPA1/pSos-NP, pMyr-PaFK506BP/pSos-NP, pMyr-PaGTPBP/pSos-NP, pMyr-PaMSRB1/pSos-NP, and pMyr-PaMTL/pSos-NP, as described above, according to the manufacturer´s instructions (Stratagene, USA).

Table 1. Primers used in full-length cDNA clone and plasmid construction

Primers	Primer sequences*	Genes	Constructs
NP-F	5′-TAGGATCCCCGGAAAGAGTCTTTGCCAAGGCATGAGGAATT-3′		
NP-R	5′-GCGGAGCTCTTTACTGCTCAAAAACATTTAATTGATTG-3′	NIa-Pro	pSos-NP
PaEIF3G-F	5′-GCCGGAATTCGCGATTGACAAAACTGA-3′		
PaEIF3G-R	5′-GGCGGTCGACCTAGTTTGCTCTAGGAGTC-3′	PaEIF3G	pMyr-PaEIF3G
PaFBPA1-F	5′-GCCGGAATTCGCCTCTGCTTCTTTCCTCAAGTCTT-3′		
PaFBPA1-R	5′-GGCGGTCGACTTAGTAAACGTAGCCCTTAACGAAC-3′	PaFBPA1	pMyr-PaFBP1
PaFK506BP-F	5′-TAGAGAATTCGCGGTCTCTGCTTTCG-3′		
PaFK506BP-R	5′-GAGGTCGACCTTTGCATTCCCAGAGT-3′	PaFK506BP	pMyr-PaFK50BP
PaGTPBP-F	5′-TAGAGAATTCGTGAAGAAGAAAGAAAAGAC-3′		
PaGTPBP-R	5′-GAGGTCGACTCAGTTGATAGGTACCTGCT-3′	PaGTPBP	pMyr-PaGTPBP
PaMSRB1-F	5′-TAGAGAATTCGCTCCTCTGCTTCTTCTC -3′		
PaMSRB1-R	5′-GAGGTCGACTGATTTCGGTTTCAGTCTC-3′	PaMSRB1	pMyr-PaMSRB1
PaMTL-F	5′-TAGAGAATTCTCGGACACCTGCGGCAA-3′		
PaMTL-R	5′-GAGGTCGACTCAGTGACCGCAGGTGC-3′	PaMTL	pMyr-PaMTL

*Restriction sites are underlined.

Results

Auto-activation of bait plasmid

Cdc25H yeast co-transformed with pSos-NP and pMyr were able to grow on SD/galactose (–UL) medium (at 25°C) and SD/glucose (–UL) medium (at 25°C) but not in SD/galactose (–UL) medium (at 37°C) or SD/glucose (–UL) medium (at 37°C) (Fig. 1). Therefore, it could be used in the screening of NIa-Pro-binding protein in the yeast SRS.

Isolation of PRSV NIa-Pro interacting host proteins

To identify host proteins interacting with PRSV NIa-Pro, a cDNA library was constructed from *C. papaya* L. seedlings. Using yeast SRS, 1.85×10^6 independent yeast transformants were screened using PRSV NIa-Pro as bait. In total, 35 positive candidate clones were identified after two rounds of testing for galactose-dependent growth at 37°C. Then, the cDNA plasmids of these 35 colonies were rescued and the specificity of the interaction was verified by retransformation into yeast strain cdc25H cells in combination with the bait pSos-NP or control plasmids. Based on sequence alignment with the relevant homologous sequence from *C. papaya, Arabidopsis thaliana, Gossypium arboreum, Ricinus communis, Populus trichocarpa, Platanus acerifolia, Oryza sativa,* and *Populus tremula,* the cDNA inserts of the 35 candidate plasmids encoded proteins which were identified as six different host proteins: EIF3G, FBPA1, FK506BP, GTPBP, MSRB1, and MTL. According to sequence alignments with whole-genome shotgun reads of *C. papaya* L. in GenBank, the full-length cDNAs of the six genes were cloned by RT-PCR and designated as PaEIF3G (GenBank Acc. No. JN008890), PaFBPA1 (GenBank Acc. No. JN008888), PaFK506BP (GenBank Acc. No. JN008892), PaGTPBP (GenBank Acc. No. JN008891), PaMSRB1 (GenBank Acc. No. JF431992) and PaMTL (GenBank Acc. No. JN008889).

Interaction of NIa-Pro with full-length papaya proteins in yeast

The full-length cDNAs of six genes were inserted into the pMyr vector. The resulting six plasmids, pMyr-PaEIF3G, pMyr-PaFBPA1, pMyr-PaFK506BP, pMyr-PaGTPBP, pMyr-PaMSRB1 and pMyr-PaMTL, were co-transformed with pSos-NP into cdc25H cells, whereas pMyr-PaEIF3G/pSos, pMyr-PaFBPA1/ pSos, pMyr-PaFK506BP/pSos ,pMyr-PaGTPBP/pSos, pMyr-PaMSRB1/pSos and pMyr-PaMTL/pSos were co-transformed into cdc25H as negative controls. Only transformants with the pMyr-PaEIF3G/pSos-NP, pMyr-PaFBPA1/pSos-NP, pMyr-PaFK506BP/pSos-NP, pMyr-PaGTPBP/pSos-NP, pMyr-PaMSRB1/pSos-NP and pMyr-PaMTL/pSos-NP co-transformed yeasts and the positive controls could grow on SD/galactose (–UL) medium (at 37°C) (Fig. 1). The result of these 15 independent complementation experiments indicates that the specific interactions between these six host proteins and PRSV NIa-Pro can occur in the yeast cells, respectively.

Discussion

In this study, the bait vector pSos-NP and papaya cDNA library were constructed using the SRS/CytoTrap two-hybrid system to screen for host proteins that interact with the PRSV NIa-Pro. In total, we isolated six functional proteins from papaya (PaEIF3G, PaFBPA1, PaFK506BP, PaGTPBP,

PaMSRB1, and PaMTL) interacting with PRSV NIa-Pro protein, respectively.

One of the first steps in translation of viral RNAs by the host machinery is the recruitment of mRNAs by the translation eukaryotic initiation factors (eIFs). Many studies showed that the interaction between eIF4E/4G and the potyviral genome-linked protein VPg is crucial for successful completion of the potyviral life cycle (Charron *et al.*, 2008; Léonard *et al.*, 2000; Michon *et al.*, 2006; Khan *et al.*, 2006, 2009) and eIF4E and its isoform, eIF(iso)4E can mediate virus resistance in several plant-potyvirus interactions (Zhang *et al.*, 2006). Recent study demonstrates that HC-Pro is also an interaction partner of eIF(iso)4E and eIF4E and contains a 4E Binding Motif (Ala-Poikela *et al.*, 2011). In this work, we identified a new eukaryotic translation initiation factor eIF3G from papaya interacting with PRSV NIa-Pro .This finding can help to understand a novel role of eIF in virus invasion and host defense.

Plants had evolved complicated mechanisms to solve problems with a variety of environmental stresses and stress-related proteins play important roles in these mechanisms. Previous reports have showed that the metallothionein-like protein (MTL) (Choi *et al.*, 1996; Hsieh *et al.*, 1995; Buchanan-Wollaston, 1994), methionine sulfoxide reductase B (MSRB) (Oh *et al.*, 2010; Tarrago *et al.*, 2009) and fk506-binding protein (FK506BP) (Lima *et al.*, 2006; Ingelsson *et al.*, 2009) play important roles in various biotic and abiotic stress responses of plants, such as heavy metals, oxidative stress and pathogen attack. In this work, we confirmed that FK506BP, MSRB1 and MTL from papaya can interact with PRSV NIa-Pro, respectively, suggesting that these interactions may be involved in plant stress response pathways and could interfere with virus infection or host defense during host-virus interaction.

Fructose-1, 6-bisphosphate aldolase (FBPA) is a key enzyme in Calvin cycle (Marsh and Lebherz, 1992) and can regulate the rate of photosynthesis in higher-plant chloroplast (Iwaki *et al.*, 1991). In this work, we identified a FBPA from papaya (PaFBPA1) interacting with PRSV NIa-Pro and propose that this interaction may be involved in some chloroplast functions. It has been previously reported that

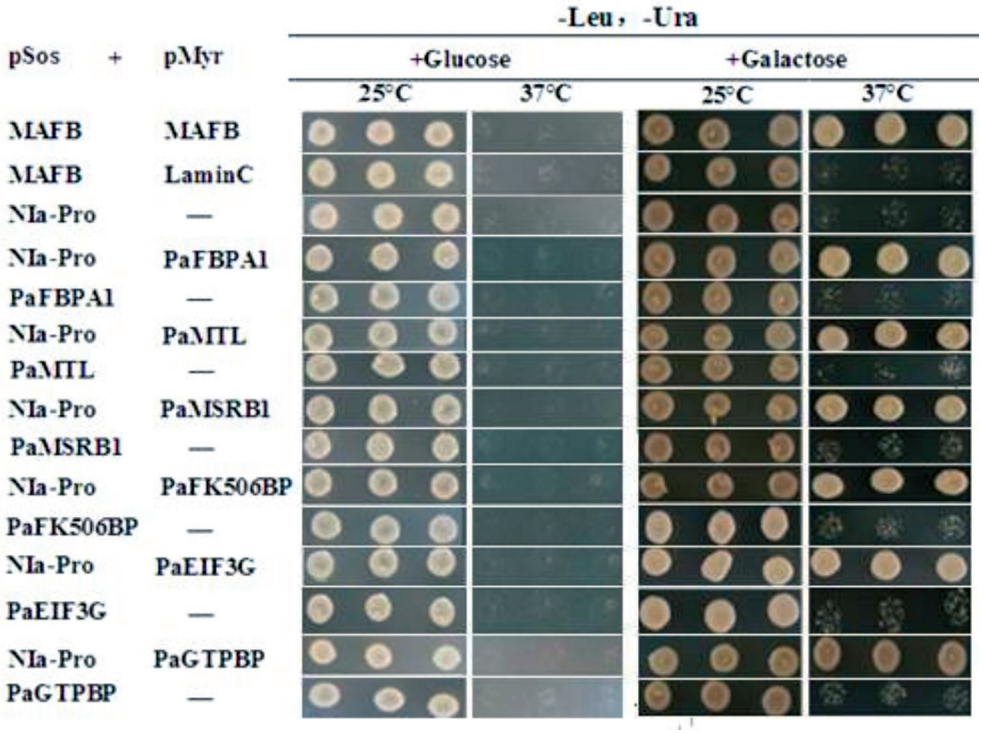

Fig. 1

Detection of interaction between PRSV NIa-Pro and host proteins (PaEIF3G, PaFBPA1, PaFK506BP, PaGTPBP, PaMSRB1, and PaMTL) by the Sos recruitment assay

S. cerevisiae strain cdc25H was transformed with the indicated plasmid combinations. Three colonies from each transformant were picked up, resuspended and diluted to optical densities at 600 nm of 0.5 in sterile water. An aliquot of 2.5 ml of each dilution was patched in rows onto each of two synthetic glucose minimal medium without leucine and uracil [SD/glucose (–UL)] and two synthetic galactose minimal medium without leucine and uracil [SD/galactose (–UL)] plates and one of each type of plate was incubated at the permissive or non-permissive temperature (25 or 37°C) for 6 days to compare the growth of yeast. pMyr-MAFB and pSos-MAFB were used as positive controls. pMyr-Lamin C and pSos-MAFB were used as negative controls.

potyviral coat protein or HC-Pro could alter chloroplast numbers, morphology and inhibit their functions in infected plants by interacting with some host proteins associated with chloroplasts, such as the large subunit of Rubisco (Feki *et al.*, 2005), 37-kDa chloroplast protein (McClintock *et al.*, 1998), the chloroplast division-related factor NtMinD (Jin *et al.*, 2007) and precursor of ferredoxin-5 (Cheng *et al.*, 2008; Pompe-Novak *et al.*, 2001). In addition, we demonstrated that NIa-Pro interacts with the GTP-binding family protein of papaya, namely PaGTPBP with diverse functions including signal transduction, secretion and regulation of cytoskeleton (Ma, 1994; Tuteja, 2009).

In summary, using SRS we isolated six papaya proteins, which interact with PRSV NIa-Pro specifically. Our results can help to gain insight into roles of this multifunctional protein NIa-Pro in the cycle of PRSV infection. However, the functions of all these interactors remain to be determined through the transgenic papaya plants in which the genes are over-expressed or silenced.

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