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Interaction Study of *Soybean mosaic virus* Proteins with Soybean Proteins using the Yeast-Two Hybrid System

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Interactions between viral proteins and host proteins are essential for virus replication. Especially, translation of viral genes completely depends on the host machinery. In potyviruses, interactions of genome-linked viral protein (VPg) with host translation factors including eIF4E, eIF(iso)4E, and poly(A)-binding protein (PABP) has previously been characterized. In this study, we investigated interactions between *Soybean mosaic virus* (SMV) viral proteins and host translation factors by yeast two-hybrid system. SMV VPg interacted with eIF4E, eIF(iso)4E, and PABP in yeast two-hybrid system, while SMV helper component proteinase (HC-pro) interacted with neither of those proteins. The interaction between SMV NIb and PABP was also detected. These results are consistent with those reported previously in other potyviruses. Interestingly, we found reproducible and specific interactions between SMV coat protein (CP) and PABP. Deletion analysis showed that the region of CP comprising amino acids 116 to 206 and the region of PABP comprising amino acids 520 to 580 are involved in CP/PABP interactions. Soybean library screening with SMV NIb by yeast two-hybrid assay also identified several soybean proteins including chlorophyll a/b binding preprotein, photosystem I-N subunit, ribulose 1,5-biphosphate carboxylase, ST-LS1 protein, translation initiation factor 1, TIR-NBS type R protein, RNA binding protein, ubiquitin, and LRR protein kinase. Altogether, these results suggest that potyviral replicase may comprise a multiprotein complex with PABP, CP, and other host factors.

Keywords : replication, SMV, soybean proteins, virus-host interactions

Compatible interactions between viral proteins and host proteins are essential for an successful infection of plant viruses. Particularly, plant viruses which have small genomes and limited protein coding capacity depend on the host machinery for completing the life cycle including translation and transcription of viral genomes (Brown and Gold, 1996; Gale et al., 2000). For RNA viruses, a majority of host proteins are found in an association with the viral replicase (Lai, 1998; Quadt et al., 1993; Wang et al., 2000; Yamaji et al., 2006). For example, the eukaryotic initiation factor (eIF) and elongation factor EF-1α interact with the replicase complexes of various RNA viruses such as Qβ phage, *Brome mosaic virus* and *Tobacco mosaic virus* (Brown and Gold, 1996; Lai, 1998; Quadt et al., 1993). In addition, several host proteins interacting with viral coat proteins (CPs) or movement proteins (MPs) have been reported and these host proteins are involved in cell-to-cell movement or subcellular localization of viruses (Chen et al., 2000; Kellmann, 2001; Malik et al., 2005; Ren et al., 2005). Moreover, 5'-capped positive-strand RNA viruses have elaborated strategies that allow the preferential translation of viral genes (Bushell and Sarnow, 2002). In potyviruses, genome-linked viral protein (VPg) substitutes for functions of the cap structure of mRNA. Hence it is interesting that VPg interacts with eIF4E and/or eIF(iso)4E, because eIF4E, a member of the eukaryotic translation initiation factor, recognizes the cap structure at the 5' end of mRNAs (Kang et al., 2005; Leonard et al., 2000; Leonard et al., 2004).

Soybean mosaic virus (SMV), one of the most economically important viruses in soybean, is a member of the genus *Potyvirus* (Mayo and Pringle, 1998). Potyviruses have a positive-stranded RNA genome of about 9.6 kb in length with a covalently bound VPg at the 5' end and a poly(A) tail at the 3' end. The genome encodes one large polyprotein, which is cleaved by viral protease to yield at least 10 mature proteins (Riechmann et al., 1992). Most potyviral proteins are remarkably multifunctional (reviewed in Urcuqui-Inchima et al., 2001), suggesting that interactions between proteins may play important roles in the life cycle of potyviruses. Actually, several interactions between potyviral proteins and their host proteins have

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been reported (Blanc et al., 1997; Hong et al., 1995; Kang et al., 2004; Leonard et al., 2004; Wang et al., 2000). We have previously showed that the C-terminal region of SMV CP may contain a domain(s) or amino acids required for CP-CP interaction and virus assembly by introducing deletion and site-directed mutagenesis followed by analysis of those mutants in yeast two hybrid assay (Kang et al., 2006). Deletion of the C-terminal region of the CP caused loss of the CP-CP self-interaction ability detected in CP mutants with the C-terminal region. Alanine substitutions that disrupted the CP self-interaction were found on six residues and were roughly located in two regions (aa 190-212 and 245-249) of the SMV CP. These regions include previously reported key amino acid residues mentioned above and may also be potential regions containing additional key residues.

In the present study, we tested interactions between several SMV viral proteins with its host proteins from soybean using the yeast two-hybrid system. Yeast two-hybrid analysis demonstrated that protein interactions between SMV and soybean were consistent with those reported previously in other potyviruses. In this study, we also found reproducible and specific interactions between SMV CP and PABP, and interaction domains of CP and PABP were characterized by using truncation mutants. Furthermore, in order to identify NIb interacting proteins, soybean cDNA library was screen by yeast two-hybrid assay.

Materials and Methods

Plasmids and strains. Plasmid pGEM-T easy vector (Promega, USA) and *E. coli* strain DH10b were used for general DNA manipulation. Yeast strain AH109 (*MATa, trp1-901, leu2-3, ura3-52, his3-200, gal4Δ, gal80Δ*) and GAL-4-based two-hybrid plasmids pACT2 and pAS2-1 were purchased from Clontech (USA). SMV viral genes and soybean genes were amplified by PCR with ex*Taq* DNA polymerase (TaKaRa, Japan) using SMV cDNA and soybean (cv. Lee68) cDNA, respectively as described (Kwak et al., 2007). PCR products were initially cloned into pGEM-T easy vector and subsequently cloned into yeast two-hybrid vectors. VPg and nuclear inclusion b-polymerase (NIb) were fused into downstream of GAL4 activation domain (GAL4-AD) by insertion between trimmed *Bam*HI site of pACT2. CP and helper component-protease (HC-pro) were cloned into *Nco*I and *Eco*RI sites of pACT2, respectively. eIF4E, eIF(iso)4E, and PABP were fused into downstream of GAL4 binding domain (GAL4-BD) using *Bam*H1 sites of pAS2-1. CP and PABP truncation mutants were made by PCR using primers designed to contain the required restriction sites so that the PCR products could be cloned in-frame into GAL4-AD or GAL4-BD. Each clone sequence was verified by sequencing.

Yeast two-hybrid assay. The yeast two-hybrid plasmids, pACT2 and pAS2-1, and AH109, all media, buffers, and protocols for the yeast two-hybrid assay were prepared according to manufacturer's instructions (MATCHMAKER system 2; Clontech, USA). Competent cells of AH109 were transformed simultaneously with cloned pACT2 and pAS2-1 using the lithium acetate method (Schiestl and Gietz, 1989). Yeast cells transformed with pACT2 or pAS2-1 clones were selected on leucine- (SD/-L) or tryptophan- (SD/-T) deficient agar media, respectively. Co-transformants expressing putative interacting proteins were selected by plating on leucine-, tryptophan-, histidine-deficient agar medium (SD/-LTH) or leucine-, tryptophan-, histidine-, adenine-deficient agar medium (SD/-LTHA). All protein-protein interactions were confirmed by α-galactosidase activity using X-α-Gal reagent. α-galactosidase activity was assayed for 1 to 2 days on the SD/-LTHA agar medium containing X-α-Gal. Newly formed fresh co-transformants were streaked on SD/-LT and SD/-LTHA media coated with X-α-Gal.

Construction of the soybean two-hybrid cDNA library. Total RNAs were isolated from leaves of soybean cultivar Geomjung 2, and mRNA was further purified by using the Oligotex® mRNA purification kit (QIAGEN, USA). Approximately 5 μg of mRNA was used for cDNA synthesis following the protocol of the HybriZAP 2.1 two-hybrid cDNA synthesis kit. cDNAs were inserted into the in λ HybridZAP-2.1 vector as *Eco*R1-*Xho*I fragments. The primary library contained about 1×10^6 PFU (plaque forming units). The primary λ cDNA library was amplified once and then converted to a plasmid (GAL4-AD) library by *in vivo* mass excision according to the Stratagene protocol.

Screening the library with SMV NIb. Competent cells of AH109 were first transformed with NIb cloned in pAS2-1 and then transformed with soybean cDNA library plasmid DNA. The transformants were plated on SD/-LTH agar media. Colonies that grew on selection medium were restreaked onto SD/-LTHA, and assayed for α-galactosidase activity. Colonies that survived in SD/-LTH and turned blue in α-galactosidase assay were considered putative positives. Plasmid DNAs were recovered from putative positive yeast colonies, and their sequences were determined by the dideoxynucleotide chain termination method using ABI Prism 3730 XL DNA Analyzer (PE Applied Biosystems, USA) located at the NICEM (Seoul National University).

Results and Discussion

Testing interactions between potyviral proteins and host proteins. Three soybean proteins, PABP, eIF4E, and eIF(iso)4E were tested for interaction with SMV viral proteins including VPg, CP, HC-pro and NIb using a yeast two-hybrid system (Fig. 1). PABP, eIF4E, and eIF(iso)4E were fused to the GAL4-BD, and VPg, CP, HC-pro and NIb were cloned to downstream of the GAL4-AD. The resulting constructs were introduced into the yeast strain AH109. Co-transformed yeast cells were selected on SD/-LT and SD/-LTHA. As expected from results of previous studies (Leonard et al., 2000; Leonard et al., 2004), SMV VPg was interacted with all three tested host proteins (Fig. 1). Several lines of studies suggest that VPg/eIF4E interaction is crucial for virus replication (Leonard et al., 2000; Schaad et al., 2000). Moreover, both eIF4E and PABP participate in initiation of protein synthesis (Le et al., 1997; Merrick, 1992). Therefore, the possible roles of these interactions could be correlated with the initiation of translation of the viral genome, and VPg may serve as a protein recruitment factor for translation initiation complex assembly. In addition, PABP/NIb interaction was detected, while neither eIF4E nor eIF(iso)4E interacted with NIb (Fig. 1). PABP/NIb interaction is to be predicted since this interaction has been reported for another potyvirus, *Zucchini yellow mosaic virus* (ZYMV) (Wang et al., 2000). In ZYMV, Wang et al. (2000) suggested that NIb/PABP interaction functions to facilitate viral replication. The interaction between SMV HC-pro and three host proteins, however, was not observed. Interestingly, we were able to detected CP/PABP interaction, which has not yet reported in any other system (Fig. 1). This interaction was also confirmed by galactosidase assay (data not shown).

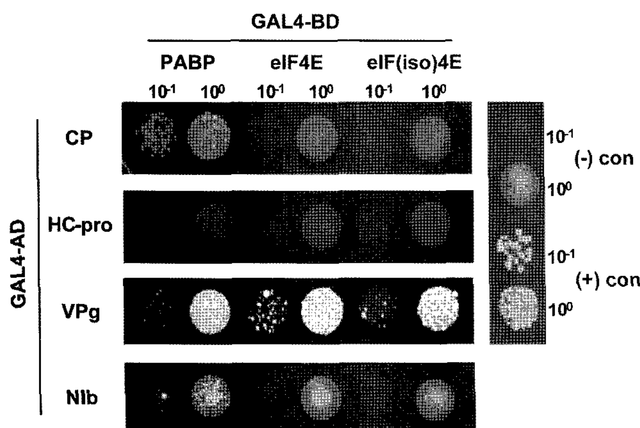

Fig. 1. Interactions between SMV viral proteins and soybean proteins in yeast two-hybrid system. CP, HC-pro, VPg and NIb were fused into downstream of GAL4-AD of pACT2. eIF4E, eIF(iso)4E, and PABP were cloned into downstream of GAL4 binding domain (GAL4-BD) of pAS2-1. Yeast cells co-transformed with pACT2 and pAS2-1 clones were selected on SD/-LTH agar media. Interactions between SV40 large T antigen$_{(84-708)}$ and murine p53$_{(72-390)}$ or human lamin C$_{(66-230)}$ were used as positive and negative controls, respectively.

Characterization of interacting domains of CP and PABP proteins. To further examine the interaction between SMV CP and soybean PABP, truncation mutants were introduced into both SMV CP and soybean PABP (Fig. 2, panels A and B, respectively). Deletions in the CP and PABP genes were made by PCR and were fused to downstream of GAL4-AD and GAL4-BD, respectively. Their interactions were tested using a yeast two-hybrid assay (Fig. 2A). The full-length CP (CP Full AD) consistently interacted with full-length PABP (PABP Full BD). CP truncation mutants comprising amino acids 1 to 81 (CP F1 AD) or amino acids 172 to 265 (CP F3 AD) failed to interact with full-length PABP. CP truncation mutant lacking the F3 region from the full-length CP (CP ΔF3 AD) also failed to interact with full-length PABP. However, the CP fragment lacking the F1 region (CP ΔF1 AD) strongly interacted with full-length PABP. These results suggest that the region comprising amino acids 81 to 265 is necessary for CP/PABP interaction. This was confirmed by the CP truncation mutant comprising amino acids 116 to 206; interactions between this mutant and PABP were detected

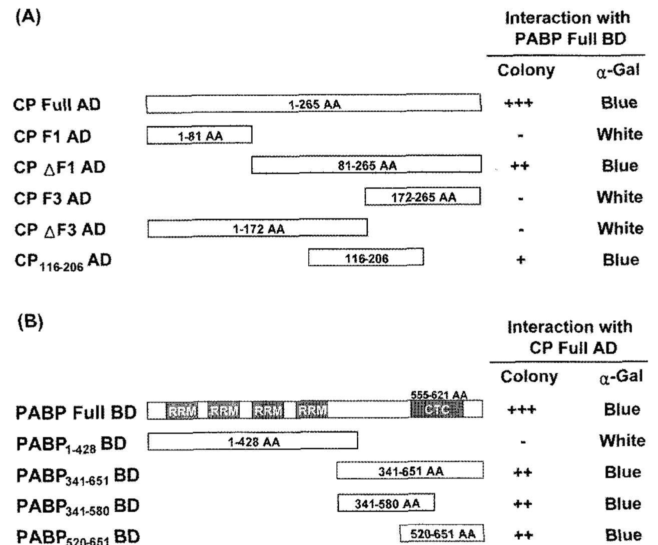

Fig. 2. Deletion analysis to determine regions in CP and PABP responsible for interaction. Interactions were measured by a yeast two-hybrid assay, and confirmed by α-galactosidase activity. The number of + symbols indicates the comparative number of colonies formed on the SD media. (A) CP truncation mutants were made by PCR and cloned in-frame into GAL4-AD. All of the CP mutants were tested against PABP Full BD in yeast two-hybrid assay. (B) PABP truncation mutants were made by PCR and cloned in-frame into GAL4-BD. All of the CP mutants were tested against CP Full AD in yeast two-hybrid assay.

on the SD/-LTHA media and by the galactosidase activity test (Fig. 2A). In this regard, it is worth noting that deletion of the C-terminal region of the CP caused loss of the CP-CP self-interaction ability detected in CP mutants with the C-terminal region (Kang et al., 2006). Alanine substitutions at the amino acid positions R190, E191, E212, R245, H246 and R249 disrupted CP-CP interaction, whereas substitutions at the amino acid positions R188, D189, D198, K205, K218 and D250 did not indicate that the C-terminal region of SMV CP may contain a domain(s) or amino acids required for CP-CP interaction and virus assembly. It will be very interesting, therefore, to determine whether these key amino acid residues mentioned above may also be potential region for PABP recognition and/or interaction.

To define regions of PABP required for CP/PABP interaction, four truncation mutants were tested for interaction with SMV CP (Fig. 2B). First, N-terminal two-thirds of PABP containing RNA recognition motif ($PABP_{1-428}$) and another one-third fragment carrying inter region and C-terminal domain of PABP ($PABP_{341-651}$) were co-transformed with full-length CP into yeast cells, and transformants were selected on SD/-LTHA media. An interaction between $PABP_{341-651}$ and full-length CP was detected on the SD media, while $PABP_{1-428}$ failed to interact with CP. Based on results of CP/ $PABP_{341-651}$ interaction, two more truncation mutants including $PABP_{341-580}$ and $PABP_{520-651}$ were constructed and tested for interaction with full-length CP (Fig. 2B). Yeast two-hybrid assay demonstrated that both $PABP_{341-580}$ and $PABP_{520-651}$ interact with CP. This suggests that the region comprising amino acids 520 to 580 in PABP is involved in the interaction with CP. At the C-terminus of soybean PBAP, there is the CTC (C-termianl conserved) domain comprising amino acids 555 to 621. This CTC domain seems to provide a scaffold on which other proteins can bind and mediate processes such as export, translation and turnover of the transcripts (Kozlov et al., 2001; Melo et al., 2003; Siddiqui et al., 2007). In this study, we found that CTC domain is important in CP/PABP interactions. Both CP and PABP are known to be involved in transcription and translation of viral genomes. As previously discussed, PABP is involved in the regulation of translation (Berlanga et al., 2006; Marissen et al., 2004). Therefore, our interaction study indicates that CP could participate in the formation of the translation initiation complex of viral genome directly or indirectly. Moreover, the interaction of PABP with NIb indicates that PBAP may be involved in viral replication (Wang et al., 2000). Interaction between SMV CP and SMV NIb, however, was not observed in our study while interaction between *Tobacco vein mottling virus* CP and SMV NIb was demonstrated (Hong et al., 1995; Kang et al., 2004). Although direct interaction between SMV CP and NIb was not observed, they might interact indirectly by recruiting other factors. Altogether, these suggest that potyviral replicase might be a scaffold protein for a multiprotein complex including NIb, PABP, CP, and other factors. In order to confirm this model, further research is needed .

Yeast two-hybrid screening for proteins interacting with potyviral NIb. To find additional soybean proteins that may interact with SMV NIb, a full-length NIb was fused to downstream of the GAL4-BD. The resulting plasmid was used as bait construct and introduced into the yeast strain AH109. The transformants did not grow on a medium lacking histidine, demonstrating that NIb does not contain an endogenous activation domain. The yeast two-hybrid cDNA library was constructed from mRNA from leaf tissues of SMV susceptible soybean (cv. Geomjung 2). Then, we proceeded to introduce a cDNA library into yeast cells expressing SMV NIb as bait. A total of 1.2×10^6 transformants were screened on SD/-LTHA media. We picked up 86 surviving colonies, which were subsequently streaked on new plates to segregate out the single positive clone. The α-galactosidase activity was also tested for each clone and selected 32 clones. Plasmid DNAs were prepared from the galactosidase positive clones, and their sequences were determined.

The nucleotide and the deduced amino acid sequences of 32 positive clones were analyzed using the BLASTN program, and also used for searched against protein databases from the National Center for Biotechnology Information (NCBI). About 30% of the sequences turned out to have homology to a chlorophyll binding protein (10 of 32). The second most abundant positive clones corresponded to a photosystem subunit protein (8 of 32). Five of the sequences showed homology to ribulose 1,5-biphosphate carboxylase, and four of the sequences encoded a ST-LS1 protein. We also found several other possible host genes that have high homology to translation initiation factor, TIR-NBS type R protein, RNA binding protein, ubiquitin, and LRR protein kinase (Table 1). Recently, we performed differential display RT-PCR and PCR-based subtractive hybridization to identify pathogenesis and defense related genes from soybean seedlings using both susceptible (Geumjeong 1) and resistant (Geumjeong 2) cultivars against SMV-G7H infection. About 570 genes that expressed differentially during SMV infection processes (Jeong et al., 2005) were identified. Interestingly, several host proteins interacting with NIb including ribulose 1,5-biphosphate carboxylase, translation initiation factor, and RNA binding protein were also differentially expressed (Jeong et al., 2005; data not shown). Further characterization and functional analysis

Table 1. Soybean cDNA clones isolated in yeast two-hybrid screening for SMV NIb-interacting proteins

Occurrence	Gene description	Accession no.
10	Chlorophyll a/b binding preprotein	CAA31419
8	Photosystem I-N subunit	AAO49652
5	ribulose 1,5-biphosphate carboxylase	NP176880
4	ST-LS1 protein	CAA28450
1	translation initiation factor 1	BAC45143
1	TIR-NBS type R protein	ABF81459
1	RNA binding protein	NP196239
1	Ubiquitin	BAA05085
1	LRR protein kinase NP175749	

of these selected genes will enhance our understanding of protein-protein interactions, defense signal transduction and biological function in soybean plants.

We are currently examining NIb interactions with translation initiation factor and RNA binding protein. We exluded the chlorophyll binding protein and photosystem subunit protein for further analysis because their subcellular localizations of the proteins were predicted to be in chloroplasts. Confirming their interactions and identification of the crucial amino acids involved in those interactions will be conducted by introducing deletions and site-directed mutations onto each target protein. Since NIb is the RNA-dependent RNA polymerase of potyviruses, it is expected that NIb may bind RNA, as was demonstrated in a systemic survey of the PVA proteins capable of interacting with RNA (Merits et al., 1998). Elucidating interactions between NIb and translation initiation factor or RNA binding protein will shed light on further understanding on viral replicase assembly.

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