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IDENTIFICATION OF HOST PROTEINS INTERACTING WITH POTYVIRAL RNA-DEPENDENT RNA POLYMERASE AND INVESTIGATION OF THE ROLES OF THE CARBOXY TERMINUS OF CUCUMBER POLY-(A)BINDING PROTEIN 1 IN POTYVIRAL REPLICATION AND CELLULAR TRANSLATION

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# IDENTIFICATION OF HOST PROTEINS INTERACTING WITH POTYVIRAL RNA-DEPENDENT RNA POLYMERASE AND INVESTIGATION OF THE ROLES OF THE CARBOXY TERMINUS OF CUCUMBER POLY- (A) BINDING PROTEIN 1 IN POTYVIRAL REPLICATION AND CELLULAR TRANSLATION

By

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# ABSTRACT

IDENTIFICATION OF HOST PROTEINS INTERACTING WITH POTYVIRAL RNA-DEPENDENT RNA POLYMERASE AND INVESTIGATION OF THE ROLES OF THE CARBOXY TERMINUS OF CUCUMBER POLY- (A) BINDING PROTEIN 1 IN POTYVIRAL REPLICATION AND CELLULAR TRANSLATION

By

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Viruses rely on host factors to complete their replication and life cycles. The Potyviridae, whose members have a positive sense, single-stranded RNA genome with a viral-encoded, 3' poly- (A) tail, is the largest and most economically important plant virus family. However, host factors involved in potyviral replication have not been identified. In this work, I sought to identify such host proteins and examine how they are involved in potyviral replication and in host cellular processes. I reported here a cucumber poly- (A) binding protein, CS-PABP1, as a promising candidate that could be involved in potyviral replication. CS-PABP1 interacted with RNA-dependent RNA polymerase (RdRp, viral replicase) of zucchini yellow mosaic potyvirus (ZYMV) in the yeast two-hybrid and *in vitro* binding assays. Since the 3' poly- (A) of potyviral genome is the site where the minus strand synthesis starts and ZYMV RdRp itself does not bind to poly- (A), we propose that one of the consequences of the interaction is to recruit RdRp or partially assembled replication complexes onto the poly- (A) of viral genome.

Deletion analysis indicated that the carboxy terminus of PABP (PABP-CT), which has not been previously well studied, was essential for the interaction. I provided evidence in this work that PABP-CT and its partners were involved in translational regulation. Cucumber PCI6 (PABP-CT interacting), PCI243 and Arabidopsis ERD15

(early responsive to dehydration) interacted with PABP-CT and contain a 12-amino-acid motif that is present in human PABP-CT interactors. Deletion and point mutation analyses of PCI6 indicated that the motif was necessary for the interaction. PCI6 inhibited translation in wheat germ and mouse ascites Krebs2 translation systems. A non-PABP interacting PCI6 mutant did not inhibit translation in wheat germ, and caused reduced translation in the Krebs2 system. PCI6 is a wound- and jasmonic acid-inducible protein. The above results suggested a possible translational regulation in gene expression upon stresses or other stimuli.

Like other higher eukaryotes, cucumber PABPs exist as a gene family. A second member of the cucumber PABP family, CS-PABP2, which shares 86% identity to CS-PABP1 and is expressed constitutively in different tissues, was amplified. While CS-PABP2 interacted with host PABP-CT interactors as well as that of CS-PABP1, it did not interact with ZYMV RdRp. Mutational analysis on a ZYMV RdRp region, which shares similarity to the 12-amino-acid motif, suggested that RdRp might interact with CS-PABP1 using a different motif than the host interactors.

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| | |
|---|---|
| BCMNV | bean common mosaic necrosis potyvirus |
| BMV | brome mosaic bromovirus |
| CI | cylindrical inclusion protein |
| CP | coat protein |
| CT | carboxy terminus |
| CTC domain | C-terminal conserved domain |
| eIF | eukaryotic initiation factor |
| ERD15 | early responsive to dehydration 15 |
| eRF | eukaryotic polypeptide chain release factor |
| HC-Pro | helper component-proteinase |
| NCBI | national center for biotechnology information |
| NIa | nuclear inclusion protein a |
| NLS | nuclear localization signal |
| NT | amino terminus |
| NTR | non-translated region |
| PABP | poly- (A) binding protein |
| Paip | PABP interacting protein |
| PAM | PABP binding motif |
| PAP | poly-A polymerase |
| PCBP | poly- (rC) binding protein |
| PCI6 | PABP-CT interacting 6 |
| PsbMV | pea seed-borne mosaic potyvirus |
| PVA | potato virus A |
| RdRp | RNA-dependent RNA polymerase |
| RRM | RNA recognition |
| TEV | tobacco etch potyvirus |
| TuMV | turnip mosaic potyvirus |
| TVMV | tobacco vein mottling potyvirus |
| VPg | viral protein-genome linked |
| ZYMV | zucchini yellow mosaic potyvirus |

# Chapter 1

## Literature Review

## Introduction

The Potyviridae, or potyvirus family, is one of the largest and most economically important group of plant viruses. This family of single-stranded RNA viruses includes approximately 200 members that collectively infect most crop species; Many species are infected by more than one potyvirus (Shukla et al., 1994). The potyvirus group has been extensively studied, and much has been learned about many aspects of potyviral replication, including identification of participating viral proteins, viral protein functions, and subcellular localization of the replication machinery (reviewed in Revers et al., 1999; Riechmann et al., 1992; Shukla et al., 1994; Urcuqui-Inchima et al., 2001). Given the limited coding capacity of viral genomes, viruses also rely on host encoded proteins for successful replication. Despite a growing list of host proteins that are involved in the replication of other RNA viruses (Lai, 1998; Strauss and Strauss, 1999), host proteins participating in potyviral replication have not been identified. My first objective was to learn more about the potyviral replication complex by identifying host proteins interacting with the potyviral RNA-dependent RNA polymerase (RdRp, viral replicase). The first two sections of the literature review will review host proteins known to be involved in viral replication and the current status of research on potyviral replication.

In the course of this project, host poly- (A) binding protein (PABP) was identified and confirmed to specifically interact with zucchini yellow mosaic potyvirus (ZYMV) RdRp. The carboxy terminus of PABP (PABP-CT) was necessary and sufficient for the

interaction. PABP is an essential cellular protein that plays important roles in mRNA stability, initiation and regulation of translation (Kahvejian and Sonenberg, 2002). While the roles of the amino terminal portion of PABP have been well defined, roles of the carboxy terminus are just now being identified. In the third section of this review, I will review structure and functions of PABP.

## Host proteins involved in viral replication

RNA viruses usually have small genomes, limited coding capacities, and depend on existing or modified host apparatuses, substrates and energy to complete their life cycle. A growing number of host proteins have been proven or implicated to be involved in viral replication, and the majority of these proteins are subverted from RNA-processing or translation machineries of host cells (Lai, 1998; Strauss and Strauss, 1999). These host proteins can be classified into three groups based on how they were identified; some of them were found in different viral replication complexes or directly interact with viral replicase, some were identified as binding protein of the cis-elements of viral genomes, and some were identified via genetic approaches.

The first evidence showing the requirement of host proteins for viral replication was from studies on Qβ phage (Blumenthal and Carmichael, 1979). The Qβ viral holoenzyme contains three host proteins; EF-Tu (Elongation Factor), and EF-Ts, which are counterparts of eukaryotic EF-1α and EF-1β respectively, and 30S ribosomal protein S1. Removal of them will inactivate the enzymatic activity (Blumenthal and Carmichael, 1979). Two decades later, similar results were found in vesicular stomatitis virus (VSV, Das et al., 1998). The purified VSV RNA-dependent RNA polymerase (RdRp) was found inactive unless cellular extracts, specifically EF-1β and EF-1χ, were added in the

reaction. It was further found that EF-1α was co-purified with VSV RdRp and EF-1αβχ subunits were packed in the purified virion, clearly indicating the necessity of host proteins (Das et al., 1998). Besides EF-1α, different subunits of eIF-3 (eukaryotic Initiation Factor), along with other undefined host proteins, were found in the brome mosaic virus (BMV, Quadt et al., 1993) and tobacco mosaic virus (TMV, Osman and Buck, 1997) replication complexes isolated from BMV and TMV infected plants, respectively. It was further shown that subunit P41 of eIF-3 bound to BMV RdRp (2a protein) *in vitro*, and addition of wheat germ eIF3 or subunit P41 stimulated BMV viral replication *in vitro* by three-fold, suggesting eIF3 was directly involved in viral replication (Quadt et al., 1993). Given the difficulty of isolating active replication complexes, other biochemical and genetic methods also have been employed to identify host proteins directly interacting with viral RdRp. Human protein Sam68 (Src-associated in mitosis, 68kDa) was identified to interact with polioviral RdRp protein $3D^{pol}$ in the yeast two-hybrid assay (Mcbride et al., 1996). Sam68 was further found to be relocated from the nucleus to poliovirus-induced vesicular compartments in the cytoplasm where viral RNA replication occurs, suggesting that Sam68 could be involved in replication (Mcbride et al., 1996). How Sam68 involved in viral replication is not clear yet.

Deletion and point mutation analyses have defined cis-elements of the viral genomes that are important for replication. These are usually located at the 5' and 3' ends, but sometimes in the internal region of a viral genome. Viral and host proteins binding to these cis-elements could be involved in viral replication, either by recruitment of RdRp onto these cis-elements, or by the interactions between cis-element-binding proteins. Host proteins such as: poly- (rC) binding protein (PCBP, Gamarnik and Andino,

1997; Parsley et al., 1997), La antigen (reviewed in Lai, 1998), polypyrimidine tract-binding protein (PTB, reviewed in Lai, 1998), nucleolin (Waggoner and Sarnow, 1998), and poly- (A) binding protein (Spagnolo and Hogue, 2000) have been found to bind to different cis-elements of different viruses. Host PCBP binds to the stem-loop B of the cloverleaf structure at the 5' end of poliovirus and plays at least two roles, stimulating viral translation (Gamarnik and Andino, 1997, 2000; Parsley et al., 1997) and is also required for viral replication (Herold and Andino, 2001; Parsley et al., 1997). Besides host PCBP, polioviral $3CD^{Pro}$ also binds to the cloverleaf structure to inhibit viral translation and initiate the synthesis of minus strand (Andino et al., 1990). These two proteins could control the transition from translation to replication (Gamarnik and Andino, 1998).

Janda and Ahlquist (1993) developed a genetic approach to identify host proteins involved in or necessary for viral replication. A yeast-BMV system was developed so that replication of BMV RNA3 could be supported by yeast proteins in combination with BMV 1a and 2a proteins whose coding sequences were inserted into the yeast genome (Janda and Ahlquist, 1993). At least three recessive mutants that failed to support BMV replication were identified and the corresponding host genes and proteins were investigated. These proteins revealed that host proteins are involved in a broad range of viral replication steps, from translation of the viral replicase gene, to replication complex assembly, to initiation of replication. Yeast Lsm1p (small RNA-like binding protein), which is related to the RNA splicing complex, but found predominately in the cytoplasm, inhibited an early replication step of template selection mediated by viral protein 1a and a cis-element located within the tRNA-like structure at the 3' end the RNA3 (Diez, et al.,

2000). Yeast Ded1p is an essential translation-associated protein with ATP-dependent RNA helicase activity. When Ded1p was mutated, BMV replicase 2a translation was specifically inhibited, suggesting Ded1p specifically promotes 2a translation (Noueiry et al., 2000). It has been observed that viral replication complexes are associated with intracellular membranes (Molla et al., 1993; Restrepo-Hartwig and Ahlquist, 1996, 1999; Schaad et al., 1997). Mutation at the OLE1 gene, which encodes Δ9 fatty acid desaturase, caused decreased unsaturated fatty acids synthesis and 90% inhibition of BMV replication, suggesting the composition of ER membrane is critical for viral replication (Lee and Ahlquist, 2001).

## Replication of potyviruses and related viruses

The Potyviridae is the largest plant virus family with about 200 members that cause significant losses in a wide range of crops (Riechmann et al., 1992; Shukla et al., 1994; Revers et al., 1999; Urcuqui-Inchima et al., 2001). Replication complexes are just beginning to be defined for this important group of viruses. Potyviruses have a positive sense, single-stranded RNA genome, which is approximately 10 kb long, with a covalently attached viral protein (VPg, Viral Protein-genome linked) at the 5'end, and a poly- (A) tail at the 3' end. The RNA contains a 5'NTR, a 3'NTR, and a long open reading frame that is expressed as a polyprotein of approximately 340-370 kD (Fig. 1-1). The polyprotein is cleaved into nine individual proteins by three viral encoded proteases (reviewed in Riechmann et al., 1992); P1 (the first protein), HC-Pro (Helper Component-proteinase), and NIa (named Nuclear Inclusion protein a because of its tendency to accumulate in the nucleus). P1 protein cleaves the linkage between P1 and HC-Pro and frees P1 from the polyprotein (Verchot et al., 1991); HC-Pro cleaves the junction

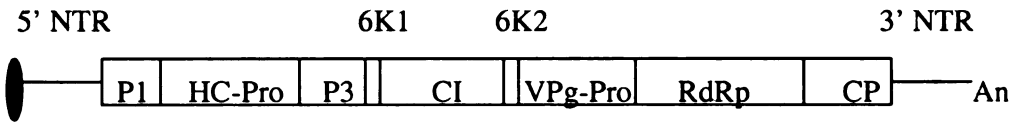


**Fig. 1-1. Organization of the potyvirus genome.** Potyviruses have a positive sense, single-stranded RNA genome, approximately 10 kb long, with a poly- (A) tail at the 3' end, and a viral protein VPg covalently linked to the 5'end. At least nine proteins are produced: protein 1 (P1), helper component protease (HC-Pro), protein 3 (P3), 6K1 protein, cylindrical inclusion protein (CI), 6K2 protein, VPg-Pro (Viral protein-genome linked- proteinase, also termed as NIa, nuclear inclusion protein a,), RdRp (RNA-dependent RNA polymerase, also termed as nuclear inclusion b NIb), and coat protein (CP).

between HC-Pro and P3 (the third protein, Carrington et al., 1989); NIa is responsible for the remaining cleavages in the C-terminal two-thirds of the polyprotein (Dougherty and Carrington, 1988). Several intermediate proteins such as CI/6K2, 6K2/NIa, and 6K2/VPg have been observed during TEV infection and may play very important roles (Restrepo-Hartwig and Carrington, 1994; Schaad et al., 1997). Based on their genome structure and gene expression strategy, potyviruses are classified as members of Picornavirus superfamily including the animal-infecting Picornaviridae family, which contains several well-studied viruses, such as: poliovirus, encephalomyocarditis virus and coxsackievirus (Kerekatte et al., 1999).

The functions of potyviral proteins have been extensively studied, and we now know that most potyviral proteins play a role in replication. These include P1 protein, HC-pro, P3 protein, CI (Cytoplasmic Inclusion protein), 6 K2 (second 6 kD protein), NIa, and NIb (Nuclear Inclusion protein b, functions as RdRp or replicase) (Klein et al., 1994; Li and Carrington, 1995; Meritus, 1999; Murphy et al., 1996; Restrepo-Hartwig and Carrington, 1994; Schaad et al., 1996; Schaad et al., 1997; Verchot and Carrington, 1995). Among the above listed proteins, P1, P3 and HC-Pro function as accessory factors, while CI, NIa and NIb (RdRp, see below) are possibly core components of the viral replication complex (Schaad et al., 1997). For example, when the coding sequence of P1 was deleted from the TEV genome, the ΔP1 mutant accumulated in protoplasts to approximately 2-3% the level of parental genome. In addition, the accumulation of ΔP1 mutant was stimulated in transgenic plants expressing P1 protein. These data suggested that P1 protein is not strictly required for TEV replication, but functions *in-trans* to enhance replication (Verchot and Carrington, 1995).

The CI protein of PPV (plum pox virus) has been shown to have a nucleic acid–stimulated ATPase activity and a helicase activity (reviewed in Riechmann et al., 1992). This helicase activity unwound double-stranded RNA molecules and was dependent on hydrolysis of NTP (reviewed in Riechmann et al., 1992). It was also shown in TEV that mutations affecting highly conserved helicase motifs eliminated detectable genome replication, confirming the necessity of the CI protein in potyviral replication (Carrington et al., 1998). Given the helicase activity, CI protein probably destabilizes secondary structures in potyviral genomes or/and unwinds double-stranded RF (Replication Form) to make minus strand accessible for the replication complex to make progeny RNA genomes. TEV 6K2 protein was first found to be necessary for TEV replication by point mutation and insertional analysis (Restrepo-Hartwig and Carrington, 1994). It was further found that 6K2 was integrated into the ER membrane and to the vesicular compartment derived from the ER membrane in TEV infected plants and in 6K2 transgenic plants (Schaad et al., 1997). Deletion analysis showed that the central hydrophobic region is necessary for the targeting (Schaad et al., 1997). Several intermediate proteins with 6K2 have been observed, including CI/6K2, 6K2/NIa, 6K2/VPg (Restrepo-Hartwig and Carrington, 1994; Schaad et al., 1997). When fused to NIa (the cleavage site between 6K2 and NIa was mutated), the 6K2 protein could override the ability of NIa to be targeted to the nucleus and instead targeted NIa to the ER membrane (Restrepo-Hartwig and Carrington, 1992). It was proposed that CI, NIa and VPg might be targeted to the ER membrane by 6K2 during TEV infection and assembly along with NIb to make functional replicase.

NIa is a multifunctional protein that has RNA binding activity (Daròs and Carrington, 1997), interacts with viral replicase NIb (RdRp, Li et al., 1997; Daròs 1999; Hong et al., 1995), and is the major proteinase responsible for cleaving the polyprotein (reviewed in Riechmann et al., 1992). NIa is a modular protein; the N-terminus encodes the VPg and the C-terminus encodes the proteinase. NIa has an internal less-optimal cleavage site for auto-cleavage and can be inefficiently cleaved into N-terminal VPg and C-terminal proteinase. The nature of the inefficient cleavage might be essential for viral replication, since mutations removing the site or accelerating cleavage led to debilitated replication, suggesting the cleavage between two parts at the right time of replication is necessary (Carrington et al., 1993; Schaad et al., 1996).

The VPg protein resulting from the cleavage of NIa is covalently attached to the 5' end of RNA genome through the amino acid tyrosine (Tyr) at position 1860 (Murphy et al., 1991). Mutation of the Tyr residue abolished TVMV (Tobacco Vein Mottling Virus) replication in protoplasts (Murphy et al., 1996), indicting the importance of the VPg in viral replication. It has been shown in poliovirus that VPg functions as a primer for the initiation of minus strand synthesis (Paul et al., 1998, and see below). Recently, TuMV (Turnip Mosaic Virus) VPg was found to interact with Arabidopsis eIF(iso)4E in yeast two-hybrid and *in vitro* assays (Wittmann et al., 1997). A VPg mutant, which lost the ability to interact with eIF(iso)4E, was identified and replaced the wild type VPg in full-length TuMV cDNA (Léonard et al., 2000). The mutated TuMV was not infectious, indicating the interaction is necessary for infection (Léonard et al., 2000). Besides Arabidopsis eIF(iso)4E, Arabidopsis eIF4E and wheat eIF(iso)4E (Léonard et al., 2000) interacted with TuMV VPg in a similar degree to that of Arabidopsis eIF(iso)4E. It was

also found that tomato eIF4E and tobacco eIF4E interacted VPg of TEV (Schaad et al., 2000), suggesting the interaction between VPg and eIF(iso)4E and/or eIF4E is common and important for host-potyvirus interaction, although the precise role of the interaction remains unknown.

NIb was first identified along with NIa since they are located in the TEV infected host nucleus as inclusion body in a 1:1 ratio and were named as nuclear inclusion proteins a and b (Knuhtsen et al., 1974; Dougherty and Hiebert, 1980). It was further found that TEV NIb has two independent NLS (Nuclear Localization Signal) responsible for the targeting to nucleus and that the conformation of NIb is important, because any major deletions in the NIb protein abolished the nuclear localization (Li and Carrington, 1993). However, the function of NIb in the nucleus is not clear. Although most of NIb proteins remain in the nucleus, NIb functions as an RNA dependent RNA polymerase in the cytoplasm (Riechmann et al., 1992; Revers et al., 1999; Shukla et al., 1994; Urcuqui-Inchima et al., 2001). All potyviral NIbs have a GDD motif that is the hallmark of an RdRp (Poch et al., 1989; O'Reilly and Kao, 1998), suggesting that it might serve as the catalytic component in the potyviral replication complex. It was later shown *in vitro* using recombinant NIb protein overexpressed in *E.coli* that TVMV NIb has RNA-dependent RNA polymerase activity (RdRp, Hong and Hunt, 1996). RdRp apparently functions *in-trans*, because replication of TEV with the RdRp mutation in the GDD motif can be partially or fully restored in transgenic tobacco protoplasts expressing wild type TEV RdRp (Li and Carrington, 1995).

The crystal structure of potyviral RdRps has not been obtained, however, data are available from polioviral RdRp $3D^{pol}$ protein (reviewed in O'Reilly and Kao, 1998).

Polioviral RdRp has a similar structure as other polymerases such as Klenow fragment (*E.coli* DNA-dependent DNA polymerase), HIV reverse transcriptase, and T7 polymerase (T7 phage DNA-dependent RNA polymerase) (reviewed in O'Reilly and Kao, 1998). The overall shape resembles a 'right hand' with finger, palm and thumb subdomains. Comparison of the predicted secondary structure of different RdRps with that of polioviral RdRp has revealed that all RdRps have a similar structure. The palm domain is responsible for the catalysis activity, the finger may determine preference for RNA templates and the thumb domain is likely involved in formation of the clamp on template binding. Besides these three domains, poliovirus and other RdRps have a unique motif at the N-terminus proposed to be involved in oligomeriztion of polioviral RdRp (Hansen et al., 1997). However, there is no evidence that oligomerization is necessary for every RdRp, suggesting that the RdRp unique domain might be involved in other functions.

Interactions between potyviral RdRp and other viral proteins, or with itself have been studied in TEV (Daròs et al., 1999; Li et al., 1997), TVMV (Fellers et al., 1998; Hong et al., 1995), PVA (Potato Virus A) and PsbMV (Pea seed-borne Mosaic Virus) (Guo et al., 2001). In TVMV, RdRp was found to interact with coat protein (CP) in the yeast two-hybrid system (Hong et al., 1995). The interaction between CP and RdRp may suggest that CP can regulate the relative levels of plus and minus strand TVMV RNA in infected cells (Hong et al., 1995). However, surprisingly, an RdRp mutant ADD, which lost the polymerase activity, was not able to interact with CP. One explanation could be that the region in RdRp responsible for the interaction is close to the GDD motif. Another possibility is that the ADD mutant was not stable, however, it is not likely since the ADD

mutant interacted with NIa at a comparable level to that between WT RdRp and NIa (Hong et al., 1995). No interaction between RdRp and CP of PVA or PsbMV was observed in yeast (Guo et al., 2001). Self-interaction of TVMV RdRp was reported (Hong et al., 1995), which is consistent with poliovirus RdRp where oligomerization of RdRp is critical for replication (Hobson et al., 2001). However, similar RdRp self-interaction was not found in yeast two-hybrid assays for TEV, PVA or PsbMV (Guo et al., 2001; Li et al., 1997). Thus it is not clear whether oligomerization of RdRp is important for potyviral replication. Interaction between NIa and RdRp was found in TEV (Li et al., 1997; Daròs et al., 1999), TVMV (Hong et al., 1995), PVA and PsbMV (Guo et al., 2001). Disruption of the interaction between NIa and RdRp by mutation in NIa disabled ability of mutant TEV to infect tobacco protoplasts. RdRp mutants recovering the interaction with the NIa mutant partially restored ability to infect, indicating the interaction is necessary for TEV infection (Daròs et al., 1999).

Potyviral replication occurs in the cytoplasm in tight association with the host cell membrane (Martin and Garcia, 1991; Schaad et al., 1997). For TEV, the replication complex is localized to the vesicular compartment derived from ER membrane that collapsed into discrete aggregated structures upon TEV infection (Schaad et al., 1997). It was proposed that TEV 6K2 protein initiates assembly of the TEV replication complex by targeting the proteolytic precursors, such as 6K2/NIa, 6K2/VPg, and CI/6K2, directly to ER membrane, and by anchoring the replication complex on the ER membrane (Restrepo-Hartwig and Carrington, 1994; Schaad et al., 1997).

Replication of positive sense, single strand RNA viruses starts with the synthesis of the minus strand intermediate using the positive strand as template, followed by

positive strand synthesis. The synthesis of the minus strand of potyviruses and picornaviruses is initiated at the 3' end poly- (A) tail of genomic RNA. The poly- (A) tail is necessary for picornavirus replication (Cui et al., 1993; Herold and Andino, 2001) and recent evidence suggested that poly- (A) tail is necessary for potyvirus replication (Tacahashi and Uyeda, 1999). Herold and Andino (2001) investigated the minimal length of poly- (A) necessary for polioviral replication using *in vitro* transcribed poliovirus replicons with different numbers of A residues ranging from 0 to 17. When the last nuleotide in the 3' NTR or poly- (A) tail was ribonucletide, the replicon could eventually replicate even without any A residue, although it did not replicate until several hours later. It was further found that progeny viruses all contained long poly- (A) tails, indicating the restoration of the tail. However, if the last A residue in the poly- (A) was a deoxyadenosine, replicons with 5 A residues could not replicate, confirming that poly- (A) is necessary and more than 5 A residues are required for polioviral replication (Herold and Andino, 2001). Initiation of poliovirus replication was postulated to start from the uridylytion of VPg using poly- (A) tail as template, and uridylated VPg in turn served as primer for synthesis of minus strand (Paul et al., 1998; Aglo et al., 1999). It has been shown *in vitro* that poliovirus VPg can be uridylated by RdRp ($3D^{pol}$ protein) using poly- (A) as template (Paul et al., 1998).

RdRps of poliovirus and other picornavirueses do not bind to the poly- (A) tail (Cui et al., 1993; Paul et al., 1994). The poliovirus RdRp is recruited by polioviral 3AB protein onto a secondary structure, which includes the 3' NTR, and several adenosine residues of the poly- (A) tail (Harris et al., 1994; Agol, 1999). In potyviruses, it appears that important secondary structure exists at the 3' end of viral RNA genome, which

includes part of the coat protein coding sequence, and the 3'NTR (Mahajan et al., 1996; Haldeman-Cahill et al., 1998). It was proposed that potyviral NIa recruits RdRp onto viral RNA since NIa has RNA and RdRp binding activity (Schaad et al., 1997). However, how host proteins are involved in recruiting poty and picorna viruses onto the 3' NTR is not clear.

# Poly- (A) binding protein (PABP)

## 1. Functions of Poly- (A) binding protein

Most eukaryotic mRNAs have a cap structure, $m^7$GpppN (N is any nucleotide) at the 5' end, and a poly- (A) tail at the 3' end (Gingras et al., 1999). Both structures are added post-transcriptionally in the nucleus and are necessary for export of mature mRNAs to the cytoplasm. The poly- (A) is about 50-70 bases long in yeast and 200-250 bases in higher eukaryotes (Amrani et al., 1997; Jacbosen, 1996; Minvielle-Sebrastia et al., 1997). Poly- (A) tail in the cytoplasm is bound by poly- (A) binding protein (PABP, termed Pab in yeast and humans, PABP is used through this dissertation), which is an essential cellular protein for eukaryotes (Sachs et al., 1987; Coller et al., 1998). PABP, together with poly- (A), plays a broad range of roles: pre-mRNA processing (Armani, et al., 1997; Minvielle-Sebrastia et al., 1997), mRNA stability (Caponigro and Parker, 1995; Coller et al., 1998), poly- (A) metabolism (reviewed in Jacobsen, 1996; Sachs and Davis, 1989), and initiation and stimulation of translation (Gallie, 1998; Gray et al. 2000; Jacobsen, 1996; Otero et al., 1999; Sachs et al. 1997).

PABP has four tandem RRMs (RNA Recognition Motif) located in the N-terminal two-thirds of the protein, an approximately 75-amino-acid-long C-terminus Conserved (CTC) domain, and a linker rich of methionine and proline in between (Sachs

et al., 1986; Kahvejian and Sonenberg, 2002). The RRM, which is the hallmark for a large number of RNA-binding proteins, is composed of about 90 to 100 amino acids with 2 short stretches of conserved amino acids named as RNP1 (Ribonuclearprotein) and RNP2 (reviewed in Burd and Dreyfuss, 1994). The three-dimensional structure of RRMs has been determined as a four-stranded antiparallel β sheet flanked by two perpendicularly oriented α helices. The RNP1 and RNP2 are located in the central two β sheets and directly contact RNA (reviewed in Burd and Dreyfuss, 1994). Comparison of the sequences of PABP from different organisms indicated that individual RRMs in a single PABP are more divergent from each other than from the counterpart RRMs in PABPs from different species, suggesting that the individual RRMs are not functionally equivalent (Burd et al., 1991). Deletion analysis indicated that the C-terminus of PABP (PABP-CT, including the linker and CTC domain) is not necessary for poly- (A) binding; however, one individual RRM of yeast PABP was not able to bind to poly- (A). It was also found that RRM1-2 bound to poly- (A) as efficiently and specifically as the RRM1-4 or full-length PABP. RRM3-4 bound to non-poly- (A) RNA and might bind to either a different part of the same mRNA or other RNA (Burd et al., 1991). Point mutation analysis indicated that RRM2 was responsible for the poly- (A) and RRM4 for non-poly- (A) binding (Deardorff and Sachs, 1997).

Polyadenylation is one of the posttranscriptional modifications of pre-mRNA and includes the cleavage of the mRNA precursor near the polyadenylation site (AAUAAA) and addition of adenosines to the 3' end (Wahle and Keller, 1992). The cleavage process requires yeast CF I (Cleavage Factors I) and CF II, and polyadenylation involves CF I, PF I (Polyadenylation Factor I), and Pap1p (poly- (A) polymerase, Amrani et al., 1997;

Minvielle-Sebrastia et al., 1997). It was found that PABP was co-purified with CF I (Minvielle-Sebrastia et al., 1997) and specifically interacted with Rna15, a key component of CF I (Amrani et al., 1997). Extracts from temperature sensitive PABP mutants yeast cells grown in non-permissive temperature, were able to cleave the pre-mRNA properly but failed to achieve a poly- (A) tail with proper length (Amrani et al., 1997; Minvielle-Sebrastia et al., 1997). Instead of 50-70 bases, up to hundreds of adenosines were added. When PABP protein was added into the extract, normal length of poly- (A) tail was produced, suggesting that yeast PABP controls the poly- (A) length of newly synthesized mRNAs (Amrani et al., 1997; Minvielle-Sebrastia et al., 1997). In mammalian systems, PABP II, which is a ca.49 kD, nuclear protein with only one RRM and distinct from the major member PABP1, was responsible for the 3' end processing (Whale et al., 1993).

Several lines of evidence have indicated that PABP is involved in mRNA stability. Firstly, PABP is negatively involved in decapping (Caponigro and Parker, 1995). One of the major mRNA decay pathways in eukaryotes (yeast) is initiated by poly- (A) shortening, followed by the cleavage of 5' cap structure (decapping) by Dcp1p, and degradation of the rest of mRNA by Xrn1p, which is a 5' to 3' exonuclease (Decker and Parker, 1993). PABP is an inhibitor of decapping because in yeast cells lacking a functional PABP gene (yeast strain pab1Δ), mRNAs were decapped prior to deadelynation, indicating that PABP protein but not deadelynation is necessary to protect the cap structure (Caponigro and Parker, 1995).

Secondly, it was shown that stabilization of mRNA is an intrinsic property of PABP *in vivo* (Coller, et al., 1998). A system was developed to uncouple other functions

of PABP from its poly- (A) binding activity. PABP was tethered to the 3' NTR of reporter mRNAs by fusing PABP to MS2 coat protein and placing MS2 binding sites in the 3' NTR of the reporter gene. Deletion of both RRM1 and RRM2 did not destabilize mRNA, suggesting that poly- (A) binding activity is not required for PABP to stabilize mRNA (Coller et al., 1998). Deletions of either RRM3, or RRM4 or 90 amino acids from PABP-CT abolished the stability of reporter mRNA, suggesting that RRM3, 4 and PABP-CT are necessary for the stabilization activity of PABP (Coller et al., 1998; Gray et al., 2000). The poly- (A) tail of reporter mRNAs was shortened similar to other cellular mRNAs, but reporter mRNAs had a longer half-life, suggesting that poly- (A) is not required for stabilization of mRNA. Collectively, Coller et al (1998) concluded that the primary function of poly- (A) is to recruit PABP onto mRNA, and poly- (A) binding and mRNA stabilization activity of PABP are controlled by different portions of the protein.

Thirdly, it was found that PABP is positively involved in deadelynation (Caponigro and Parker, 1995; Sachs and Davis, 1989; Sachs and Deardorff, 1992). The poly- (A) tail is gradually shortened after mRNAs are transported to the cytoplasm and this shortening is also dependent on the nature of mRNA, especially the sequence of the 3' NTR (Non-Translated Region, reviewed in Jacobson, 1996). In yeast cells that have been depleted of PABP protein, mRNAs with longer poly- (A) tails were observed (Caponigro and Parker, 1995; Sachs and Davis, 1989). A PABP-dependent poly- (A) ribonuclease (PAN) that shortened the poly- (A) to about 20 bases only in the presence of PABP was identified (Sachs and Deardorff, 1992). Furthermore, longer poly- (A) tails were also found in yeast cells with PAN mutations, confirming that both PAN and PABP are required for poly- (A) tail shortening (Sachs and Deardorff, 1992).

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It has also been documented that translational efficiency of mRNAs with caps and poly- (A) tails was much more than the sum of translational efficiency with either cap or poly- (A) alone. This synergistic stimulation suggests that both ends may communicate to lead to much higher translation (Gallie, 1991; Tarun and Sachs, 1995). Subsequent studies showed that yeast PABP interacted with yeast eIF4G I (eukaryotic Initiation Factor) and eIF4G II *in vitro* and *in vivo* (Tarun and Sachs, 1996, eIF4G is a subunit of eIF4F that contains eIF4E, eIF4G, eIF4A). It was also found in plants that not only wheat eIF4G and eIFiso4G, but also eIF4B, interacted with PABP (Le et al., 1997a). Similar results were also found in mammalian systems (Imataka et al., 1998; Piron et al., 1998).

Binding of PABP to poly- (A) and binding of eIF4E to the cap was highly enhanced by the binding between PABP and eIF4G (Le et al., 1997b; Wei et al., 1998). The interaction between eIF4G and PABP provided evidence for the closed-loop model: initiation factor eIF4E, binds to the cap (reviewed in Gingras, 1999); PABP binds to 3' poly- (A); eIF4G is a platform for the interaction and assembly of multiple initiation factors: eIF4E, PABP, eIF4A, eIF3. The interactions between PABP and eIF4G, eIF4E and eIF4G bring two ends of mRNA close to each other and result in a circular molecule (reviewed in Gallie, 1998; Kahvejian and Sonenberg, 2002; Jacobson, 1996: Sachs et al., 1997). It was observed with the atomic force microscopy that purified yeast PABP, eIF4G and eIF4E circularized capped and polyadenylated RNA *in vitro*, confirming the closed-loop model (Wells et al., 1998). At least two advantages could result from circularization: 1. The 40S ribosome subunit could be easily re-recruited from 3' end to 5' end and start another round of translation; and 2, intact mRNA with both cap and poly- (A) will be preferentially translated, avoiding production of truncated proteins (reviewed

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in Gallie, 1998). It was recently shown that the interaction between PABP and eIF4G indeed was necessary for efficient translation (Tarun et al., 1997; Kessler and Sachs, 1998). For eIF4G mutants that lost ability to bind to PABP, or PABP mutants that lost ability to bind to eIF4G, efficient translation was abolished, suggesting the interaction between these two proteins, or the maintenance of circular molecules were necessary for the efficient translation of mRNA (Tarun et al., 1997; Kessler and Sachs, 1998). PABP deletion analysis has indicated the RRM2 of PABP was necessary and RRM1-2 was sufficient for the interaction with eIF4G and efficient translation (Kessler and Sachs, 1998).

## 2. C-terminus of poly- (A) binding protein

The N-terminus of PABP (PABP-NT) is responsible for most, if not all, of the above functions. The C-terminal third of PABP is not as conserved as that of the N-terminus, however, a CTC domain approximately 75 amino acids long is conserved among all PABPs from different organisms (Deo et al., 2001; Kozlov et al., 2001; Kahvejian and Sonenberg, 2002). The roles of the C-terminus have drawn increasing attention recently. PABP-CT was found to be capable of forming oligomers in the presence of poly- (A). Deletion of Xenopus PABP-CT abolished the gel retardation supershift band, which resulted from auto-polymerization of PABP on the poly- (A) tail (Kühn and Pieler, 1996). It was further found that deletion of PABP-CT diminished the poly- (A) organizing activity (Kühn and Pieler, 1996), which renders PABP able to form a higher order complex on poly- (A) with multiple, regularly spaced copies of PABP on a single RNA molecule (Baer and Kornberg, 1983). Coller et al (1998) observed that a yeast PABP mutant with a deletion of 50 amino acids from CT lost the ability to rescue

the inviability of yeast strain pab1Δ, suggesting that CT is necessary for yeast to survive. Furthermore, yeast PABP with a deletion of 90 amino acids from CT lost the ability to stabilize mRNA, suggesting that PABP-CT may be directly involved in mRNA stability which might be necessary for cellular survival (Gray et al., 2000). It was also found that deletion of human PABP-CT caused the accumulation of human PABP1 in the nucleus, suggesting PABP-CT was necessary for PABP1 to shuttle between the nucleus and cytoplasm (Afonia et al. 1998).

## 3. Poly- (A) binding proteins in plants

Although there is only a single PABP gene in yeast, higher eukaryotes have multiple PABPs. In humans, there are at least three members in the PABP gene family. PABP1 is the major member and responsible for the cytoplasmic functions. An inducible PABP (iPABP), which shares 77% amino acid identity with PABP1, is expressed mainly in activated T cells and platelets (Houng et al., 1997; Yang et al., 1995). PABP3 has 92% identity to PABP1 and is only expressed in round spermatids (Féral et al., 1999). PABP II is a small nuclear protein (49 kD) necessary for polyadenylation but distinct from PABP1 in that it has only one RRM (Brais et al., 1998).

PABP genes have been isolated from Arabidopsis, wheat, and tobacco, and available data show that PABPs also occur in plants as a gene family (Hilson et al., 1993; Le et al., 1997b; Le and Gallie, 2000; Belostotsky and Meagher, 1993). In Arabidopsis, cDNAs of at least four members have been isolated: AtPABP1, AtPABP2, AtPABP3, AtPABP5 (Hilson et al., 1993; Belostotsky and Meagher, 1993). Two cDNAs from tobacco were also identified (Le and Gallie, 2000). Investigations of these PABPs have

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found that they have divergent sequences, differential expression patterns, and potentially different functions.

Different members of PABPs in Arabidopsis showed quite different gene sequences and expression patterns. AtPABP2 is expressed in all organs; stems, flowers, leaves, roots, siliques and pollen based on northern and western analysis (Hilson et al., 1993; Palanivelu et al., 200b), suggesting that AtPABP2 is a major member in Arabidopsis. Detailed study of AtPABP2 expression indicated that it is spatially and temporally regulated in different organs; it is expressed strongly in the stele and meristem region of roots, and its expression dramatically decreased in ovules after fertilization. AtPABP2 was also strongly expressed in the transmittal tissues, and possible involvement in pollination-dependent poly- (A) tail shortening was proposed (Palanivelu et al., 200b). AtPABP1 is expressed mainly in roots, with a lower expression in immature flowers. AtPABP5 and AtPABP3 share only 55% and 65% identity with AtPABP2 and both are only expressed in immature flowers (Belostotsky and Meagher, 1993). Detailed study on AtPABP5 found that the expression was restricted to pollen and ovule development and early emryogenesis (Belostotsky and Meagher, 1996).

Comparison of amino acid sequences of PABPs from available data suggested that NtPABP3 (tobacco PABP3), NtPABP7 and TsPABP (wheat PABP) are most closely related to AtPABP2; identity among them ranges from about 64% (wheat vs. Arabidopsis) to 72% (tobacco vs. Arabidosis). The two tobacco PABPs share 80% identity (Le and Gallie, 2000). AtPABP5 and AtPABP3 are less related not only to AtPABP2, but also to available PABPs from other species, suggesting either that their

counterparts have not been identified or they are evolutionally more divergent from others.

Functions of Arabidopsis PABPs have been studied in a yeast strain lacking a functional yeast PABP gene. Both AtPABP5 and AtPABP2 can partially restore the poly-A shortening, the initiation of translation, and the viability of yeast. However, AtPABP5 could not restore the coupling between mRNA deadnylation and decapping, but AtPAB2 could (Belostotsky and Meagher, 1996; Palanivelu et al., 2000a), suggesting a functional difference of these two members. However, the different functions of different members *in planta* are currently unknown, partially owing to the inability to identify PABP knock-out Arabidopsis. Presumably as in yeast, PABPs are essential in plants (Palanivelu et al., 2000b).

## 4. Poly- (A) binding protein and viral infections

Viruses rely on hosts for energy, substrates, and key proteins. To make full use of the host translational apparatus, viruses often shut down host translation (Pe'ery and Mathews, 2000). Given its important roles in translation and viability of eukaryotes, PABP is one of the major targets for host shut-off during viral infections (Chen et al., 1999; Joachims et al., 1999; Kerekatte et al., 1999; Piron et al., 1998). The NS1 protein of influenza virus A binds to human PABP II, which is necessary for polyadenylation, in the nucleus and sequesters PABP from binding to the oligo- (A) tail of host pre-mRNAs. This prevents further adenylation of pre-mRNAs, disrupts the export of mRNAs from nucleus to cytoplasm, and leads to the accumulation of pre-mRNAs and PABP in the nucleus (Chen et al., 1999). Human PABP1 was also cleaved at a site in between RRM4 and PABP-CT by the picornaviral 2A protein during enterovirus and coxsackievirus

infections and the cleavage of PABP correlated with the host shut-off (Joachims et al., 1999; Kerekatte et al., 1999). The viral NSP3A protein from Rotavirus was found to have much higher affinity to human eIF4G I than human PABP1 has to eIF4G I, and NSP3A could replace PABP proteins that associated with eIF4 complex. This led to two related results: host translation was shut down because PABP no longer participated in host translation, and viral translation was stimulated, because Rotavirus mRNAs are capped but not polyadnylated. NSP3A binds to the 3' end of the rotaviral genome and bridged the 5' and 3' end of viral mRNAs by interacting with the eIF4 complex that binds to viral 5' cap (Piron et al., 1998).

## Objectives of the dissertation

The interactions between a virus and its hosts are critical for successful infection. This is particularly true for RNA viruses that have a small genome and do not encode enough functions to independently replicate and perform other critical processes. Successful replication requires adaptation of host machinery as is evidenced by the growing number of host factors that have been identified to be involved in viral replication.

The potyvirus family, or Potyviridae, is the largest and one of the most economically devastating plant virus families. Replication of potyviruses has been extensively studied and it is known that most of potyviral proteins are involved in replication. However, host components of the replication complex had not yet been identified. In this dissertation work, I sought to determine whether there are host proteins associating with ZYMV RdRp and determine whether they are involved in potyviral replication. As studies of viral infection often provide new insight into host processes, I

also sought to examine the normal cellular functions of the ZYMV RdRp-interacting host proteins and their partners.

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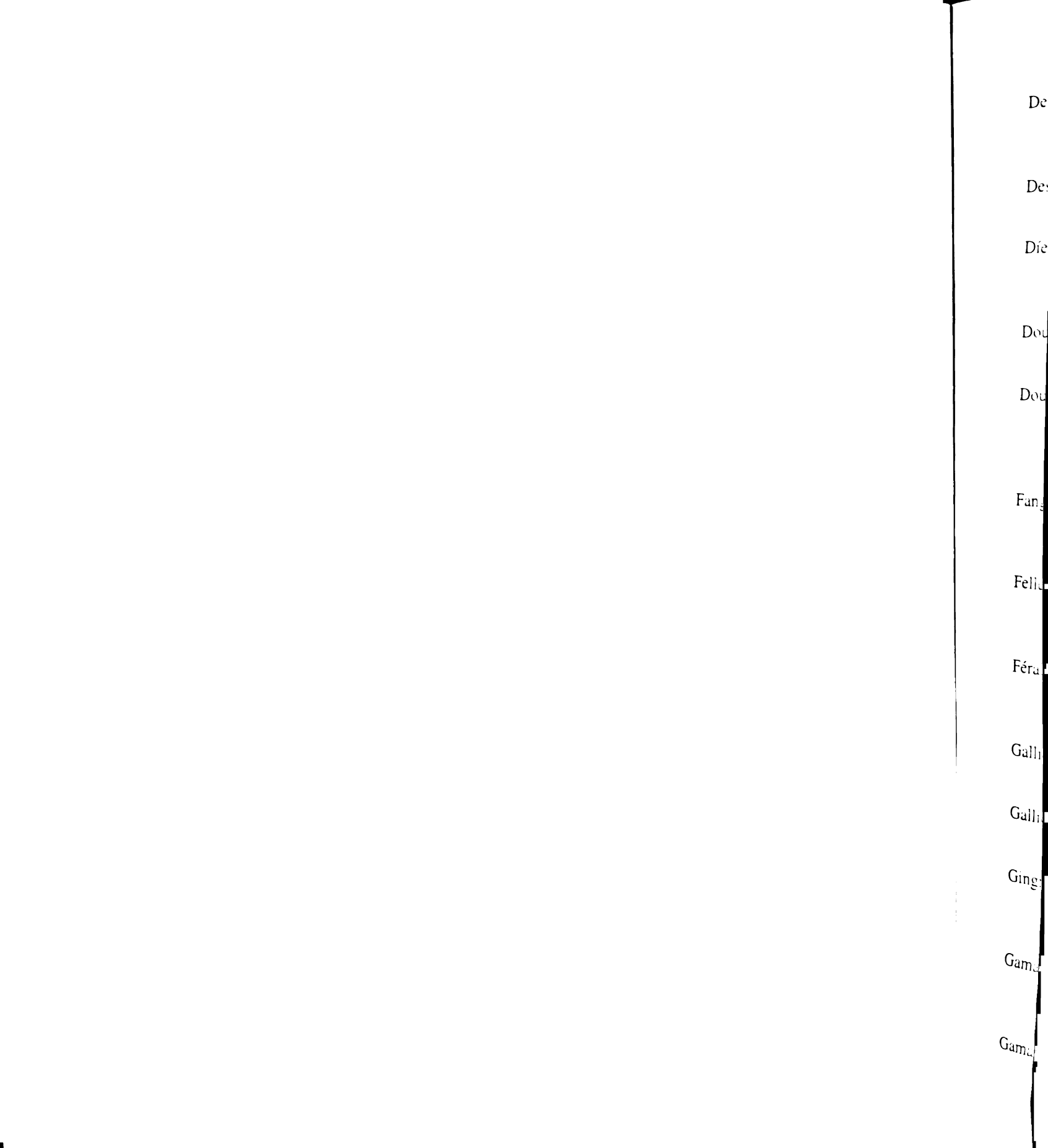
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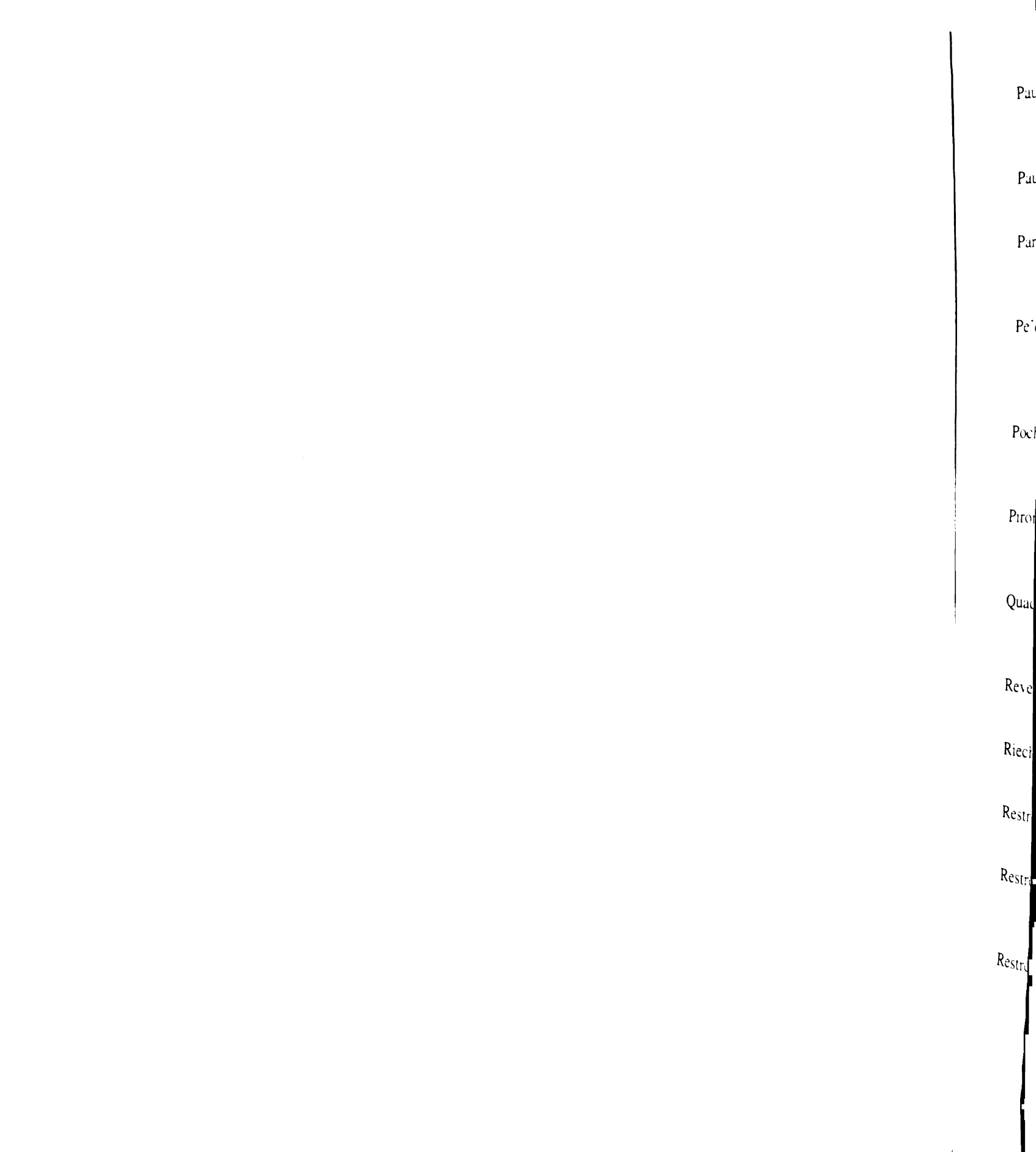
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# Chapter 2

## Interaction between zucchini yellow mosaic potyvirus RNA-dependent RNA polymerase and host poly-(A)-binding protein

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Ullah, Z contributed to Fig. 2-3

## Abstract

Viral replication depends on compatible interactions between a virus and its host. For RNA viruses, the viral replicases (RNA dependent RNA polymerases; RdRps) often associate with components of the host translational apparatus. To date, host factors interacting with potyvirus replicases have not bee identified. The Potyviridae, which form the largest and most economically important plant virus family, have numerous similarities with the animal virus family, the Picornaviridae. Potyviruses have a single stranded, plus sense genome; replication initiates at the viral-encoded, 3' poly (A) terminus. The yeast two-hybrid system was used to identify host plant proteins associating with the RdRp of zucchini yellow mosaic potyvirus (ZYMV). Several cDNA clones representing a single copy of a poly-(A) binding protein (PABP) gene were isolated from a cucumber (*Cucumis sativus* L.) leaf cDNA library. Deletion analysis indicated that the C-terminus of the PABP is necessary and sufficient for interaction with

the RdRp. Full-length cucumber *PABP* cDNA was obtained using 5' RACE; *in vitro*- and *E. coli*-expressed PABP bound to poly-(A)-sepharose and ZYMY RdRp with or without the presence of poly (A). This is the first report of an interaction between a viral replicase and PABP, and may implicate a role for host PABP in the potyviral infection process.

# Introduction

Successful systemic infection by a pathogen depends on compatible interactions between the pathogen and its host. This is particularly true for viral pathogens which have extremely small genomes and limited protein coding capacity. Several host proteins are involved in replication of RNA viruses, either as components of the viral replication complex, or by binding directly to the viral genome (reviewed in: Lai, 1998; Strauss and Strauss, 1999). For RNA viruses, a majority of the factors found in association with the viral replicase, the RNA dependent RNA polymerase (RdRp), are subverted from the host RNA-processing and translational machinery. For example, elongation factors EF-1$\alpha$ and different subunits of eIF3 are associated with the replicase complexes of an array of bacterial, plant, and mammalian RNA viruses such as QB phage, brome mosaic virus, tobacco mosaic virus, vesicular stomatis virus, measles virus and poliovirus (Lai, 1998; Strauss and Strauss, 1999). The exact roles of the translational machinery proteins in viral replicase complexes are not fully understood; virus replication and translation might be coupled, or the host proteins may play different roles in virus replication than they do in host translation.

The Potyviridae, which resemble the animal virus family, the Picornaviridae, form the largest and one of the economically most important families of plant viruses;

approximately 200 members cause serious diseases in a wide range of crop plants (Shukla et al. 1994). The members of this family have a plus sense, single stranded RNA genome of approximately 10 Kb, a VPg (viral protein genome linked) covalently linked to the 5' end, and a poly-(A) tail at the 3' end. The RNA encodes a single polyprotein, which is subsequently cleaved into nine proteins by viral-encoded proteases (Dougherty and Semler, 1993); function of the various potyviral proteins has been a active area of investigation. Among the potyviral proteins, NIb (nuclear inclusion b; originally named for its tendency to accumulate in the nucleus), functions as an RNA-dependent RNA polymerase (RdRp) (Hong and Hunt, 1996; Li and Carrington, 1995). Potyviral RdRps of tobacco etch virus (TEV) and tobacco vein mottling virus (TVMV) interact with other potyviral proteins including P1, P3, and the NIa (nuclear inclusion a) protein, which is composed of the VPg at the amino terminus and the main viral proteinase at the carboxy terminus (Hong et al. 1995; Li et al. 1997; Fellers et al. 1998; Merits et al., 1999; Daros et al. 1999).

Replication, which proceeds by copying of the plus strand to the complementary minus strand intermediate, followed by plus strand synthesis, occurs in membrane associated, cytoplasmic fraction (Martin and Garcia, 1991; Schaad et al. 1997). Cytoplasmic localization of the replication machinery is thought to be achieved by direction of a subset of the potyviral NIa protein proteolytic precursor (including the amino terminal adjacent 6 kDa hydrophobic protein) toward the endoplasmic reticulum rather than the nucleus (Schaad et al. 1997). The RdRp is, in turn, postulated to be recruited to the membrane fraction via interaction with the 6 kDa - VPg - proteinase (NIa) complex.

Replication of the minus strand is initiated at the 3' poly (A) tail of the plus sense, genomic RNA. Presence of a poly (A) tail is essential for replication of picornaviruses such as poliovirus, encephalomyocarditis virus and rhinovirus (Cui et al. 1993, Todd and Semler, 1996 Todd et al. 1997; Agol et al. 1999) and recent evidence suggests it is also necessary for potyvirus replication (Tacahashi and Uyeda, 1999). Initiation of poliovirus replication requires uridylylation of the VPg which serves as a primer for minus strand synthesis (Agol et al. 1999). The uridylylation is performed by the poliovirus RdRp ($3D^{pol}$ protein) and requires the presence of poly (A) template (Paul et al. 1998). It is not known whether viral poly (A) tails exist as free poly (A)s within the host, or if like eukaryotic mRNAs, they are found in association with host poly (A) binding protein (PABP). Given the reported high abundance of PABPs, studies have indicated an approximately 75-95% fold excess of free PABP over binding sites on cytoplasmic poly (A) (Drawbridge et al., 1990; Gorlach et al. 1994), association of PABP with viral RNAs seems likely. PABP-dependent translation of viral genomes, analgous to the PABP-dependent translation of eukaryotic mRNAs, has been proposed (Gallie, 1998).

Poliovirus and other picornavirus RdRps do not bind to poly (A) directly (Cui et al. 1993; Paul et al. 1994). The poliovirus $3D^{pol}$ is recruited to a complex secondary structure in the 3' non-translated region (NTR) upstream of the poly (A) tail via interaction with the RNA-binding 3AB proteins (analagous to the potyviral 6 kDa-VPg; 3A is involved membrane association; 3B is the VPg) (reviews: Xiang et al. 1997; Agol et al. 1999). These interactions are thought to provide template specificity. Potyviruses appear to have important secondary structure near the 3' end of the genome which may include sequences within both the 3' NTR and the adjacent coat protein coding region

(Mahajan et al. 1996; Haldeman-Cahill et al. 1998). Although mutations in these regions interfere with replication, specific binding of viral proteins to these sequences has not been demonstrated for potyviruses.

Specific host factors interacting with potyviral replicases have not been identified yet. In this study we examined interactions between zucchini yellow mosaic potyvirus (ZYMV) and its cucumber (*Cucumis sativus* L.) host, by identifying cucumber proteins that interact with ZYMV RdRp. Yeast two-hybrid analysis demonstrated reproducible and specific interactions between ZYMV RdRp and cucumber poly (A)-binding protein.

# Materials and Methods

## Strains and plasmids

Plasmid pBluescript KS and *Esherichia coli* strain XL1-Blue (Stratagene) were used for general DNA manipulation. Yeast (*Saccharomyces cerevisiae*) strain YRG2 and the GAL-4 based two-hybrid plasmids pBD-GAL4, p53, pLAMINC, pSV40, and pAD-GAL4 were purchased from Stratagene. pBD-GAL4 and pAD-GAL4 (binding domain and activation domain, respectively) were used to express coat protein (CP), helper component-proteinase (HC-Pro), RNA-dependent RNA polymerase (RdRp), and poly-(A) binding protein (PABP) in yeast as GAL4 binding domain- or activation domain-fusion proteins. pGEM®-T EASY vector (Promega) was used for cloning 5' RACE (rapid amplification of cDNA ends) products. pGEX-5x-1 (Phamacia) was used to produce fusion proteins GST-PABP or GST-RdRp in *E. coli* strain Xa 90, and pET-28a-1 (Novagen) was used to produce His-PABP in *E. coli* strain BL21(DE3).

The ZYMV RdRp gene was amplified by PCR with Vent DNA polymerase (New England Biolabs) using ZYMV cDNA [Connecticut (CT) isolate; Grumet and Fang,

1990] as template. The amplified product was inserted as an *EcoRI - SalI* fragment into pBD-GAL4, pAD-GAL4 and pGEX-5x-1 vectors in frame to form pBDRdRp, pADRdRp, and pGEXRdRp respectively, and sequenced. The primers were designed as follows (restriction sites are italicized): 5' end primer, 5' CCG*GAATTC*AGCAAGCGA GAAAGATG 3', and 3' end primer 5' AGA*GTCGAC*TTGGAGCATCACAGTGT 3'.

The helper component-protease (HC-Pro) gene was amplified as above using the ZYMV NAA isolate cDNA (Gal-On et al., 1992) as template, inserted as an *EcoRI - SalI* fragment into pBD-GAL4 and pAD-GAL4 vectors in frame to form pBDHC and pADHC, and sequenced. The primers were: 5' end primer 5' CCG*GAATTC*AGCG AAGTTGACCAC 3' and 3' end primer 5' AGA*GTCGAC*ACCAACTCTGTAATG 3'.

The ZYMV-CT isolate coat protein (CP) gene in the pTL37-CP construct (Fang and Grumet, 1993), was digested with *NcoI*, filled in with Klenow fragment (Gibco BRL), and then digested with *PstI*. The blunt-*PstI* CP fragment was then ligated to pUC119, which had been digested with *EcoRI*, filled in with Klenow, then digested with *PstI*. The *EcoRI - PstI* CP fragment was then inserted into pBD-GAL4 and pAD-GAL4 vectors to form pBDCP and pADCP, and sequenced.

The yeast two-hybrid full-length cucumber PABP clones were generated as follows. PCR (Vent polymerase) was used to amplify the 5' end of the full length PABP clone (obtained by 5' RACE as described below) and to add an *EcoRI* site to the start codon. The 5' end primer was 5' TA*GAATTC*ATGGCTCAGGTTCCACC 3'. The 3' end primer was the GSP2 primer used for 5' RACE (see below). The PCR product was digested with *EcoRI* and *ClaI*, ligated to the *EcoRI - ClaI* digested plasmid pADNI8, which had the partial PABP clone, to form pADPABP. The full length PABP was also

inserted as an *EcoRI* - *SalI* fragment into pBD-GAL4, pGEX-5x-1, and pET-28a-1 to form pBDPABP, pGEXPABP, and pETPABP.

## Construction of the cucumber leaf two-hybrid cDNA library in λ HybriZAP-2.1 vector

Total RNA was isolated from young leaves of cucumber cultivar 'Straight 8' as described by Chomczynski and Sacchi (1987); mRNA was further purified using the Promega PolyATract® mRNA isolation system II. Approximately five μg mRNA was used for cDNA synthesis following the protocol of the HybriZAP® 2.1 two-hybrid cDNA synthesis kit, and cDNAs (ranging from 0.5 Kb to 2.5 Kb) were inserted into the λ HybriZAP-2.1 vector as *EcoRI* - *XhoI* fragments. The primary library contained ~ $5\times10^6$ PFU (plaque forming units). The primary λ cDNA library was amplified once and then converted to a plasmid (pAD-GAL4) library by *in vivo* mass excision according to the Stratagene protocol.

## Screening the library with RdRp and testing for interactions between viral proteins

Yeast transformation, growth media, and X-gal (5-bromo-4-chloro-3-indolyl B-D-galactopyranoside) filter assays were performed following the supplier's procedures (Stratagene). Yeast YRG2 cells were first transformed with pBDRdRp, and then transformed with CsCl-purified cDNA library plasmid DNA. The transformants were plated onto SD (synthetic dropout) medium without leucine, tryptophane, or histidine (SD-L-T-H). Colonies that grew on selection medium were restreaked onto SD-L-T-H, transferred to nitrocellulose membrane (Schleicher & Schuell), and assayed for

expression of β-galactosidase activity (Lac Z) by X-Gal filter assay. Colonies that survived in medium without histidine and turned blue in the X-Gal assay were considered as putative positives. Plasmids were recovered from putative positive yeast colonies using Zymoprep yeast plasmid miniprep (Zymo Research, CA), transformed to *E. coli*, and amplified. The recovered plasmids were then transformed back to YRG2 yeast either alone, or in combination with pBD-GAL4, p53, pLamin C, or pBD-RdRp. The transformants were plated onto SD-H or SD-L-T-H, and assayed for Lac Z activity. Those that can turn on two reporter genes only in the presence of pBD-RdRp, but can not turn on reporter genes, either alone, or in combination with pBD-GAL4, p53 or pLamin C were considered to be postitives.

## 5' RACE (rapid amplification of cDNA ends)

A 5' primer, GSP1, 5'-TCATTCTTCCATT CATCTCAGCAA-3') and 3' primer, GSP2 (5'-TTGTCATCATCGATGCTATCAT) complementing the 5' end of the longest PABP cDNA (NI 8) near the *SacI* and *ClaI* sites respectively, were synthesized as gene specific primers for 5'RACE. The fragments were then amplified according to the 5' RACE protocol (Gibco BRL), and cloned to pGEM®-T EASY vector using AT cloning (Promega protocol). The two longest cDNA fragments, 1.2 and 1.4 kb, were sequenced. The overlapping sequences of the two cDNAs were the same. One had a 397 bp 5' NTR, the other had a 216 bp 5' NTR.

## RdRp and PABP deletions

All the PABP deletions were made from the longest cDNA obtained from the two hybrid screen, NI8 (Fig. 2-3). NI8Δ300 was amplified by PCR, using RG110 and RG157 as 5' and 3' terminal primers. RG110 was located at the 5' end of NI8, and included an

*EcoRI* site, 5'-AAA*GAATTC*GGCTTTGTAAATTTTGAG-3'. RG 105 complemented the region from position 1801 to 1819 (position denoted according to full-length *Cs-PABP* cDNA) of NI8 and included a *XhoI* site, 5'-TCT*CTCGAG*CAAATGTAGAACC TCAGT-3'. The PCR product was inserted into pADGAL4 as an *EcoRI-XhoI* fragment to form pADNI8Δ300. NIΔmlu was amplified with RG110 and RG115, which complemented the region from position 1476 to 1495, with *XhoI*, *SphI*, and *SacI* sites at the 5'end, 5'- GTA*CTCGAGCATGCGAGCTC*AAAGGTACAGGCTGCTGG-3'. The PCR product was inserted as an *EcoRI-XhoI* fragment into pADGAL4 to form pADNI8Δmlu, and into pBluescript to form pBSNI8Δmlu. pBSNI8Δmlu was then digested with *SphI*, and religated to form pBSNI8Δsph. The *EcoRI-XhoI* NI8Δsph fragment was then subcloned to pADGAL4 to form pADNI8Δsph.

All the RdRp deletions were made from full-length RdRp (Fig. 2-3). RdRpΔ1, Δ4, Δ5, Δ6, Δ7, and Δ8 were amplified by PCR using primers as indicated in Fig. 2-3; all the PCR products were inserted into pBDGAL4 as *EcoRI-SalI* fragments. The sequences of primers RG100, 101, 121, 125, 126, and 127 were: 5'-AGA*GTCGAC*CCTGACTTTCT CAAGC-3', 5'-CCG*GAATTC*TGC GCTGCGATGATT-3'; 5'-ACT*GAATTC*CTCGAG AAAGAGAGAAT-3'; 5'-GTG*GAATTC*CCAATTCTTGCTCCTGA-3'; 5'-TTA*GAA TTC*GAGCTCAGGCCGCTT-3', and 5'-TTC*GTCGAC*TCTCGAGTTTTGGAGTG-3', respectively. RdRpΔ2 was generated as follows: pBDRdRp was digested with *NcoI*, filled in with Klenow fragment, and digested with *EcoRI*. This RdRp fragment was ligated to pBDGAL4, which had been digested with *SalI*, blunted, then cut by *EcoRI* to form pBDRdRpΔ2. pBSRdRp (RdRp in pBluescript) was cut with *EcoRI* and *XhoI*, the *EcoRI-XhoI* RdRpΔ3 fragment was then subcloned to pBDGAL4 as pBDRdRpΔ3.

## In vitro expression and poly-(A)-sepharose binding of PABP and RdRp

Full-length PABP and RdRp were cloned to pET28a-1 to produce histidine-tagged PABP or RdRp. Each pET plasmid, and the Promega TNT® system control construct expressing luciferase without a histidine tag, was expressed in a 50μl volume using 2μg plasmid DNA in the Promega TNT® quick coupled transcription/translation system with the addition of 0.4 mM magnesium acetate, 30 mM KCl, and $^{35}$S-methionine. 5 μl of 50 ul reaction product was incubated with poly-(A)-sepharose (Sigma) for 1 hour on ice in 100 μl binding buffer [100 mM sodium acetate, 5 mM $MgCl_2$, 5 mM KCl, 40 mM HEPES, pH 7.6, 10% glycerol, protease inhibitor cocktail (Sigma), 0.2% Triton X-100, 5 mM β-mercaptoethanol]. The beads were washed six times with 500 μl binding buffer plus 200 mM NaCl. The beads were then boiled in 24 μl loading buffer and analyzed by SDS-PAGE followed by autoradiography overnight.

## Expression of PABP and RdRp in *E. coli*

The *E. coli* stain Xa 90 harboring pGEXPABP was grown at 37 C in LB with 50 mg/l ampicillin to $O.D._{600}$ = 1.0. IPTG (β-D-thiogalactoside) was added to 0.1 mM and the cells grown for another 3 hours at 37 C, then harvested. The *E. coli* stain Xa 90 harboring pGEXRdRp was grown at 37 C in LB with 50 mg/l ampicillin to $O.D._{600}$ = 0.3-0.4. IPTG was added to the medium and the cells were then grown at 16 C overnight prior to harvesting. GST-tagged PABP and RdRp were purified as recommended by the supplier (Phamacia); yield of protein was estimated by Biorad assay. *E. coli* strain BL21 (DE3) harboring pET-PABP was grown at 37 C in LB with kanamycin to $O.D._{600}$ = 0.6. After addition of IPTG to 1.0 mM, the cells were grown for 3 h at 37 C. His-tagged PABP was purified as recommended by the supplier (Novagen).

## In vitro analysis of protein-protein interaction

Five μg GST, or GST-PABP purified from *E. coli* was immobilized on 30 μl glutathion-sepharose (Pharmacia), and incubated with 5 μl of *in vitro* $^{35}S$ labeled RdRp or luciferase (prepared using Promega TNT quick coupled transcription/translation system) in 90 μl binding buffer plus 10 μl bovine serum at room temperature for 1 hour. The beads were then washed 6 times with 500 μl binding buffer plus 200 mM NaCl, boiled in 24 μl loading buffer, and analyzed by SDS-PAGE followed by autoradiography overnight. For binding to poly (A), 5 μg His-PABP was immobilized on poly-(A)-sepharose, 5 μl in vitro $^{35}S$ labeled luciferase or RdRp were added. Binding, washing, SDS-PAGE and autoradiography were as above.

## DNA and RNA hybridization

Genomic DNA was extracted from young cucumber leaves according to the protocol of Dellaporta et al. (1985). Ten μg restriction enzyme-digested DNA was subjected to electrophoresis on a 1% agarose gel and transferred to nylon membrane (Micron Separations) by capillary blotting (Sambrook et al, 1989). Total RNA was isolated from cucumber leaf, root, immature and mature flowers as described by Chomczynski and Sacchi (1987); 10 μg of each was used for northern blot (Sambrook et al., 1989). Full-length *CS-PABP1* was labeled using the DIG DNA labeling and detection kit (Boeringer Mannheim). High stringency hybridization and wash conditions were done according to Sambrook et al. (1989). Low stringency hybridization and wash conditions were done following Hilson et al. (1993).

# Results

## Screening of the cucumber yeast two-hybrid library with ZYMV RdRp

The yeast two-hybrid cDNA library was constructed from mRNA from leaf tissue of ZYMV- susceptible cucumber (cv. Straight 8). The library contained 5 X $10^6$ pfu; greater than 90% of the excised plasmids encoded cDNA inserts ranging in size from 0.5-2.5 kb. Quality of the library was assessed by successful PCR amplification of the low copy cucumber gene, *CS-ACS1* (ACC synthase; Trebitsch et al. 1996). Following two separate screenings of the library using ZYMV RdRp expressed in the GAL4 binding domain vector as bait, eleven cucumber clones were found to have specific, reproducible interaction with the RdRp. Each clone induced the two reporter genes allowing for growth in the absence of histidine and expression of β-galactosidase activity. In each case, plasmids encoding putative interactors were isolated and retransformed back to yeast either alone, or in combination with ZYMV RdRp, human p53, Lamin C, or binding domain vector alone. Each interacting clone failed to induce the reporter genes alone, or in any combination except with RdRp.

Of the eleven verified clones showing interaction with RdRp, three enocoded distinct, unknown proteins, one had significant sequence homology to DNA J, one to protein phosphatase, and six encoded a protein having high homology (ca. 70% nucleotide identity) to poly-(A) binding protein (PABP) of *Arabidopsis thaliana* and wheat. The PABP-homologous cDNAs ranged in size from 0.5 kb to 1.5 kb, the longer the cDNA, the stronger the observed interaction with RdRp. The six RdRp-interacting cDNAs were reverse transcribed from at least three different mRNAs: cDNA NI 8, 60, and NI 351 have the same polyadenylation sites149 nucleotides downstream of the stop

codon; NI 7 has a poly-(A) addition site 201 nucleotides from the stop codon; and NI 439 and 447 have poly-(A) addition sites 250 nucleotides from the stop codon. Although PABPs are encoded by a multigene family in *Arabidopsis*, and multiple isoforms have been observed in wheat (Belostotsky and Meagher, 1993; Hilson et al. 1993; Le et al., 1997), the overlapping sequence of all six cDNAs showed complete sequence identity, indicating that all of the clones were transcribed from the same cucumber *PAPB* gene.

The putative *PABP* clones were tested for interaction with two other ZYMV proteins, coat protein (CP) and helper component protease (HC-Pro) (Table 1-1). Although both the CP and HC clones were capable of self-interaction, as would be predicted based on ability of CP to polymerize to form capsids, HC-Pro to dimerize, and previous yeast two-hybrid studies (Hong et al. 1995; Thornbury et al. 1985; Ureuqui-Inchima et al. 1999; Wang and Pirone, 1999), neither the ZYMV CP nor HC-Pro interacted with cucumber PABP. These results further suggest a specific interaction between cucumber PABP and the potyviral RdRp. The interaction between PABP and ZYMV RdRp did not occur when tested with RdRp fused to the GAL4 activation domain and PABP to the DNA binding domain. One way interactions have been observed in numerous other two-hybrid combinations and may be due to conformational changes arising from the fusion protein (Fields and Sternglanz, 1994). PABP also did not interact with itself; this is consistent with previous studies indicating that multimerization of PABP requires the presence of poly (A) (Kuhn and Pieler, 1996). The ZYMV RdRp did not interact with itself, CP, or HC-Pro.

**Table 2-1. Interactions among ZYMV coat protein (CP), helper component-proteinase (HC-Pro), RNA dependent RNA polymerase (RdRp), and cucumber poly-(A)-binding protein (PABP) in the yeast two-hybrid system.**

| | BD-CP | BD-HC-Pro | BD-PABP | BD-RdRp |
|---|---|---|---|---|
| AD-CP | +++[a] | - | ND | - |
| AD-HC-Pro | - | ++ | ND | - |
| AD-PABP | - | - | - | +++ |
| AD-RdRp | - | - | - | - |

ND: Not determined.

[a] The strength of the interactions were based on the X-Gal filter assay. +++, colonies turned dark blue within 3 hours; ++, colonies turned dark blue within 6 hours to overnight; +, colonies turned light blue overnight; -, did not turn blue. All controls, including the clones by themselves or in combination with human p53 or Lamin C, remained white.

## Characterization of the cucumber PABP gene and interacting domains of the PABP and RdRp proteins

To further examine the interaction between the ZYMV RdRp and the putative cucumber PABP, the full length cDNA was obtained by 5' RACE (5' rapid amplification of cDNA ends). In total, a 2597bp cDNA sequence was obtained for the cucumber *PABP* gene (*CS-PAPB1*; submitted to GenBank, accession no. AF240679). In the 397bp 5' NTR of the cDNA, there are 9 oligo-(A) clusters, ranging from 4 A to 9 A. These A-rich sequences were also found in PABPs from *Arabidopsis*, wheat and other organisms (Belostotsky and Meagher, 1993; Hilson et al. 1993; Le et al., 1997). The 3' NTR is 250bp and three polyadenylation signal sequences are located at positions 2099, 2151, and 2201 bp.

The coding sequence is 1950 bp and predicts a 649 amino acid protein with a molecular weight of 70.5 kD which is similar to other plant and animal PABPs (Belostostsky and Meagher, 1993; Le et al. 1997). This gene (*CS-PABP1*) is most similar to the shoot-expressed *Arabidopsis PABP2* gene (Belostotsky and Meagher, 1993; Hilson et al. 1993). Comparison of percent amino acid identity shows greater homology between the putative cucumber *Cs-PABP1* gene and *Arabidopsis PABP2* (69.7%), than between *Arabidopsis PABP2* and *Arabidopsis PABP5* (49.4%). Consistent with apparent transcription from a single gene, Southern blot analysis of cucumber genomic DNA indicated that the cucumber *PABP* clone hybridized with a single distinct genomic band with most restriction enzymes (Fig. 2-1); additional bands were not observed in low stringency washes (data not shown). Although there is no *EcoRI* site within the coding region of the *CS-PABP1* gene, the two bands observed in the *EcoRI* digest are likely due

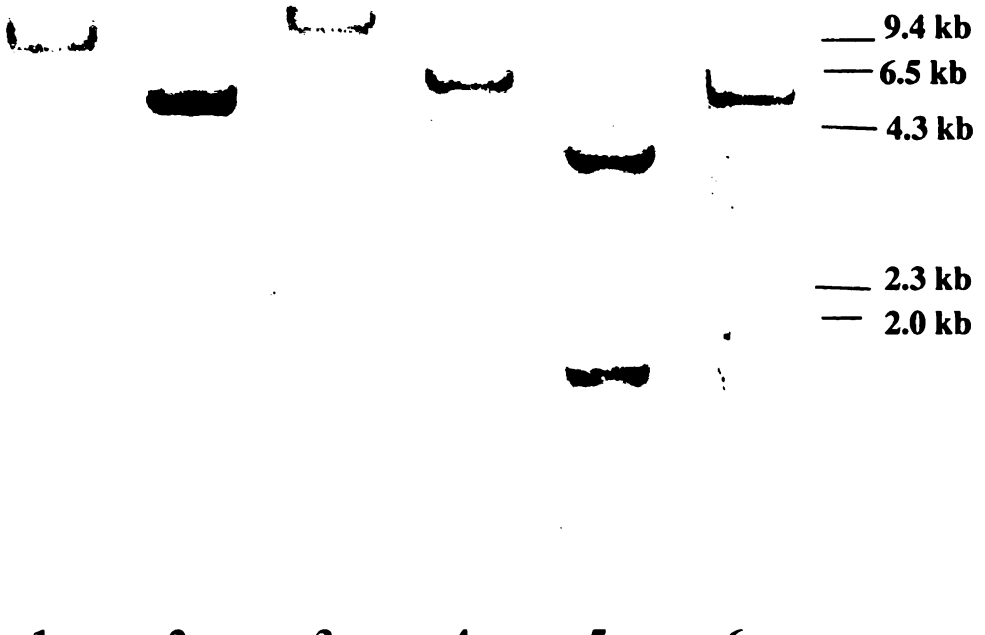


**Fig. 2-1. PABP is a single or low copy number gene.** Cucumber DNA (~10μg) was digested with *XhoI* (1), *XbaI* (2), *PstI* (3), *HindIII* (4), *EcoRI* (5), and *BamHI* (6), transferred to nylon membrane, and hybridized with Dig-labeled PABP coding sequence. PABP cDNA sequence does not have internal recognition site for these restriction endonucleases.

an *EcoRI* site within an intron. Northern blot analysis of leaves, roots, immature and mature flowers from susceptible 'Straight 8' plants, and leaves from the resistant lines 'TMG-1' and 'Dina-1', showed expression of *CS-PABP1* in all tissues tested; in each case hybridization was with a single band (data not shown). Higher expression was observed in leaves and immature flowers; there was no difference in expression between the susceptible and resistant genotypes.

The amino terminal two-thirds of the predicted PABP contains 4 RRMs (RNA recognition motifs), which are found in PABPs from all sources, including yeast and animals (Le et al., 1997) (Fig. 2-2). Each RRM in turn has two conserved sequences, RNP-1 and RNP-2, which come in direct contact with RNA (Kuhn and Pieler, 1996). RRM-1 is responsible for the interaction with eIF4G in yeast (Kessler and Sachs, 1998). The two smaller cucumber cDNAs (NI439 and NI359) lacked these RRMs, suggesting that the RRMs are not involved in binding with ZYMV RdRp.

The carboxy-terminal third of the PABP protein is not as highly conserved as the amino terminus, and functions of the C-terminus are less well defined. At the C-terminus (amino acids 553-624), there is a 71 amino acid region that is conserved among *Arabidopsis,* wheat, and cucumber. This CTC (C-terminal conserved) domain was also found within other characterized PABPs (e.g. yeast, vertebrate) and has been implicated in homodimerization and efficient poly (A) binding (Kuhn and Pieler, 1996). The smallest PABP cDNA obtained from the two-hybrid screen (NI 351) encodes the last 130 amino acids and interacts with the ZYMV RdRp. This suggests that the CTC domain might be involved in the interaction with RdRp. Consistent with these results, deletion of the C-terminal 50 amino acids of PABP abolished the interaction in yeast, indicating that

A.

MAQVPPQPQVPNSGADPAANGGANQHVI**TSLYVGDLDVNVTDSQLYDLFNQ**
**VGQVVSVRVCRDLTSRRSLGYGYVNYSNPVDASRALDVLNFTPLNGNPIRV**
**MYSHR**DPSVRKSGSGNIFIKNLDKAIDHKALHDTFSAFGSILSCKVATDSSGQ
**SKGFGFVQFDTEEAALKAIEKLNGMLLNDKQVFV**GPFLRKQERESVSEKTKF
**NNVFVKNLAETTSEEDLKNMFGEFGPITSVVVMRDGEGKSKCFGFVNFENA**
**DDAARSVEALNGKKVDGKEWYVGKAQK**KSEREVELKSRFEQSVKEAADKYQ
**GANLYVKNLDDSIDDDKLKELFTGFGTITSCKVMRDPNGISRGSGFVAFSSPE**
**EAARALAEMNGRMIVSKPLYVALAQR**KEDRIARLQAQFSQMQPMAMASSVA
PRGMPMYPPGGPGIGQQIFYGQAPPTIISSQPGFGYQQQLMPGMRPGGGPMPNFF
VPMVQQGQQGQRSGGRRAGAIQQTQQPVPLMQQQMLPRGRVYRYPPGRGLPD
LPMPGVAGGMFSVPYEMGGMPPRDAVHPQPVPVGA**LASALANATPDQQRTM**
**LGENLYPLVEQLEPDNAAKVTGMLLEMDQTEVLHLLESPEALKAKVAEAM**
**EVLRSVAQQSGNAADQLASLSLTDNLDS**

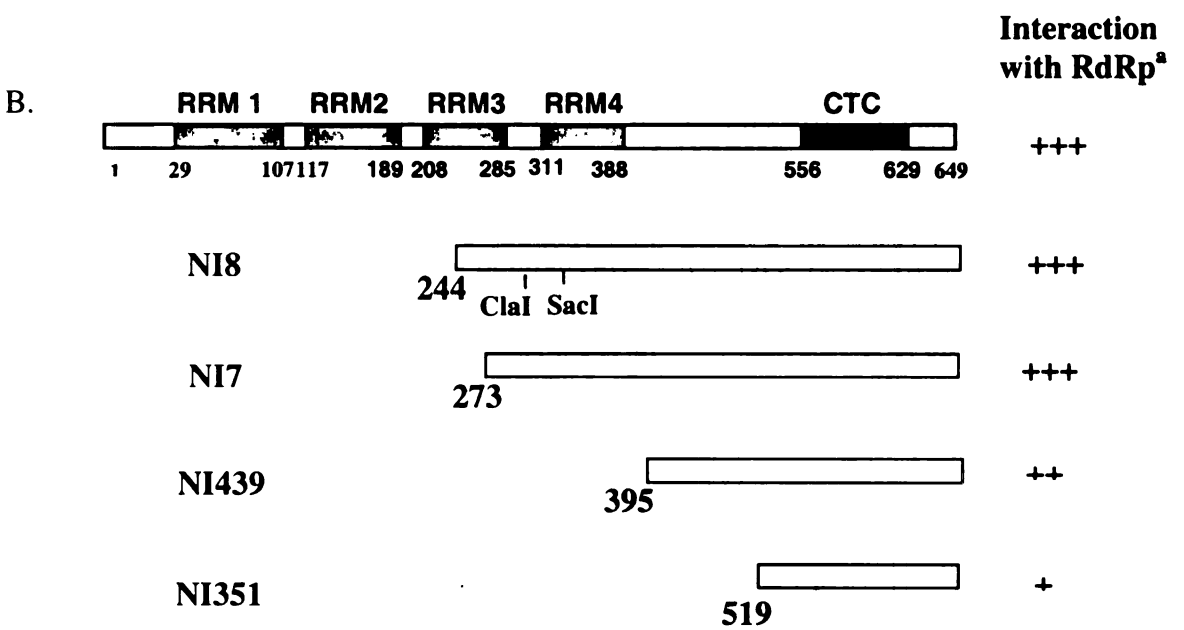


**Fig. 2-2. Predicted amino acid sequence of cucumber poly- (A) binding protein.** A. Amino acid sequence predicted from the full-length cDNA. The first 4 regions in bold are RNA recognition motifs (RRM), the last region in bold is the C-terminal conserved domain (CTC domain). B. Schematic presentation of full-length PABP protein and products for two-hybrid interacting PABP cDNAs (numbering of the clones is as in Table1).

the C-terminus of PABP is essential for the binding with RdRp (Fig. 2-3A); we cannot rule out, however, the possibility that the failure to detect interaction with the C-terminal deleted protein is due to lack of stability of the deleted protein.

Deletion analysis was used to examine regions of the RdRp responsible for interaction with PABP (Fig. 2-3B). Deletions from both the amino and carboxy termini of the RdRp abolished the interaction in yeast. Although we cannot eliminate the possibility that certain deletion products were unstable, it may be that a large portion of the RdRp is necessary for the interaction with PABP.

## *In vitro* verification of the RdRp - PABP interaction

The *in vitro* expression product of the full-length PABP cDNA migrated in SDS-PAGE gels as predicted for an ca. 70 KDa protein. The labeled cucumber PABP was able to bind to poly-(A)-sepharose *in vitro* (Fig. 2-4A, lane 7), confirming that the cloned cDNA encodes a true PABP. Like other picornaviral RdRps (Cui et al. 1993; Paul et al. 1994), ZYMV RdRp alone did not bind directly to poly (A) (Fig. 2-4A, lane 6). To demonstrate that the cucumber PABP can bind to ZYMV RdRp *in vitro*, full length PABP was expressed in *E. coli* as either a His-PABP fusion protein or GST-PABP fusion protein and was immobilized on poly-(A)-sepharose or glutathione-sepharose. Approximately 10-20% of the input RdRp bound to these fusion proteins (Fig. 2-4B, 4C), but none bound to poly-(A)-sepharose (Fig. 2-4B) or GST-sepharose alone (Fig. 2-4C). Luciferase did not bind to the PABP bound to polyA- or glutathione-sepharose. These results suggested that the RdRp and PABP bind to one another specifically *in vitro*. In analogous experiments where RdRp was expressed in *E. coli* as a GST-RdRp fusion protein and immobilized on glutathione-sepharose, approximately 10-20% of the *in vitro*

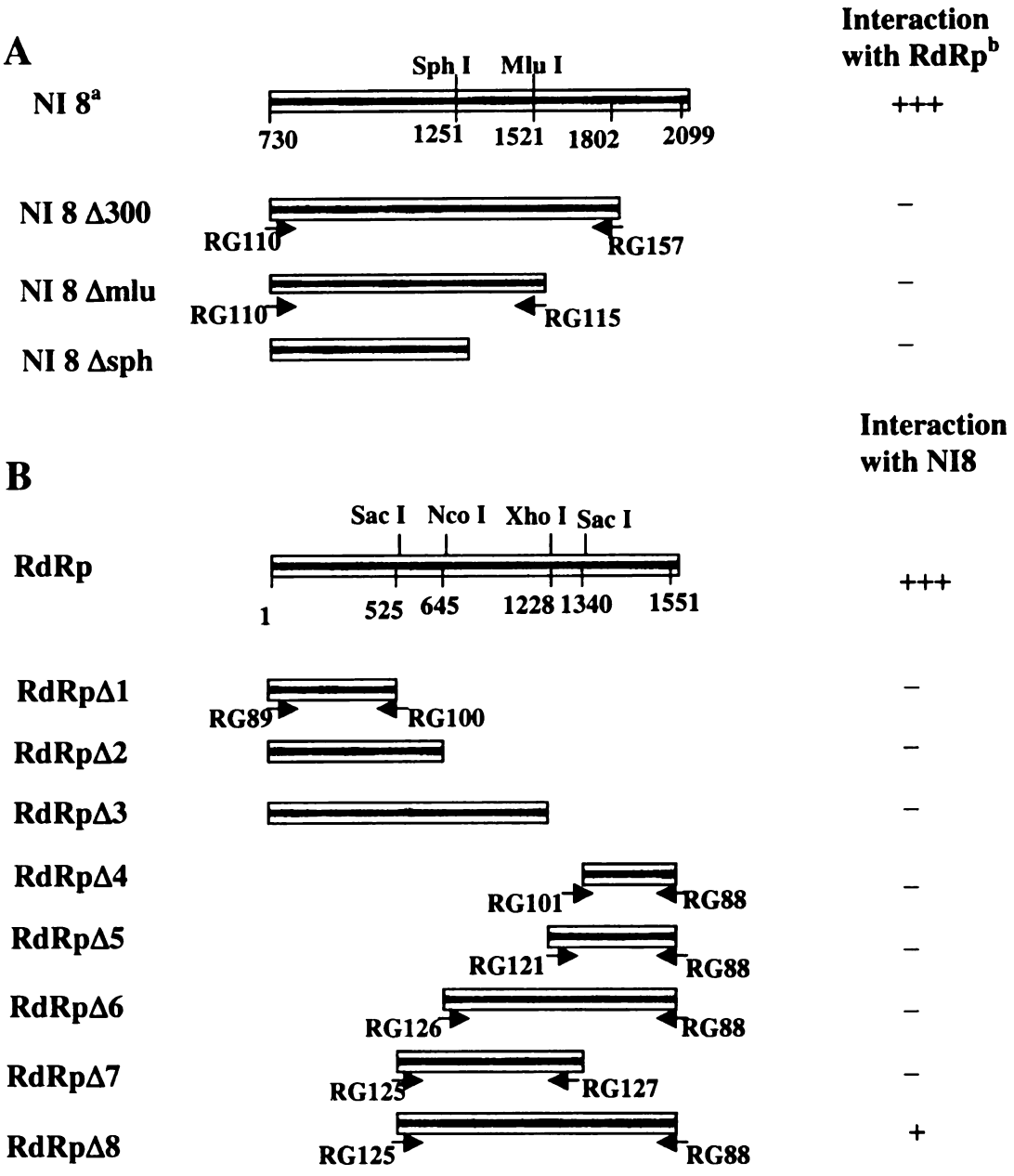


**Fig. 2-3 Deletion analysis to determine regions in PABP and RdRP responsible for the interaction**. A. Different sizes of cDNA NI 8 were amplified by PCR or generated by restriction enzyme digestion. All the deletions were tested against full-length RdRp in the yeast two-hybrid system. B. Different sizes of RdRp were amplified by PCR or generated by restriction enzyme digestion. All the deletions were tested against NI 8 in the yeast two-hybrid system. Numbering in each case corresponds to the full length cDNA sequence.

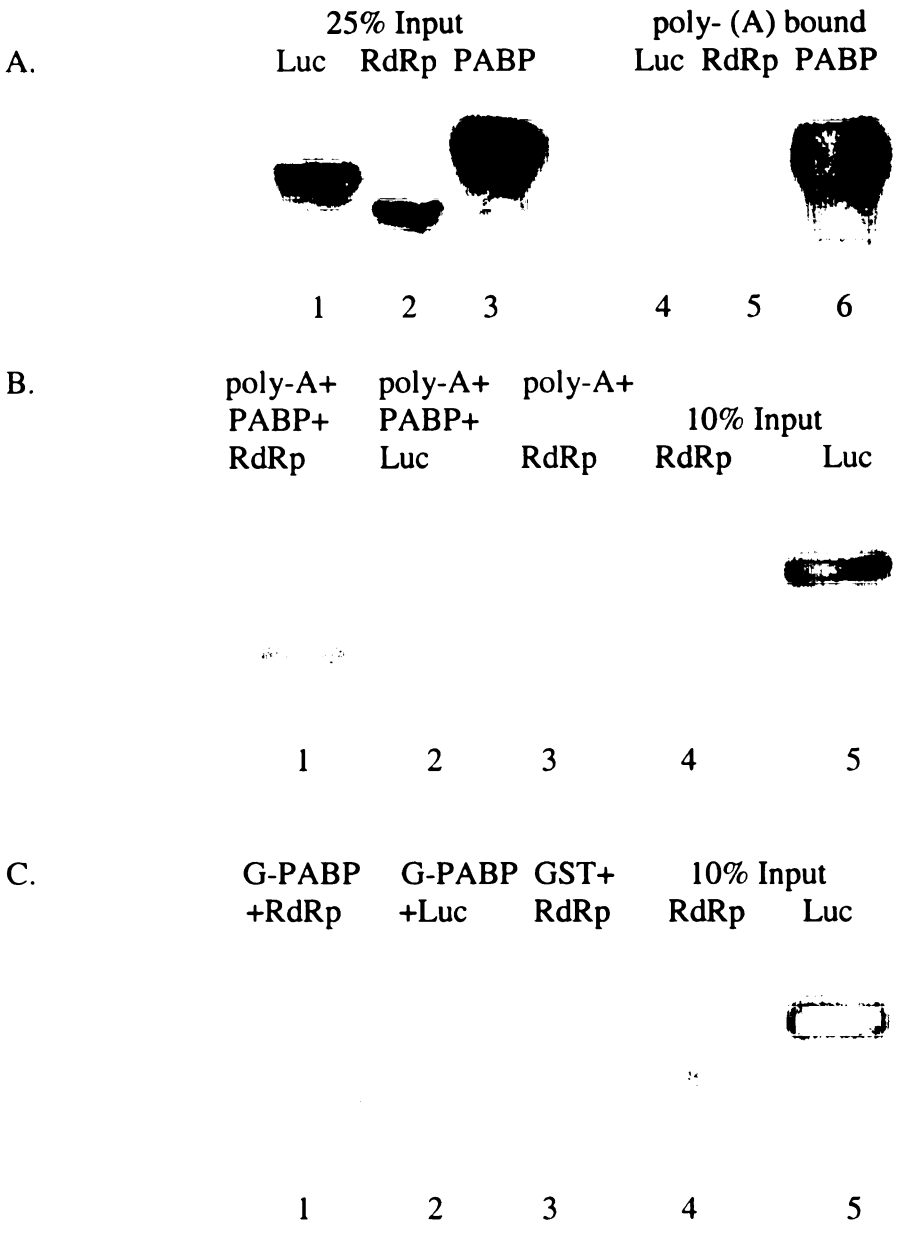


**Fig 2-4. Cucumber PABP can bind to poly-(A) and RdRp *in vitro*.** A. PABP can bind to poly-(A)-sepharose *in vitro*. Lane 1-Lane 3: 25% input of *in vitro* labeled luciferase (Luc), RdRp, and PABP. Lane 5- Lane 7: proteins on poly-(A)-sepharose after six washes. B. PABP can bind to RdRp *in vitro* in the presence of poly-(A). Lanes 1 and 2: His-PABP purified from *E. coli* was immobilized on poly-(A)-sepharose, and incubated

with $^{35}$S-RdRp (Lane 1) or $^{35}$S-Luc (Lane 2), followed by six washes. Lane 3: RdRp was directly incubated with poly-(A)-sepharose without His-PABP. Lanes 4 and 5:10% input of $^{35}$S-RdRp and $^{35}$S-Luc. C. PABP can bind to RdRp *in vitro* without the presence of poly-(A). GST-PABP (Lane 1, 2 ) or GST (Lane 3) was immobilized on glutathione-sepha-rose, and then incubated with $^{35}$S-RdRp (Lane 1, 3) or $^{35}$S-Luc (Lane 2) followed by six washes. Lanes 4 and 5 are 10% input of $^{35}$S-RdRp and $^{35}$S-Luc.

labeled PABP was retained (data not shown). Since a comparable percentage of binding was observed whether the RdRp or PABP was immobilized and whether RdRp was expressed *in vitro* or in *E. coli* suggests a modest affinity between the two molecules. Whether this modest affinity reflects affinity *in planta* where other interacting factors may be involved, is not known.

Consistent with the ability of the C-terminal portion of the PABP to interact with the RdRp in yeast, the binding *in vitro* did not require the presence of poly-(A) (Fig. 2-4C). The NI 8 and NI 439 clones also weakly bound RdRp *in vitro*, but the shortest clone, NI 351, did not show detectable binding (data not shown). The difference between these results with the short clones *in vitro,* and the yeast two hybrid assay may reflect differences in sensitivity of the two methods. The shorter clones also showed reduced interactions in the yeast two-hybrid system as assayed by speed and intensity of blue color development.

## Discussion

The above-described results indicate that the ZYMV potyviral RdRp is capable of specifically interacting with host PABP in the yeast two-hybrid system and in *in vitro* binding assays. To our knowledge, PABP has not been implicated as a component of any viral replicase complex, nor identified to play a role in virus replication (Lai, 1998; Strauss and Strauss, 1999). The repeated and high frequency isolation of PABP (6 of 11 clones) indicated that the interaction is reproducible. The interaction of the RdRp with PABP is particularly intriguing since potyviruses are polyadenylated at the 3' end of the positive strand which is the site of initiation of minus strand synthesis.

Interaction was abolished by either amino or carboxy terminal deletions of the RdRp. This may reflect complex secondary or tertiary structure of the RdRp. RdRps, like other types of DNA and RNA polymerases, consist of finger-palm-thumb domains resulting in intramolecular interactions between the amino- and carboxy-terminal portions of the molecule (O'Reilly and Cao, 1998; Lesburg et al. 1999); such interactions also may be important for association with the PABP. Similar problems in assigning functions to specific RdRp domains were observed with the tobacco etch potyvirus (TEV) RdRp. Loss of interaction between the TEV RdRp and the NIa protein were observed with both amino and carboxy terminal deletions of the TEV RdRp, and nuclear localization capacity was eliminated by deletions from either terminus and by small insertions at several positions in the protein (Li and Carrington, 1993; Li et al. 1997).

In this study ZYMV RdRp did not interact with itself. Previous two-hybrid analysis with TEV and tobacco vein mottling virus (TVMV) potyviral RdRps have provided different results (Hong et al., 1995; Li et al. 1997). Self-interaction was observed for TVMV RdRp but not TEV RdRp. The reasons for these differences among the potyviral RdRps is not known, it may reflect real differences or may be an artifact due to an interfering effect of the GAL4 domains in one or both fusion proteins. We also did not observe the interaction between RdRp and CP as was reported for TVMV (Hong et al., 1995). In that study interaction was observed only when the RdRp was fused to the binding domain. Despite known interactions between HC-Pro and CP *in vivo* (e.g., for aphid transmission), and demonstrations of interaction using *in-vitro* binding assays (Blanc et al. 1997; Peng et al. 1998), we did not observe interaction between HC-Pro and CP in our yeast two-hybrid assay. The failure to bind in the yeast two-hybrid assay may

reflect burying of the amino terminus of the CP in the fusion protein construct. The CP amino terminus is normally externally located on the virion and is critical for the CP/HC-Pro interaction.

PABP belongs to a large family of RNA-binding proteins that contain highly conserved RNA recognition motifs (RRMs). Although PABPs generally exist as multigene families, complete sequence identity in overlapping regions of the interacting cucumber clones indicated that a single cucumber PABP was interacting with the ZYMV RdRp. Southern blot analysis showed only a single hybridizing band, which may reflect the high sequence variability among different members of the PABP family (Belsotosky and Meagher, 1993; Hilson et al. 1993). These results suggest specificity in the RdRp-PABP interaction, although the possibility of developmental or tissue specific expression of the different PABPs such that only a single gene family member was expressed in leaves at the time they were harvested for cDNA synthesis, cannot be eliminated; within *Arabidopsis* different members of the PABP gene family are expressed in different tissues (Belostosky and Meagher, 1993; Hilson et al. 1993). Northern analysis indicated that the *CS-PABP1* gene was expressed in all tissues tested including leaves, roots, immature and mature flowers.

PABP has been the subject of a good deal of recent research indicating that it plays a critical role in eukaryotic translation (Jacobson, 1996; Gallie, 1998; Sachs et al. 1997). PABP is an essential component of eukaryotic cells; deletion of PABP in yeast can cause lethality (Sachs et al. 1987) and reduction in PABP levels, either by cleavage or sequestration by viral proteins, can result in shut down of host translation (Chen et al. 1999; Joachims et al. 1999; Piron et al. 1998). Recent evidence has shown that PABP

also facilitates initiation and maintenance of efficient translation by promoting interactions between the 5' and 3' termini of messenger RNAs (reviews: Gallie, 1998; Sachs et al. 1997). A typical mRNA molecule has a 5' cap and 3' poly-(A) tail, the two termini function synergistically to promote translation through protein-protein interactions: at the 5' end, eIF4E, which is a subunit of eIF4F (containing subunits eIF4E, eIF4G, and eIF4A), binds to the cap structure; at the 3'end, PABP binds to the poly-(A) tail, PABP then binds to eIF4G. These interactions result in a circular mRNA molecule. By bringing the 5' end and 3' ends close to each other, translation of full-length message is promoted and re-initiation of translation is facilitated. Although not capped, the potyviral 5' NTR of TEV conferred synergistic enhancement of translation when in combination with a poly (A) tail (Gallie et al., 1995).

Viral RdRps frequently have been shown to interact with components of the host translational apparatus (Lai, 1998; Strauss and Struass, 1999). The results presented here showing interaction of PABP with the ZYMV RdRp suggests that an additional component of the host translational machinery associates with a viral replicase and raises some intriguing questions. Perhaps, similar to its role in eukaryotic translation, PABP facilitates intramolecular interactions relevant to potyviral replication. Cellular proteins binding viral RNA may serve to bring spatially separate regions, including 3' and 5' termini, of viral RNA template together to form replication complexes. The joining of plus and minus strand leader sequences is a critical step in mouse hepatitis virus RNA synthesis (Lai, 1998). Perhaps the interaction between RdRp and host PABP serves to promote interaction between RdRp and the viral poly (A) tail, either by helping to recruit RdRp to the poly (A) tail, or by facilitating removal of PABP from the poly (A) tail and

allowing access of the RdRp for initiation of replication. Deletion analyses and partial clones obtained in the yeast two-hybrid screen indicated that interaction with the RdRp occurred via the carboxy terminus and not the RNA binding motifs in the amino terminal half of the protein. Studies with *Xenopus* PABP showed that the conserved CTC domain was important for polymerization of PABP leading to enhanced PABP binding (Kuhn and Pieler, 1996). Perhaps association of the RdRp with PABP interferes with the PABP polymerization and facilitates removal from the poly (A) tail.

Recent studies have shown that viral-induced shut down of host protein synthesis, which is thought to facilitate viral infection by increasing accessibility of host factors for viral purposes, can be mediated, at least in part, by sequestration or cleavage of PABPs. PABPs were sequestered by NS1 protein during influenza A virus infection (Chen et al., 1999), were removed from interaction with eIF4F during rotavirus infection (Piron et al., 1998), and were cleaved by viral proteases during Picornavirus infection (Joachims et al. 1999). In each case there was an associated reduction in host protein synthesis that could be related to an effect on PABP. Interestingly, the interaction between influenza A NS1 and human PABPII, which takes place in the nucleus and results in hnRNAs with poly (A) tails that are too short to allow for export, occurs via the carboxy terminus of PAPBII.

Shut down of host translation is less well studied for plant viruses. In the one system that has been examined, infection by pea seed borne mosaic potyvirus (PSbMV), inhibition of host gene expression and virus-mediated mRNA degradation occurred in a reversible manner during the course of infection (Wang and Maule, 1995; Aranda et al. 1996; Aranda and Maule, 1998). There appears to be a widespread loss of many host

mRNAs, indicating degradation. If RdRp serves to remove PABP from the poly (A) tail, this could result in decreased mRNA stability. Such an observation would not be inconsistent with sequestration of PABP. During potyvirus infection, RdRp is expressed in large quantities and can accumulate in the nucleus as an inclusion body (NIb). Its function in the nucleus is unknown since the viral life cycle is completed within the cytoplasm. It is possible that the NIb/RdRp sequesters PABP and keeps it from binding to hnRNA in the nucleus, inhibiting RNA processing.

The possible involvement of the RdRp-PABP interaction in viral replication by recruitment to the poly (A) tail or participation in translational inhibition are not necessarily mutually exclusive. RdRp may interact with PABP to facilitate viral replication, while at later stages, increasing quantities of RdRp might inhibit host translation. Our future research will examine possible functions of the interaction between potyviral RdRp and cucumber PABP.

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# Chapter 3

## Identification and characterization of a wound inducible protein that interacts with the carboxy terminus of poly- (A) binding protein and inhibits translation


## Abstract

A cucumber protein, PCI6 (PABP-CT Interacting), was identified based on its ability to interact with the carboxy terminus (CT) of poly- (A) binding protein (PABP) both in yeast two-hybrid and *in vitro* binding assays. PCI6 shares a conserved amino acid domain (**S**m**L**N**p**n**A**pl**F**vp) in common with a second cucumber PABP-CT interactor (PCI243), human PABP-CT interactors, and with Arabidopsis ERD15 (Early Responsive to Dehydration). Deletion analysis and point mutations indicate that the presence of this domain is necessary for the interaction. PCI6 expression is inducible by both wounding and jasmonic acid; mRNA levels increased sharply 4-8 hours post-treatment in both wounded and non-wounded upper leaves, indicating systemic induction. Recent studies with human PABP interacting proteins have indicated a newly identified mechanism of translational regulation. PCI6, like the human PABP-CT interactors, influenced translation in mouse ascites Krebs2 and wheat germ translation systems. Wild type PCI6 inhibited translation in wheat germ, whereas the non-interacting mutant, PCI6-23A, did not. These results demonstrate an interaction between PABP and a wound responsive protein, with possible implications for translational regulation.

# Introduction

Poly- (A) binding protein (PABP), which binds to the poly- (A) tail of mRNA, is an essential component for a broad range of eukaryotic cellular functions (Sachs et al, 1987). Together with poly- (A), PABP participates in pre-mRNA processing (Amrani et al, 1997; Minvielle-Sebasia et al. 1997), regulation of mRNA stability (Caponigro and Parker, 1996; Coller et al, 1998), and initiation and stimulation of translation (Gallie, 1998; Gray et al, 2000; Jacobsen, 1996; Otero et al, 1999; Sachs et al. 1997). Deletion of the PABP coding sequence in yeast leads to a lethal phenotype (Sachs et al. 1987) and reduction of PABP levels, either by cleavage or sequestration during viral infection can inhibit host translation (Chen et al. 1999; Kerekatte et al. 1999; Joachims et al. 1999; Piron et al. 1998). PABP facilitates initiation and maintenance of efficient translation by promoting interactions between the 5' and 3' ends of mRNAs (Jacbson, 1996; Gallie, 1998; Sachs et al, 1997; Tarun and Sachs, 1996; Le et al, 1997b). A typical mRNA has a 5' cap and 3' poly- (A) tail; the two termini function synergistically to promote translation through protein-protein interactions. At the 5' end, eIF4E (eukaryotic Initiation Factor 4E), which is a subunit of eIF4F complex (containing subunits of eIF4E, eIF4A eIF4G), binds to the cap structure (reviewed in Gingras et al, 1999); at the 3' end, PABP binds to the poly- (A) and then to eIF4G. These interactions result in a circular mRNA molecule (Wells et al, 1998). By bringing the 5' and 3' ends close together, translation of full-length mRNA is promoted and re-initiation of translation is facilitated (Jacbson, 1996; Gallie, 1998; Kahavejan et al, 2002; Sachs et al, 1997).

A key distinguishing feature of PABP is the presence of four highly conserved RNA recognition motifs (RRMs) found in PABPs from all sources, including plants,

animals, and yeast (Belostotsky and Meagher, 1993; Hilson et al, 1993; Le and Gallie, 2000). The RRMs, which are located in the amino terminal two-thirds of the protein, include two conserved sequences that come in direct contact with RNA (Burd and Dreyfuss, 1994). The second RRM is responsible for interaction with eIF4G (Kessler and Sachs, 1998). The carboxy terminal (CT) third of the PABP protein is not as highly conserved as the amino terminus, and functions of the CT have been less well defined. Coller et al. (1998) observed that deletion of the C-terminal 50 amino acids of yeast Pab1p (yeast PABP) abolished the ability to rescue yeast cells lacking a functional *PAB1* gene. Cleavage of PABP between the RRMs and CT by coxsackievirus or poliovirus 2A proteinases diminished host translation, and the cleaved fragments were unable to stimulate translation of capped, polyadenylated mRNAs *in vitro* (Kerekatte et al. 1999). These experiments indicate the importance of PABP-CT in cellular functions. PABP-CT also has been implicated in homo-dimerization and proper nuclear shuttling (Kühn and Pieler, 1996; Afonia et al. 1998).

PABP-CT has drawn increasing attention recently and roles played by PABP-CT are emerging due to the identification of its interactors. Several proteins recently have been identified to interact with PABP-CT; three human proteins, eukaryotic polypeptide chain releasing factor 3 (eRF3, Hoshino et al, 1999), Paip 1 (PABP-interacting protein 1, Craig et al, 1998), and Paip 2 (Khaleghpour et al, 2001a, 2001b); a yeast protein Pbp1p (PABP binding protein, Mangus et al, 1998); and a viral protein, zucchini yellow mosaic potyvirus RNA-dependent RNA polymerase (RdRp) (Wang et al, 2000). A conserved 12-amino-acid motif was found among Paip1, Paip2, and eRF3 and the 15 amino-acid region containing this 12 amino acids was termed PAM2 in Paip1 and 2 (PABP interacting

Motif) (Roy et al, 2002). Deletion analyses and binding studies with a short peptide containing the PAM2 domain showed that this region is sufficient for the binding to PABP-CT (Khaleghpour et al, 2001b; Kozlov et al, 2001; Roy et al, 2002). Searching the NCBI (National Center of Biotechnology Information) gene bank with the motif showed several *Arabidopsis* proteins with this motif, however, functions of these proteins are not known and the interaction with PABP-CT was not verified (Khaleghpour et al, 2001; Kozlov et al, 2001).

Gene expression can be regulated not only transcriptionally, but also post-transcriptionally, for example, at translation steps (Mathews et al, 2000). Translational regulation is rapid and direct and plays important roles in the control of cell growth, development, and stress responses (Reviewed in Conlon and Raff, 1999; Gingrus et al, 1999; Schneider, 2000). Modulation of translation is usually exerted at the initiation step (Mathews et al, 2000); a body of evidence indicated that ribosome recruitment step is the major target (Reviewed in Gingras et al, 1999). Recent studies with human PABP1 interactors Paip1 and Paip2 provide the first demonstration of a proposed mechanism of translational regulation involving the 3' end of mRNA, in which a co-activator (Paip1) and a repressor (Paip2) modulate translation (Kahvejian and Sonenberg, 2002). The two molecules compete with each other for binding to PABP, and either promote or inhibit translation (Craig et al, 1998; Khaleghpour, 2001a). It is not known if this mechanism occurs more broadly.

We previously identified an interaction between a potyviral RNA-dependent RNA polymerase (RdRp, viral replicase) and cucumber PABP-CT (Wang et al, 2000) and proposed that the interaction could be involved in potyviral replication. To address the

possible roles of PABP-CT in host functions, we sought to identify and examine host proteins interacting with PABP-CT. Plant proteins interacting with PABP-CT had not yet been identified. I report here the identification of two novel proteins, PCI6 (PABP-CT Interacting) and PCI243, that interact with PABP-CT and full-length PABP. Both proteins share the 12-amino-acid motif common to the mammalian interactors Paip1, Paip2 and eRF3 (Khaleghpour et al, 2001b; Roy et al, 2002). I further demonstrated that two amino acids, leucine at 16th and phenylalanine at 23rd positions, are critical for interaction with PABP-CT. Expression of PCI6 at the transcriptional level is up-regulated by wounding and jasmonic acid (JA). Addition of PCI6, but not a non-PABP interacting PCI6 mutant, inhibited translation in the *in vitro* wheat germ translation system.

# Materials and Methods

## Screening the cucumber leaf cDNA library using the C-terminus of PABP (PABP-C254)

The cucumber leaf two-hybrid cDNA library, yeast transformation, growth media, and X-gal (5-bromo-4-chloro-3-indolyl B-D-galactopyranoside) filter assays have been described previously (Wang et al, 2000). Yeast (Saccharomyces cerevisiae) strain YRG2 cells were first transformed with pBDPABP-C254 (referred to as NI439 in Wang et al, 2000), which produces a fusion protein with the GAL4 binding domain and the C-terminal 254 amino acids of cucumber (*Cucumis sativus L.*) PABP1, and then transformed with CsCl-purified plasmid DNA from the cucumber leaf cDNA library. The transformants were plated onto SD (synthetic dropout) medium without leucine,

tryptophan, and histidine (SD-L-T-H). Colonies that grew on selection medium were restreaked onto SD-L-T-H, transferred to nitrocellulose membrane (Schleicher & Schuell), and assayed for expression of β-galactosidase activity (Lac Z) by X-Gal filter assay. Colonies that survived medium without histidine and turned blue in the X-Gal assay were considered as putative positives. Plasmids were recovered from putatively positive yeast colonies using the Zymoprep yeast plasmid miniprep protocol (Zymo Research, CA), transformed to *E. coli*, and amplified. The recovered plasmids were then transformed back to YRG2 yeast either alone, or in combination with pBDPABP-C254, vector pBDGAL4, or two negative controls: p53 and pLamin C. The transformants were plated onto SD-H or SD-L-T-H, and further assayed for Lac Z activity. Those that expressed both reporter genes only in combination with pBDPABP-C254, but did not turn on reporter genes, either alone, or in combination with pBDGAL4, p53 or pLamin C were considered postitives.

## Deletions and mutants of PCIs, ERD15 and CS-PABP1:

The coding sequences and 3'NTRs of PCI6 and PCI243 were amplified by PCR with Vent DNA polymerase (New England Biolabs) using the original clones isolated from the yeast two-hybrid cDNA library as template and inserted as *BamHI –XhoI* fragments into pAD-GAL4 vectors in frame to form pADPCI6, and pADPCI243. The primers RG168 (ATGGATCCATGGATGTTGTTACTCAAA, PCI6 5' primer) and RG93 (CAGTATCTACGATTCATA, complementary to the sequence 935-952 on pAD vector), RG188 (AAGGATCCATGGCTCTAGCATCTGTT, PCI243 5' primer) and RG93 were used to amplify PCI6 and PCI243, respectively. The PCR fragments were digested with *BamHI* and *XhoI* and inserted into the pAD vector. To make pBDPCI6, the plasmid DNA

recovered from yeast was digested with *XhoI* thoroughly and *EcoRI* partially and ligated to the pBDGAL4 vector. This full-length cDNA insert contains 5' and 3' NTRs and coding sequence.

Primers RG189 (AAGGATCCCCCATGGCCTATAGAACG, which has the PCI6 nucleic acid sequence from 76 to 94 as 5' primer) and RG93 were used to amplify PCI6d1. The *BamHI-XhoI* fragment was inserted into the pAD vector to form pADPCI6d1. Plasmids pADPCI6d2 and pADPCI6d3 (encoding first 83 and 101 amino acids, respectively) were constructed as follows: pADPCI6 was digested thoroughly with *BamHI* and partially with *EcoRI*, two *BamHI-EcoRI* PCI6 fragments were recovered and subcloned to the pADGAL4 vector. Fragments PCI6d4 and PCI6d5, which encoded the first 56 and 45 amino acids, respectively, were amplified using RG168 and RG190 (AACTCGAGGCGTTCTTGAAGCCAT), or RG168 and RG191 (AACTCGAGG GAGGACTGGATGAGC).

Point mutations were introduced into PCI6 with PCR based site-directed mutagenesis (Ho et al, 1989). A typical process is as follows: RG168 and an antisense primer with the intended mutation (RG237, RG244, RG246, or RG248, Table 3-1) were used to amplify the 5' end of PCI6 with the introduced mutation; RG93 and a sense primer (RG236, RG 243, RG245, or RG247, Table 3-1) were employed to amplify the 3' end of PCI6 with the same mutation. The PCR products were then gel-purified and 100 ng of each of two ends were used as templates to amplify full-length PCI6 with the specific mutation. RG236 (CGCTCCTTTG*GC*CGTTCCCAT) and RG237 (CATGG

GAACG***GC***CAAAGGAGCG), RG243 (GTTTCCATGT***C***GAATCCCAAC) and RG244 (GTTGGGATTC***G***ACATGGAAAC), RG245 (GTTGAATCCC***GC***CGCTCCTTTG) and RG246 (CAAAGGAGCG***GC***GGGATTCAAC), RG247 (GAATCCCAAC***T***CTCCTTTG TT) and RG248 (AACAAAGGAG***A***GTTGGGATTC) were used to mutate Phe-23 to ala, Leu-16 to Ser, Ala-19 to Ser, and Glu-20 to Ala, respectively.

The coding sequence of Arabidopsis ERD15 was amplified with Vent using plasmid 157B7T7 from the Ohio Stock Center as template and inserted as an *EcoRI-SalI* fragment into pBD-GAL4 and pAD-GAL4. The primers RG 205 (AAGAATTCATGGC GATGGTATCAGG) and RG 206 (AAGTCGACTCAGCGAGGCTGGTGG) were used.

Plasmids pBDPABP, pBDPABP-C406 (encoding the C-terminal 406 amino acids of CS-PABP1, termed NI8 previously), pBDPABP-C254, pBDPABP-C131 (encoding the C-terminal 131 amino acids of CS-PABP1, termed NI351 previously) and pBDPABPΔC50 (deletion of 50 amino acids from the CT, termed PABPΔ320 previously) were constructed by digesting the corresponding activation vector pADGAL4 plasmids and inserting the cDNA fragments into the pBDGAL4 vector as *EcoRI-XhoI* fragment (Wang et al, 2000).

## *In vitro* analysis of protein-protein interaction:

Full-length PCI6, PCI-23A mutant, and PCI243 were cloned to pET28a-1 (Novagen) to produce histidine-tagged PCI6, PCI6-23A, or PCI243. All genes were expressed using the Promega TNT$^{-}$ quick coupled transcription/translation system with 2 ug DNA, 0.4 mM magnesium acetate and 30 mM KCl in the presence of $^{35}$S-methionine. His-PABP was over-expressed and purified from *E.coli* strain BL21 (DE3) as described before (Wang et al, 2000). Five μg of His-PABP was immobilized on 30 ul of poly-(A)-

sepharose (Sigma), 5 μL of in vitro $^{35}S$ labeled PCI6, PCI6-23A, PCI243, ZYMV RdRp or luciferase was added and incubated in 90 μl binding buffer (100 mM NaAc, 5 mM $MgCl_2$, 5 mM KCl, 40 mM HEPES, pH 7.6, 10% glycerol, protease inhibitor cocktail (Sigma), 0.2% Triton X-100, 5 mM β-mercaptoethanol) plus 10 μl bovine serum at room temperature for 1 hour. The beads were then washed six times with 500 μl binding buffer plus 200 mM NaCl, boiled in 24 μl loading buffer, and analyzed by SDS-PAGE followed by auto-radiography overnight.

## β-Galactosidase activity assay:

β-Galactosidase activity of yeast cells was measured following the protocol from Clontech 'Yeast Protocols Handbook'. The yeast colonies were picked and cultured overnight in 2.5 ml SD-L-T. Either 0.2 ml or 1.0 ml yeast cells (depending on the affinity of two proteins according to the X-gal filter lifting assay) were harvested at OD600 of 0.6-1.0. Cell pellets were then resuspended in 100 μl Z-buffer (60mM $Na_2HPO_4$, 40mM $NaH_2PO_4$, 10mM KCl, 1mM $MgSO_4$) without β-mercaptol ethanol (β-ME), frozen in liquid nitrogen and thawed in a 30°C water bath three times. Z-buffer (0.7 ml) with β-ME, 0.16 ml 4 mg/ml o-nitrophenol-β-galactopyranoside (ONPG) was added and incubated in a 30°C water bath for 15 minutes or 45 minutes. The reaction was then stopped by adding 0.4 ml 1M $Na_2CO_3$ and absorbance at 420 was measured. The β-galactosidase activity was calculated as described by Miller (1972).

## Plant materials and treatment:

Cucumber (cv. Straight 8) seeds were germinated and grown in containers (10×10×7.5cm) containing commercial soil mix (sphagnum peat 70-80%, pH5.5-6.5)

(Baccto, Michigan Peat Company, Houston, TX) in a growth chamber with 14-h (24°C)/10-h (20°C) light/dark cycles. For wounding treatment, plants were grown to the 4-leaf stage and the tips of the second leaves were wounded with forceps. Tissues were harvested from the wounded second leaves and non-wounded third leaves at 0, 1, 2, 4, 8, and 24 hours after wounding. For drought treatment, two types of treatments were used. For the half-leaf drought treatment, the second leaves were detached from the plant and cut into two pieces along the main vein. One piece was put in between wet Whatman filter papers and sealed in petri dish as control. The other half was put between two dry Whatman filter papers. Control and treated half-leaves were frozen in liquid nitrogen at 0, 1, 2, 4, 24 hours after treatment. For whole-leaf drought treatment, whole leaves were detached and put either in wet or dry filter papers. For methal jasmonate (MeJA) treatment, plants were put in a lucite box (31 cm × 27 cm × 14 cm). One μl of MeJA was diluted into 60 μl ethanol and 10 μl solution was dropped on each of 5 cotton swabs that were distributed evenly in the box. The box was then sealed, one pair of plants was removed from the box at the time defined and the third and fourth leaves were harvested. Control plants were placed in a lucite box and either left untreated or treated with treated with 50 μl of ethanol.

## Northern Analysis:

Total RNA was isolated using 'Concert Plant RNA reagent' from Invitrogen. Five μg or 10 μg of RNA were used for northern blots with PCI6 as probe and 15 μg was used with PCI243 as probe. Both PCI6 and PCI243 RNA probes were made using the 'MAXIscript' kit from Ambion and labeled with Digoxigenin-11-UTP (Dig, Roche). Pre-hybridization and hybridization were conducted at 50 °C in hybridization buffer

containing 50% formamid (Roche protocol). The membrane was washed twice (15 minutes each) at room temperature using 2×SSC/0.2% SDS, and 4 times at 68 °C (15 minutes each) using 0.1×SSC/0.2% SDS. The hybridization signal was detected using Dig antibody conjugated with AP (alkaline phosphatase) and substrate CDP-Star (Roche). The membrane was then exposed to X-ray film for 1 to 15 minutes.

## *In vitro* translation:

Capped Luciferase (Luc) transcripts with (capLUCpA) or without (capLUC) poly- (A) and mouse ascites Krebs2 cell-free translation system were generously provided by the lab of Dr. Sonenberg (McGill University). His-PCI6 and His-PCI6-23A were over-expressed in *E. coli* and purified following the protocol of Novagene. The purified proteins were dialyzed against dialysis buffer (20mM Tris, pH 7.5, 100mM KCl, 0.1mM EDTA, pH 8.0, 1mM DTT, and 10% glycerol) overnight. The quality of the proteins was checked with SDS-PAGE. Nuclease-treated wheat germ extract (Promega) and mouse ascites Krebs2 cell-free translation system was used for *in vitro* translation assay. In each reaction, 25 ng of capLuc or capLUCpA was used, along with 0, 50, 100, or 200 ng (0.25, 0.5, 1.0μM respectively) of PCI6 or PCI6-23A. The dialysis buffer was added to bring the final volume to 12.5 μl. Translation was programmed at 25 °C for wheat germ, and 32°C for Krebs2 extract for 1 hour. Following the reactions, 3 μl of Krebs2 extract was assayed for luciferase activity following the producer's (Promega) protocol. For the wheat germ extract, samples were diluted 30-fold and 3 μl of the diluted samples were used to measure luciferase activity.

# Results

## Identification of cellular proteins interacting with the C-terminus of poly- (A) binding protein 1 (CS-PABP1-CT)

To identify cellular proteins interacting with the C-terminus of PABP (PABP-CT), a cucumber leaf two-hybrid cDNA library (Wang et al, 2000) was screened with the CS-PABP1 clone PABP-C254 that encodes the C-terminal 254 amino acids. Approximately $2\times10^6$ yeast colonies were screened, 133 survived the medium without histine and turned X-Gal to blue. Of these, 44 were randomly selected for further characterization. In each case, plasmids encoding putative interactors were isolated and retransformed back to yeast either alone, or in combination with PABP-C254, human p53, Lamin C, or binding domain vector without insert. Each interacting clone listed in Table 3-1 failed to induce the reporter genes alone, or in any combination except with PABP-C254, indicating specific interaction with PABP-C254.

Sequence analysis indicated that among the 28 clones verified as positive interactors, 24 cDNAs, which were named PCI6 (PABP-CT Interacting), encoded the same protein. Although PCI6 clones did not show homology to known genes based on DNA sequence, they have high amino acid similarity to an Arabidopsis protein ERD15 (Early Responsive to Dehydration 15, Kiyosue et al, 1994); 38 % identity and 50% similarity occur over a 108 amino acid region at the N-terminus (Table 3-1 and Fig.3-1A). PCI6 encodes a 141-amino-acid protein with a predicted molecular weight of 16.6 kD. It is an acidic protein (PI of 4.28) containing 9 aspartic acids, 17 glutamic acids and 16 serines. As has been observed for other acidic proteins (Khaleghpour, 2001a), PCI6 ran at a higher position than predicted in SDS-PAGE. when expressed either *in vitro*,

**Table 3-1. The cDNAs identified to interact with PABP-CT.**

| Clone | Length | Number of clones | Proteins similar (at the amino acid level) | Length of match / Percent identity |
|---|---|---|---|---|
| PCI6-1 | 0.8 kb | 20 | EDR15 Arabidopsis | 108 a.a. / 38% |
| PCI6-2 | 0.8 kb | 4 | EDR15 Arabidopsis | 108 a.a. / 38% |
| PCI243 | 0.8 kb | 1 | EDR15 Arabidopsis | 87 a.a. / 41% |
| PCI82 | 0.7 kb | 1 | Unknown | |
| PCI142 | 2.0 kb | 1 | Unknown | |
| PCI296 | 1.9 kb | 1 | Unknown | |

**A.**

```
1   M A M V S G R - R S T - - - - L N P D A P L F I P A A V R Q V E D F S P E W W Q  AtERD15.pro
1   M A L A S V E G S S K - - - - L N P N A P L F I P A A Y - Q V E D F S P Q W W Q  CS-PCI243.pr
1   M D V V T Q R N S S S S V S M L N P N A P L F V P M A Y R T V E D F S D Q W W E  CS-PCI6.pro

36  L V T T S T W Y P D Y W I S Q Q Q Q G A D G F Y D N G E N E N G G G H I D V A D  AtERD15.pro
36  L V T T S T W Y R D Y W L S Q H Q E E S D - F Y I D G E G E D D F S N - D I A E  CS-PCI243.pr
41  L I Q S S P W F R E Y W L Q E R - - - - - - - F Q D P Q N E L S F G E - N E E F  CS-PCI6.pro

76  L L P E S F D F - - - - - - - D D M E D F F D - - - - - - - - - - - - - - - - T  AtERD15.pro
74  F L P E A F D L - - - - - - - D A N E E - L R - - - - - - - - - - - - - - - - T  CS-PCI243.pr
73  I L P D L E S F F D D F T R Q Q E E E E - L E F S K D L V P M G A F K W Q K A R  CS-PCI6.pro

93  D A A E F D Q G F D G R M Y Y Q A P S E F G F - - - - - - - - - G K N G E M V K  AtERD15.pro
90  M E A E F E E - - - - - - F I Q A S L N E G Y - - - - - - - - - - - - - - - -  CS-PCI243.pr
112 S G A E V P K - - - - - - Y A Q K A P K I V N V K V S E S E N D - - - - - - - -  CS-PCI6.pro

124 K S S G N R S P R S I V E P A K Y A E K P A K W G N Q R V A A A P R N I H Q P R  AtERD15.pro
107 — - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - H P E K  CS-PCI243.pr
138 - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - S P P R  CS-PCI6.pro

Decoration 'Decoration #1': Shade (with black at 10% fill) residues that match the Consensus
exactly.
```

**B.**

| | | |
|---|---|---|
| CS-PCI6* | 14 | **S**M**LN**PN**A**PL**F**VP |
| CS-PCI243* | 10 | **S**K**LN**PN**A**PL**F**IP |
| At-ERD15* | 9 | **STLN**PD**A**PL**F**IP |
| Le-ERD15 | | **STLN**PN**A**PL**F**VP |
| Human Paip1* | | **S**K**L**SVN**A**PE**F**YP |
| Human Paip2* | | **S**N**LN**PN**A**KE**F**VP |

**Fig. 3-1 Amino acid sequence alignment among Arabidopsis ERD15, cucumber PCI6 and PCI243.** A. Sequence alignment among AtERD15, CS-PCI6, and CS-PCI243. The shaded amino acids are common to at least two sequences. B. The 12-amino-acid motif conserved among PABP-CT interacting proteins from cucumber, Arabidopsis, and human. Amino acids marked in bold are the most conserved.

* The interaction with PABP-CT has been verified.

or purified from *E.coli* as a His-tagged or GST-tagged protein (data not shown), PCI6 ran at approximately 28 kD position in SDS-PAGE (Fig.3-2, His-PCI6 ran at 31 Kd position). The acidic nature could also explain why PCI6 also activated reporter genes when expressed from the binding domain vector rather than the activation vector (data not shown).

We have identified two types of PCI6 cDNAs, PCI6-1 and PCI6-2. PCI6-1 was inserted into the vector as an *EcoRI-XhoI* fragment, as is typical for most cDNAs synthesized using the Stratagen cDNA synthesis kit. However, the PCI6-2 insert did not have an *EcoRI* site at the 5' end of cDNA, but was still in frame with AD domain. The other difference was that PCI6-2 is 4-bp longer than that of PCI6-1.

In contrast to 24 clones of PCI6, one cDNA, which was named PCI243, also encoded a protein having high amino acid similarity to *Arabidopsis* ERD15 (Fig.3-1 and Table 3-1). Like PCI6, PCI243 did not show DNA homology to any known genes including ERD15 and PCI6, and PCI6 and PCI243 did not cross-hybridize in Southern analysis to each other (data not shown). The overall identity to ERD15 in the N-terminal 87 amino acid region is about 57% and similarity is approximately 73%. PCI243 encodes a polypeptide with 99 amino acids and a predicted molecular weight of 12.8 kD. Similar to PCI6, PCI243 is an acidic protein with PI of 3.82. It consists of 9 aspartic acids and 16 glutamic acids, which account for 22.7 % of the total amino acids. PCI243 ran as a 20 kD protein in SDS-PAGE (Fig. 3-2, His-PCI243 ran as a 23 Kd protein).

We also identified another three distinct PABP-CT interactors, which encoded unknown proteins and were not further pursued.

## *In vitro* verification of the interaction between CS-PABP1 and PCIs

To verify the interactions between PABP and PCIs *in vitro*, full-length PABP was expressed and purified from *E.coli* as a His-PABP fusion protein and immobilized on poly- (A)-sepharose. *In vitro* labeled luciferase, ZYMV RdRp, PCI6, or PCI243 was separately incubated with poly- (A)-sepharose or PABP-poly-(A)-sepharose and then extensively washed (Fig. 3-2). The negative control, luciferase, did not bind to poly-A-sepharose in the presence of PABP (Lane 6). Consistent with previous observations (Wang et al, 2000), approximately 10% of the ZYMV RdRp (Lane 7) bound to PABP-sepharose. Neither PCI6 nor PCI243 proteins bound to poly- (A)-sepharose (Lane 8 and 12). Approximately 20% to 30% of input PCI6 and PCI243 were maintained on the poly-A-sepharose in the presence of PABP (Lane 9 and 13). These results indicated that PCI6 and PCI243 interacted with PABP1 *in vitro*, and PCI6 and PCI243 do not have poly-A binding activity. In this *in vitro* assay, the affinity between the PCIs and PABP is higher than that between RdRp and PABP. This agreed well with the yeast two-hybrid results where yeast strain harboring both PABP and PCI6 or PCI243 grew faster on the medium without histidine and gave much higher β-galactosidase activity (about 108 and 182 Miller Units respectively, Table 3-2 and Fig. 3-3) than those of the strain with PABP and ZYMV RdRp (about 2 Miller Units, Wang and Grumet, unpublished data).

## Identification of the regions responsible for the interaction in PCI6 and PABP

To identify the region of PABP that is responsible for the interaction, different fragments of PABP cDNAs, including full-length PABP, PABP-C406 (C-terminal 406 amino acids), PABP-C254 (C-terminal 254 amino acids), PABP-C131 (C-terminal

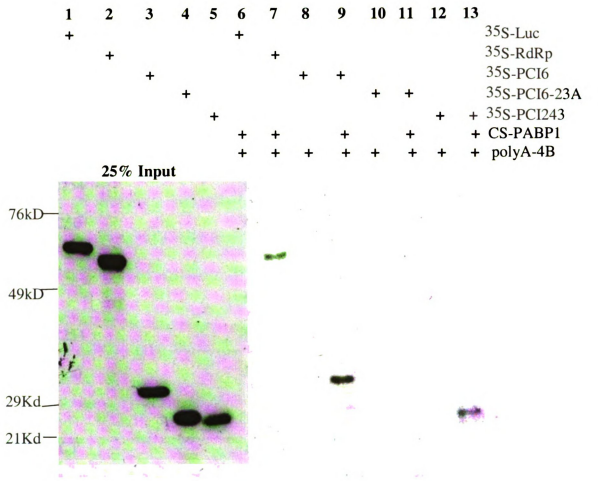



**Fig. 3-2. Cucumber PCI6 and PCI243 interacted with CS-PABP1 *in vitro*.** His-PABP1 was immobilized on poly-(A)-sepharose (lane 6, 7, 9, 11, and 13). $^{35}S$ labeled Luciferase (Luc, Lane 6), RdRp (Lane 7), PCI6 (Lane 9), PCI6-23A (Lane 11), or PCI243 (Lane 13) was then incubated with the PABP-sepharose and washed six times. Lane 1, 2, 3, 4, and 5: 25% input of Luc, RdRp, PCI6, PCI6-23A, and PCI243 respectively. Lane 8, 10, 12: PCI6, PCI6-23A, or PCI243 was incubated with poly-(A) sepharose alone.

**Table 3-2. Quatitative β-galactosidase assay for protein-protein interactions in the yeast two-hybrid system.**

| Protein fused to BD | Protein fused to AD | β-galactosidase activity |
|---|---|---|
| PABP | PCI243 | 145±18 |
| PABP-C254 | PCI243 | 143±17 |
| PABPΔC50 | PCI243 | 1.0±0.3 [a] |
| PABP | ERD15 | 135±16 |
| PABP-C254 | ERD15 | 187±19 |
| PABPΔC50 | ERD15 | 1.3±0.2 [a] |
| hPABP-CT | PCI6 | 21±3 |
| hPABP-CT | PCI6-23A | 0.4±0.1 [a] |

Data presented for PCI243 and ERD15 are the mean of four (PCI243) and three (ERD15) experiments with three replications per experiment. The data for hPABP1-CT are the mean of three replications.

[a] The yeast cells did not grow on the medium without histidine.

131 amino acids), and PABPΔC50 (lacking C-terminal 50 amino acids) were tested by yeast two-hybrid assay (Fig.3-3). Both PCI6 and PCI243 interacted with all fragments with intact C-termini (full-length PABP, PABP-C406, PABP-C254, and PABP-C131), indicating that the C-terminal 131 amino acids was sufficient for the interaction (Fig.3-3 and Table 3-2). However, deletion of only 50 amino acids from the CT of PABP abolished the interaction with both PCI6 and PCI243 (Fig.3-3 and Table 3-2), demonstrating that the C-terminus of PABP1 is necessary for the interaction, at least in yeast (Table 3-2). This interaction pattern is similar to that of ZYMV RdRp in which RdRp interacted with full-length, PABP-C406, PABP-C254 and PABP-C131, but not the PABPΔC50 (Wang et al, 2000).

Deletion of the N-terminal 25 amino acids of PCI6 eliminated the interaction with PABP, indicating the necessity of the N-terminus of PCI6 for the interaction (Fig.3-4, PCI6d1). However, C-terminal deletion of PCI6, as long as 85 amino acids, had the ability to interact with PABP (Fig.3-3, PCI6d4), suggesting that the C-terminus of PCI6 is not required for the interaction. The N-terminal 56 amino acids of PCI6 (Fig.3-3, PCI6d4) still interacted with PABP1, indicating this region is sufficient for the interaction. However, as another nine amino acids were deleted, the interaction was almost abolished. This could indicate that either the deletion of the nine amino acids could destabilize the protein, or that critical amino acids for the interaction were located in that region. Another deletion mutant, PCI6d3, only had 33% of the wild type activity. This result was rather surprising since PCI6d3 was 22 amino acids longer than PCI6d2, which had 99% of wild type activity.

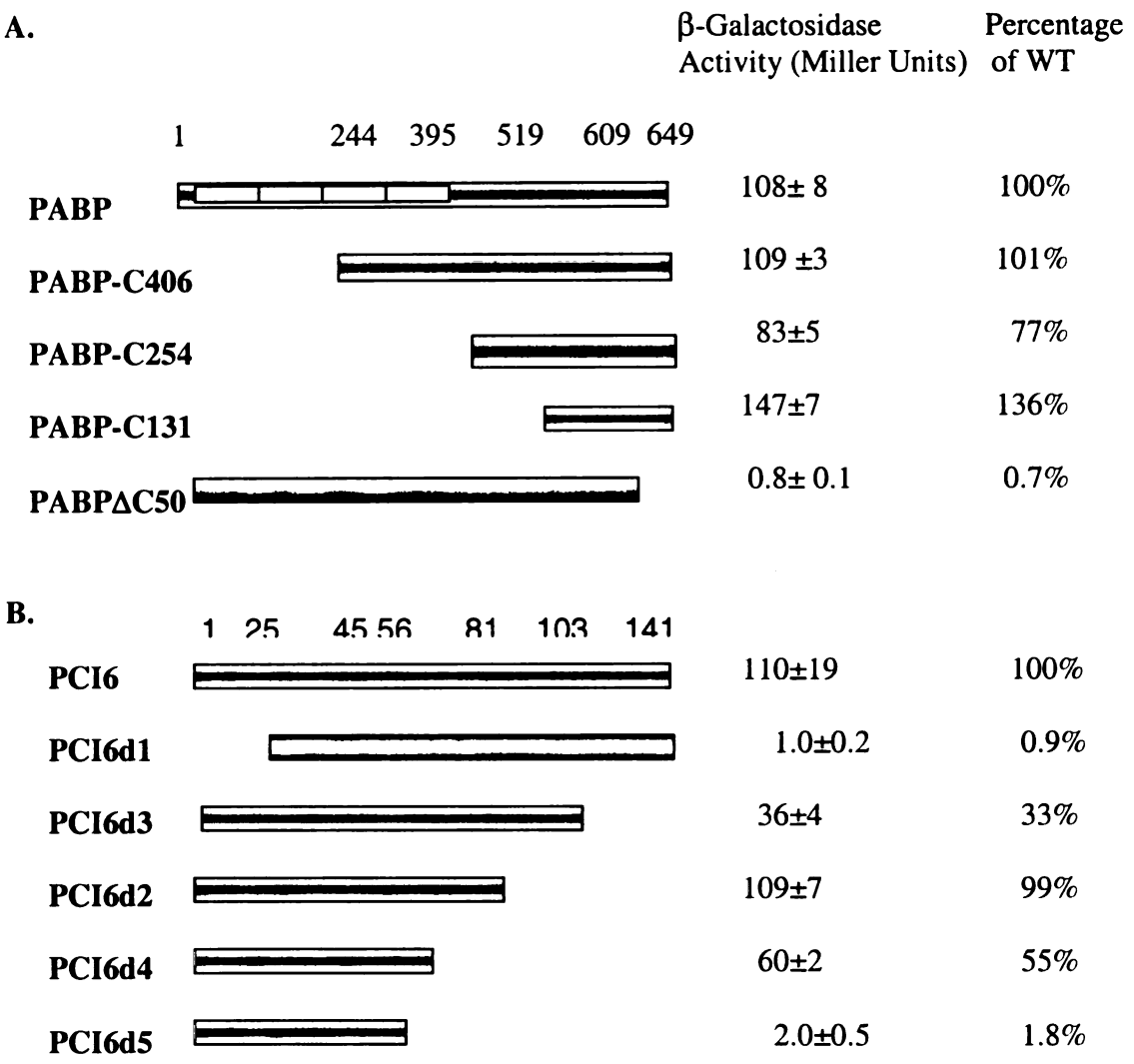



**Fig. 3-3. Deletion analysis to determine regions in PABP (A) and PCI6 (B) responsible for the binding.** Different sizes of PABP and PCI6 were generated by PCR or digestions. All fragments were tested against full-length PCI6 or PABP1, respectively, in the yeast two-hybrid system. The strength of interactions was determined by the β-galactosidase activity. Numbering corresponds to the full-length cDNA sequence. A. PABP deletions. All PABP fragments were in pBDGAL4 vector. The 4 white boxes represent PABP RRMs. Data are the mean of two experiments with three replication per experiment. The PABP and PABP-C131 with PCI6 combinations were repeated a third time with similar results. B. PCI6 deletions. All PCI6 fragments were in pADGAL4 vector. Data are the mean of three experiments with three replications per experiment.

Comparison of the PCI6, PCI243 and ERD15 protein sequences shows that the region of high similarity is located at the N terminus (Fig.3-1A). This region contains a 12-amino-acid motif that is also present in three human PABP-CT interactors: eRF3, Paip1, and Paip2 (Kozlov et al, 2001, Deo et al, 2001. Fig.3-1B) and was termed PAM2 (PABP interacting Motif 2) in Paip1 and Paip2 (Roy et al, 2002). Since ERD15 has this motif, we reasoned that ERD15 might have the ability to interact with PABP-CT. ERD15 coding sequence was then amplified and inserted into binding domain and activation domain vectors respectively. As expected, since ERD15 is an acidic protein (PI is 4.36), it turned on two reporter genes when inserted into the binding domain vector. When inserted into the activation domain vector, ERD15 specifically interacted with PABP, but not unrelated proteins such as p53, Lamin C, and ZYMV RdRp (data not shown). ERD15 interacted with PABP as well as, and in a similar pattern, to those of PCI6 and PCI243 with PABP (Table 3-2).

In the 12-amino-acid-motif, 4 of the amino acids are identical among all the identified PABP-CT interactors, including Leu-16, Gln-19, Ala-20, and Phe-23 (Fig.3-1). To identify the amino acids critical for the binding to PABP-CT, we mutated Leu-16 to Ser (PCI6-16S), Gln-19 to Ala (PCI6-19A), Ala-20 to Ser (PCI6-20S), and Phe-23 to Ala (PCI6-23A). Yeast cells with PABP and PCI6-23A did not survive the medium without histidine, and while cells with PABP and PCI6-16S grew very slowly on the medium lacking histidine (Table 3-3). When the β-galactosidase activity was measured, yeast strains either with PCI6-16S and PABP, or with PCI-23A and PABP gave only 6% and 1.2% of that with wild type PCI6 and PABP, respectively. In agreement with the result in yeast, PCI6-23A did not interact with PABP in the *in vitro*

**Table 3-3. Quatitative β-galactosidase assay for protein-protein interactions in the yeast two-hybrid system.**

| Protein fused to BD | Protein fused to AD | Growth on SD-L-T-H | X-gal filter lift assay | β-galactosidase activity |
|---|---|---|---|---|
| PABP | PCI6 | +++++ | +++++ | 112±14 |
| PABP | PCI6-16S | ++ | ++ | 7±2 |
| PABP | PCI6-19A | +++++ | +++++ | 78±8 |
| PABP | PCI6-20S | +++++ | +++++ | 41±4 |
| PABP | PCI6-23A | _ | _ | 1.3±0.3[a] |

All yeast cells were grown in SD-L-T until a cell concentration of OD600=0.6-1.0 was reached. β-galactosidase activity data presented are the mean of two experiments with three replications per experiment.

[a] The yeast cells did not grow on the medium without histidine.

binding assay (Fig.3-2, Lane 11).

## PCI6 is a wounding and JA responsive protein

Northern analysis showed that PCI6 was expressed in flowers and leaves, less in roots and stems (Fig. 3-4A). PCI243 was expressed mainly in flowers and stems, and barely expressed in leaves and roots (Fig. 3-4A). These expression patterns agreed with our leaf library screening results that 24 clones of PCI6 and only one clone of PCI243 was isolated.

Since PCI6 and PCI243 have high similarity with ERD15 of *Arabidopsis*, we tested whether PCI6 and PCI243 are drought-responsive proteins. When the leaf was cut in half with one portion kept moisted and the other portion allowed to dehydrate, the mRNA levels of PCI6 and PCI243 increased after 1 hour treatment for both the control and dyhydration treated samples (data not shown). If the experiment was performed with whole leaf samples, mRNA levels did not change. This suggested that both PCI6 and PCI243 were regulated by wounding treatment, and that cutting of leaves led to accumulation of the transcripts.

To test this possibility, cucumber plants were grown to the 4-leaf stage and the tips of leaf 2 were wounded with forceps. The wounded second leaves and non-wounded third leaves were harvested separately at 0, 1, 2, 4, 8 and 24 hours and tested for expression of PCI6. Northern blot analysis showed that the PCI6 transcripts in the wounded leaves increased at 2 hours after wounding, peaked at 4-8 hours and returned to basal level by 24 hours treatment (Fig.3-4). An analogous pattern was also observed with the upper, non-wounded leaves; PCI6 transcripts peaked at 4 and 8 hours and went down after 24 hours post wounding treatment. These northern results suggest that PCI6 is up-

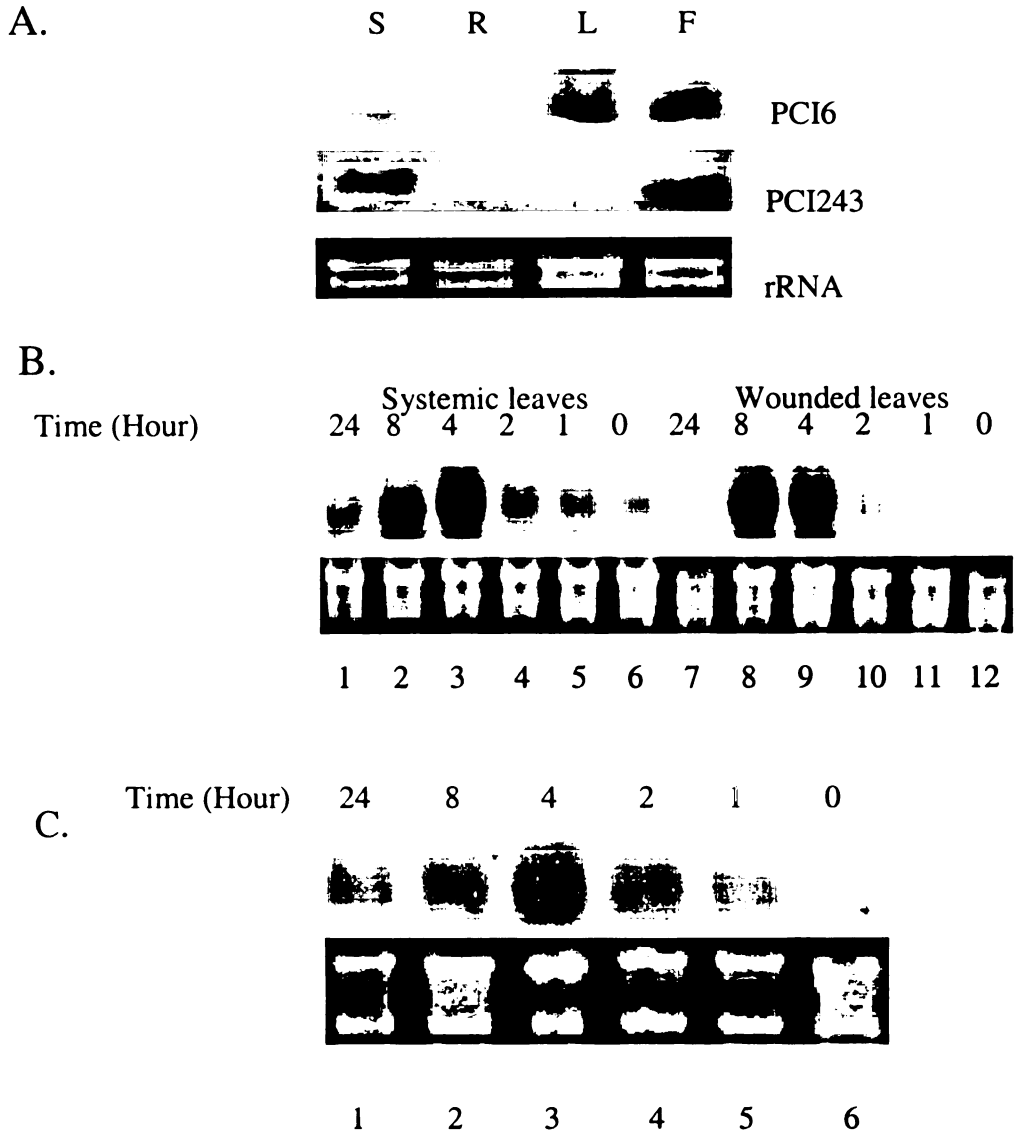



**Fig. 3-4. PCI6 expression is up regulated by wounding and MeJA.** A. Expression patterns of PCI6 and PCI243 in flowers, leaves, roots, and stems. B. Cucumber plants at 4-leaf stage were wounded with forceps at the tips of second leaves. Wounded second leaves (Lane 7-12) and non-wounded third leaves (Lane 1-6) were harvested at defined times after wounding treatment. About 10 μg total RNA isolated from treated leaves was used for northern analysis. C. Cucumber 4-leaf stage plants were exposed to MeJA in a sealed lucite box for 0, 1, 2, 4, 8, or 24 hours. Total RNA was isolated from 3rd and 4th leaves and 5 μg was used for Northern blot analysis. Top panels shows hybridization with Dig-labeled PCI6 probe, bottom panels show ethidium bromide stained RNA samples. The wounding and JA experiments were repeated one time with similar results.

regulated by wounding, and the expression is systemically controlled.

Genes responsive to wounding can be induced through JA-dependent or JA-independent pathways (Walling 2000). To determine if PCI6 expression is responsive to JA, cucumber plants were treated with methyl-JA (MeJA) in a sealed box and leaf tissues were harvested at different time points. Northern analysis showed that PCI6 transcripts increased after 1 hour exposure to Me-JA, and peaked from 2 hours to 8 hours after MeJA treatment. This suggested that PCI6 responded to wounding through a JA-mediated pathway.

## PCI6 inhibits translation *in vitro*

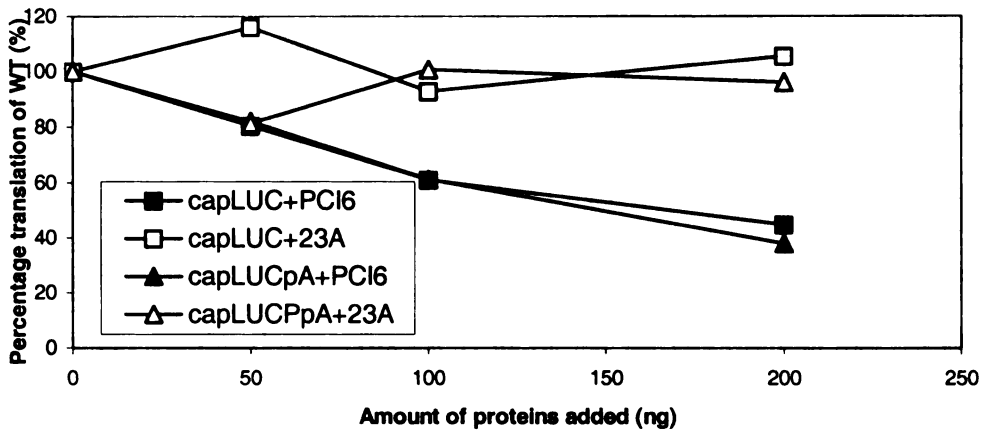
Since PCI6 shares the PAM2 with human Paip1 and Paip2 that have been demonstrated to regulate translation *in vitro* and *in vivo* (Khaleghpour, 2001a, 201b; Roy et al, 2002), we tested the possibility that PCI6 also could be involved in translational regulation. When tested using an *in vitro* wheat germ translation system, a two-fold difference (Fig. 3-5A) was observed between the translations of luciferase transcripts with (capLUCpA) or without (capLUC) poly- (A); this is in agreement with result from Iizuka et al (1994), who observed a three fold difference. The addition of increasing amount of PCI6 in the wheat germ translation system caused a progressive reduction in the translation rates of both capLUC and capLUCpA (Fig. 3-5A and B). At the highest amount of PCI6 (200ng, equal to 1 μM), there was a 62% reduction in translation as capLUCpA and 55% reduction in translation of capLUC. The reduction in translation in the presense of PCI6 was not due to lack of stability of the mRNA as verified by Northern analysis (data not shown). In contrast to the wild type PCI6, the PCI-6 mutant

A.

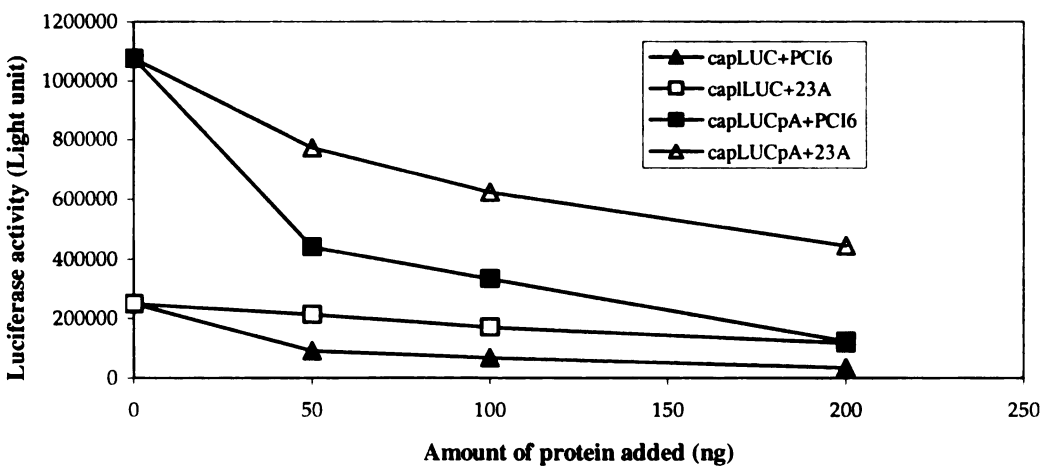
| | light unit ($\times 10^6$) | | | |
|---|---|---|---|---|
| Experiment1 | 0ng | 50ng | 100ng | 200ng |
| capLUC+PCI6 | 2.37 | 1.73 | 1.45 | 1.01 |
| capLUC+23A | 2.37 | 2.95 | 2.08 | 2.23 |
| capLUCpA+PCI6 | 4.99 | 3.67 | 2.37 | 1.97 |
| capLUCPpA+23A | 4.99 | 4.78 | 4.92 | 3.49 |

| Experiment 2 | 0ng | 50ng | 100ng | 200ng |
|---|---|---|---|---|
| capLUC+PCI6 | 0.727 | 0.640 | 0.442 | 0.339 |
| capLUC+23A | 0.727 | 0.783 | 0.716 | 0.854 |
| capLUCpA+PCI6 | 1.42 | 1.29 | 1.07 | 0.515 |
| capLUCPpA+23a | 1.42 | 0.952 | 1.46 | 1.73 |

B.




C.



D.

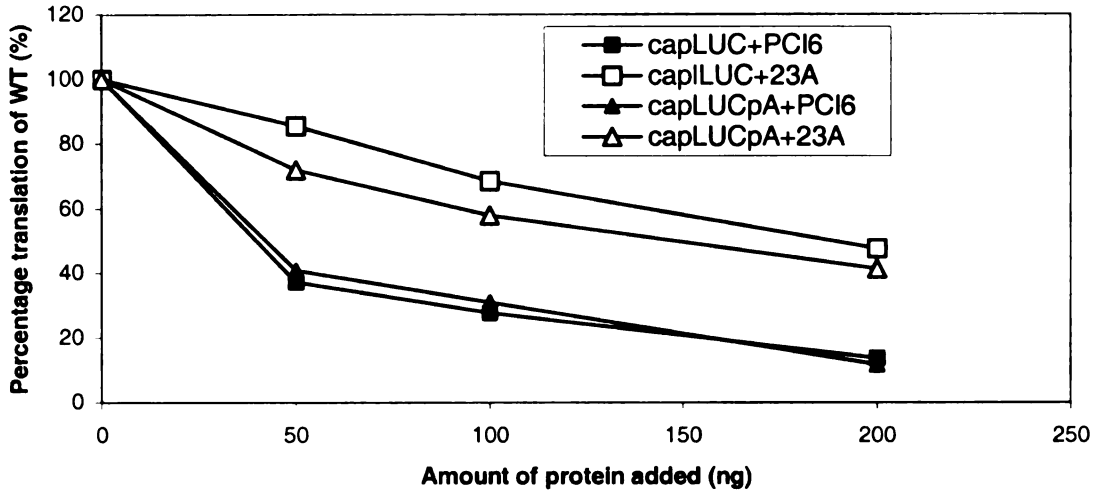



**Fig. 3-5 Effect of PCI6 on poly- (A)-dependent and –independent translation.**

Twenty five ng of capped luciferase transcript (capLUC) and an equal amount of capped, poly-adenylated luciferase transcript (capLUCpA) were used in a 12.5 µL *in vitro* wheat germ (A and B) or mouse ascites Krebs2 (C and D) translation reaction. The amount of luciferase protein translated was reflected by luciferase activity (light units). For each reaction, luciferase activity was measured twice and all reactions were repeated once. A and C. Luciferase activity following translation in the wheat germ (A) and Krebs2 system (C). B and D. Percentage in the presence of increasing PCI6 or PCI6-23A (23A) relative to control reaction without PCI6 in wheat germ (B) and Krebs2 system (D). Data shown in B, C, and D are the average of two independent experiments with two readings per experiment.

PCI6-23A that does not bind to PABP (Fig.3-2 and Table 3-3), did not inhibit translation (Fig. 3-5A and B).

The mouse ascites Krebs2 cell-free translation system, which was used to study human Paip2 functions (Khaleghpour et al, 2001a) was also used. PCI6 was verified to interact with the C-terminal 254 amino acids of human PABP to a comparable level to that of CS-PABP1 in the yeast two-hybrid assay (Table 3-2). The Krebs2 system is more sensitive to the presence of poly- (A) (an 8-fold increase in translation was observed between capLUCpA over capLUC, Khaleghpour et al, 2001a); we observed a 6-fold increase in the presence of poly- (A) (Fig. 3-5C). As for wheat germ, increasing amounts of PCI6 inhibited translation in the mouse Krebs2 system (Fig. 3-5C and D). Addition of 1 μM PCI6 inhibited translation by ca. 90% (Fig. 3-5C and D) and the percentage of translational inhibition by PCI6 was equivalent with or without poly- (A) (Fig. 3-5D). The mutant PCI6-23A, which is not able to interact with human C-terminus of PABP1 in yeast, caused some (50%), but much less inhibition than that of wild type (90%).

## Discussion

PABPs are multifunctional proteins that play important roles in mRNA stability and protein translation (reviewed in Kahvejian and Sonenberg, 2002). While functions of the amino terminal PABPs include RNA binding and interaction with translational initiation factor eIF4G, functions of the carboxy terminus are just now being defined, in part through identification of interacting factors (Kahvejian and Sonenberg, 2002). In this study, we identified two novel plant proteins, PCI6 and PCI243, that interact with PABP-CT. The interactions were first observed in the yeast two-hybrid system using a cucumber PABP cDNA (PABP-C254) that encoded the C-terminal 254 amino acids as

bait. Among 28 clones that specifically interacted with PABP-CT, 24 cDNAs encoded the same protein PCI6, indicating reproducibility of the interaction. PCI6 is an acidic protein with high amino acid similarity to Arabidopsis ERD15 at the N-terminal portion of the protein (Table 3-1). Besides PCI6, we identified PCI243, which is in many ways similar to PCI6, both encode small, acidic proteins with similarity at the N terminus. However, PCI6 and PCI243 are quite different at nucleic acid level and showed no DNA cross hybridization (data not shown).

The bait used in the screening was a cucumber PABP fragment that encodes the C-terminal 254 amino acids without any of the RRMs, suggesting the interaction does not require poly- (A) binding activity. This is supported by the fact that deletion of 50 amino acids from PABP-CT abolished the interaction (Fig.3-3 PABPΔC50), indicating the PABP-NT is not sufficient for the interaction. This is contrast to the interactions between human PABP1 and Paips where dual interactions were revealed; two regions in human PABP1 interacted with two individual regions of the Paips (Khaleghpour et al, 2001b; Roy et al, 2002). RRM1 and RRM2 are essential for the interaction with the PAM1 (PABP interacting Motif 1) of Paip1; RRM2 and RRM3 are responsible for interaction with PAM1 of Paip2. On the other hand, PABP-CT is essential to interact with PAM2 of both Paip1 and Paip2. The interaction between PABP-CT and PAM2 of Paip1 or Paip2 was not responsible for the translational regulation and functions of the interaction between PABP-CT and PAM2 are not clear at this time.

PCI6 deletions showed that the PCI6-NT is necessary and sufficient for the interaction (Fig.3-4 PCI6d1 and PCI6d4); deletion of 25 amino acids from the NT totally abolished the interaction (PCI6d1). With a C-terminal deletion of up to 85 amino acids,

PCI6 still interacted with PABP though to a lesser degree (Fig.3-4, PCI6d4). One surprising deletion was PCI6d3, which had less interaction with PABP than with the 22 amino acid shorter deletion PCI6D2. Perhaps this deletion interrupted a structural domain in a way that caused protein instability.

A 12 amino acid motif, which is a portion of the PAM2 (15 amino acids) in human Paip1 and Paip2 (Khaleghpour et al, 2001b; Roy et al, 2002), is present in the NT of PCI6, PCI243 and ERD15. Short peptides with the PAM2 fragment in Paip1 and Paip2 have been shown to be sufficient for the interaction with human PABP1 (Kozlov et al, 2000; Khaleghpour et al, 2001b; Roy et al, 2002). ERD15 was also found to interact with cucumber PABP, suggesting that the motif is predictive of ability to interact with PABP, and suggesting a possible function of ERD15. While the expression of ERD15 can be induced by several stresses such as drought, cold, and high intensity light (Kiyosue et al, 1994; Dunaeva and Adamska, 2001), and infection by plant-growth-promoting rhizobacterium *Paenibacillus polymyxa* (Timmusk and Wagner, 1999), no functions had previously been assigned to ERD15.

Two point mutations causing amino acid changes within the 12-amino-acid motif, Leu-16 to Ser and Phe-23 to Ala, inhibited the interaction by 91% and 99% respectively in yeast (Table 3-3), verifying importance of specific conserved amino acids. Both PCI6-23A and PCI6-16S are stable and have been expressed *in vitro* and in *E.coli*. The *in vitro* binding assay also showed that PCI6-23A did not bind to PABP (Fig.3-2). We also have observed that both PCI6-16S (data not shown) and PCI6-23A migrated somewhat faster in SDS-PAGE (Fig.3-2) than did wild type, suggesting that the loss of interaction with PABP may result from a conformational change.

The human PABP interactors, Paip1 and Paip2 have been demonstrated to modulate translation; Paip1 stimulates translation while Paip2 represses translation. PCI6 was examined to see whether it also could influence translation. Addition of PCI6 to the wheat germ translation system caused an inhibition of translation of both capLUC and capLUCpA. In the presence of 1 μM PCI6, translation of capLUCpA and capLUC were reduced by 62% and 55%, respectively (Fig. 3-5B). In contrast, addition of the same amount of the non-interacting mutant, PCI6-23A, did not inhibit translation (Fig. 3-5B), indicating that PCI6 exerts its function through the interaction with PABP. PABP has been shown to facilitate efficient mRNA translation by joining the 5' cap and 3' poly-(A) via interaction with eIF4G (Tarun and Sachs, 1996; Le et al, 1997) and eIF4B in plants (Le et al, 1997). The binding of PCI6 to PABP could change conformation of PABP and disrupt the interactions with initiation factors. Alternatively, the interaction could sequester PABP from binding to poly- (A) tail, as occurred for the interaction between PABP and Paip2 (Khaleghpour et al, 2001a).

In both the wheat germ and Krebs2 systems, PCI6 also inhibited translation of capped, nonadenylated RNA (capLUC), to a similar degree to that of polyadenylated RNA (capLUCpA) (Fig. 3-5B and C). Paip2 also inhibited translàtion of both polyadenylated and non-polyadenylated mRNA, although there was reduced inhibition in the absence of poly- (A) (Khaleghpour et al, 2001a). These results suggest that PABP may be also involved in cap-dependent, poly- (A) independent translation. Otero et al (1998) found that yeast Pab1p could stimulate translation of cap-dependent translation in the absence of poly- (A) and this phenomenon is called transactivation. It was proposed that interaction between PABP and other initiation factors could recruit PABP into the

translation complex and PABP could exert its stimulatory function. This is also supported by the finding in Gray et al (2000), that stimulation of translation is an intrinsic property of PABP; the poly- (A) tail and poly- (A) binding activity of PABP only serve to recruit PABP onto mRNA and they are not required for PABP to exert its stimulatory activity. In translation of capLUC, the interaction between PABP and other initiation factors could position PABP to the right place. However, recruitment of PABP by protein-protein binding might not be as effective as that of poly- (A) binding and results in weak translational stimulation.

The fact that PCI6 interacted with human PABP1-CT is not surprising given the high similarity of the CTC domain among all species (Kozlov, 2001). Presumably, then, cucumber PCI6 also interacted with mouse PABP in the mouse Krebs2 extract, since identity between mouse and human PABP is 99% and addition of PCI6 inhibited translation. However, we cannot rule out that PCI6 interacted with other initiation factors, especially since the PCI6-23A mutant still inhibited translation by 50%. It is also possible that full-length human PABP1 still interacted with PCI6-23A since human PABP1 interacted with Paip1 and 2 through both C- and N-terminal domains (Khaleghpour et al, 2001b; Roy et al, 2002). The human PABP-NT appears to interact with an acidic region of Paip1 and 2, and PCI6 also has acidic regions. Although PCI6 and Paip2 are not similar at the primary sequence, they are similar in other ways: both are small, acidic proteins, have PAM2, interact with PABP, and inhibit translation. PCI6 has homologs in *Arabidopsis* and tomato, Paip2 has homologs in *Drosophila*, *Xenopus*, and other metazons (Khaleghpour et al, 2001a). Collectively, these could suggest the

translational regulation through PABP could be a conserved mechanism among higher eukayrotes.

Northern analysis indicated constitutive expression of PCI6 in leaves, stems, flowers, and roots. Upon wounding treatment, the transcripts level of PCI6 increased dramatically and the induction not only occurred in the wounded leaves, but also in upper unwounded leaves, indicating a systemic signal has been produced. The wounding response is likely mediated by JA, since MeJA treatment could also induce the expression of PCI6, and more quickly.

Plant responses to stress can include transcriptional and post-transcriptional effects on gene expression. Transcriptional regulation has been broadly documented for an array of stresses (Creelman and Mullet, 1997), while post-transcriptional modulations in plants, for example, translational regulation, have been observed for heat shock and JA application (Creelman and Mullet, 1997; Gallie et al, 1995; Gallie et al, 1997; Reinbothe et al, 1993a, 1993b). Heat shock appears to cause a very rapid and large-scale shut down of translation of non-heat shock proteins (Gallie et al, 1995, 1997; Schneider, 2000). Translational inhibition was not due to instability of mRNA, instead, the half-lives of these transcripts increased dramatically (Gallie et al, 1995, 1997; Schneider, 2000). Inhibition occurs at the translation initiation step, the synergistic promotion of translation by the 5' cap and 3' poly- (A) was diminished (Gallie et al, 1995). Treatment with JA has been observed to cause relatively specific inhibition, for example, reduced translation of proteins related to photosynthesis (Dunaeva et al, 1999; Görschen et al, 1997; Reinbothe et al, 1993a, 1993b). Similar to heat shock treatment, JA did not affect the stability of the transcripts, but instead, affected initiation of translation (Reinbothe et al, 1993a, 1993b).

A JA inducible protein, JIP60, is a ribosome-inactivating protein (RIP) that specifically cleaved polysomes from MeJA treated barley leaves but not untreated plants (Reinbothe et al, 1994). Our results suggest that JA treatment or wounding may influence translation via a second mechanism, by increased expression of PABP-interacting translational repressor PCI6. Although not yet demonstrated, the PABP-CT binding ability of the ERD15 suggests it also influence translational efficiency and that this phenomenon may be associated with several stress conditions. It will be of interest to determine whether the effect of PCI6 on translation is general to all transcripts or is specific, perhaps via interaction with other factors.

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# Chapter 4

## Characterization of interactions between cucumber poly- (A) binding proteins (PABPs) and PABP-carboxy terminal interactors

## Abstract

A single copy of cucumber poly- (A) binding protein (PABP): CS-PABP1 was identified to interact with RNA-dependent RNA polymerase (RdRp, viral replicase) of zucchini yellow mosaic potyvirus (ZYMV) in yeast two-hybrid and *in vitro* binding assays. To examine if there are other PABP members in cucumber and if other members interact with ZYMV RdRp, RT-PCR was performed with degenerate primers to conserved PABP domains. A fragment of a second member of the cucumber PABP family (CS-PABP2), showing ca. 86% identity to CS-PABP1, was amplified. CS-PABP2 was expressed in all tissues tested: flower, leaf, root and stem. In the yeast two-hybrid assay, carboxy terminus of CS-PABP2 did not interact with ZYMV RdRp, indicating specificity for the interaction between CS-PABP1 and ZYMV RdRp and a possible functional difference between CS-PABPs. Both CS-PABPs interacted with PCI6 (PABP-CT interacting) and PCI243 and ERD15 (early responsive to dehydration), although with different affinities. Mutational analysis of an RdRp motif that shares similarity to a portion of the conserved motif present in the host PABP-CT interactors (Wang, Chapter3) suggests that RdRp-PABP1 binding occurs via a different domain than for host interactors.

# Introduction

Poly- (A) binding protein (PABP) is an essential protein for eukaryotic survival (Sachs et al, 1987). PABP binds to the 3' end poly- (A) tail of eukaryotic mRNA and functions in mRNA stability (Caponigro and Parker, 1995; Coller et al, 1998), initiation (Jacobsen, 1996; Gallie, 1998; Sachs et al. 1997) and regulation (Craig et al, 1998; Khaleghpour, 2001a) of translation. PABP is a multidomain protein with four RRMs (RNA Recognition Motifs) at the N terminus (NT), a CTC (C-Terminal Conserved) domain consisting of approximately 75 amino acids (Hilson et al, 1993; Kozlov et al, 2001) and a methionine and proline-rich region in between (Sachs et al, 1987). The PABP-NT is responsible for RNA binding, interacting with translation initiation factor eIF4G to bridge the 5' and 3' ends of mRNA (Kessler, 1998), and regulation of translation through interaction with human Paip1 and Paip2 (PABP interacting protein, Khaleghpour et al, 2001a; Roy et al, 2002).

The carboxy terminus of PABP (PABP-CT) is implicated in self binding. Deletion of Xenopus PABP-CT abolished the gel retardation supershift band, which resulted from auto-polymerization of PABPs on oligo- (A) probe, and also diminished the poly- (A) organizing activity, which allows PABP to assemble as regularly spaced copies of PABP on a single RNA molecule (Kühn and Pieler, 1996). Coller et al (1998) observed that a yeast PABP (yeast PABP is termed Pab) mutant with a deletion of C-terminal 50 amino acids lost the ability to rescue the inviability of yeast strain lacking a functional PABP gene, indicating the importance of PABP-CT. Furthermore, yeast PABP with a deletion of 90 amino acids from the CT lost the ability to stabilize mRNA (Gray et al, 2000). It was also found that deletion of human PABP-CT caused the accumulation of human

PABP1 in the nucleus, suggesting that PABP-CT was necessary for PABP1 to shuttle between the nucleus and cytoplasm (Afonia et al. 1998).

Several human and plant proteins have been identified to interact with PABP-CT, including human eRF3 (eukaryotic polypeptide chain release factor), Paip1, and Paip2 (Craig et al, 1998; Hoshino et al, 1999; Khaleghpour et al, 2001a; 2001b; Roy et al, 2002), cucumber PCI6 (PABP-CT interacting), and PCI243, and Arabidopsis ERD15 (early responsive to dehydration) (Wang, chapter 3). All of the cellular partners contain a 12-amino-acid motif that is necessary and sufficient for the interaction (Khaleghpour, 2001b; Kozlov et al, 2001; Roy et al, 2002; Wang, chapter 3). Point mutations of key amino acids in the motif eliminated interaction between cucumber PCI6 and PABP in yeast and *in vitro*, indicating the necessity of the domain for the interaction (Wang, chapter 3). The interaction between PABP-CT and eRF3 was proposed to provide a physical link between the stop codon and poly- (A) tail and facilitate the reentry of ribosome to the 5' end of same mRNA (Kahvejian and Sonenberg, 2002). As has been demonstrated for human Paip1 and Paip2 (Craig et al, 1998; Khaleghpour et al, 2001a), the interaction between PABP and PCI6 might also be involved in translational regulation, as PCI6 inhibited translation *in vitro* (Wang, Chapter 3).

The solution structure of the CTC domain of human PABP1 (546-619) was determined recently as a compact, globular structure (Kozlov et al, 2001). The CTC domain contains five α-helices that are conserved among PABP-CTC domains from different species. One distinguishing feature of the CTC is a hydrophobic pocket formed between helices 2 and 3. The Paip2 (and other PABP-CT interactors) was proposed to be oriented vertically to bind to the hydrophobic pocket. The residue phenylalanine (Phe) in

the 12-amino-acid motif was speculated to insert into the hybrophobic core (Kozlov et al, 2001). The Phe residue has been shown to be critical for the binding between PCI6 and PABP-CT (Wang, chapter3).

Two viral proteins have been identified to interact with PABP. The RNA-dependent RNA polymerase (RdRp) of the potyvirus zucchini yellow mosaic virus interacted with cucumber PABP-CT (Wang et al, 2000). The $3CD^{pro}$ (proteinase/polymerase) of the picornavirus poliovirus interacted with human PABP1 and the PABP-CT was implicated on this interaction also (Herold and Andino, 2001). Potyviruses and picornaviruses belong to the Picornavirus like superfamily, which is characterized by viruses with a positive sense, single stranded RNA genome with a covalently attached viral protein (VPg) at the 5' end and a poly- (A) tail at the 3' end (Shukla et al, 1994). The poly- (A) is necessary for poliovirus and potyviruses replication (Cui et al, 1993; Herold and Andino, 2001; Tacahashi and Uyeda, 1999). The interaction between PABP and ZYMV RdRp is proposed to be involved in potyviral replication since the poly- (A) tail is the site of initiation of the minus strand synthesis (Wang et al, 2000). Herold and Andino (2001) demonstrated that the interaction between human PABP1 and $3CD^{pro}$ is important for efficient poliovirus replication.

Although there is a single copy of PABP in yeast (Sachs et al, 1986), there are multiple copies of PABPs in animals and plants (Belostotsky and Meagher, 1993; Féral et al, 1999; Hilson et al, 1993; Le and Gallie, 2000). In *Arabidopsis*, it has been shown that different members are expressed in different tissues, and when expressed in yeast, can complement different functions (Belostotsky and Meagher, 1993, 1996; Hilson et al, 1993; Palanivelu et al, 2000a; 2000b). For example, *Arabidopsis* PABP5 is mainly

expressed in pollen, ovule and early embryo; AtPABP2 is expressed in all tissues: stem, leaf, flower, root, pollen, siliques (Belostotsky and Meagher, 1996; Palanivelu et al, 2000b). When expressed in yeast, both AtPABP5 and AtPABP2 can partially restore the poly-A shortening, the initiation of translation, and the viability of yeast lacking a functional yeast PABP. However, AtPABP5 could not restore the coupling between mRNA deadenylation and decapping, but AtPAB2 could (Belostotsky and Meagher, 1996; Palanivelu et al, 2000a). How the different members function in plants is not known.

Only a single copy of PABP (CS-PABP1), which was identified based on the ability to interact with ZYMV RdRp, was initially cloned from cucumber (Wang et al, 2000). In this study, I sought to determine whether cucumber has other PABPs, if so, to characterize their interactions with ZYMV RdRp and the host PABP-CT interactors. Here I report the cloning of a second member of the cucumber PABP gene family (CS-PABP2). Unlike CS-PABP1, CS-PABP2 does not interact with ZYMV RdRp, suggesting functional difference between the PABPs. Mutational analysis of an RdRp motif (from S62 to F73) with similarity to the conserved motif present in the host PABP-CT interactors, indicating that RdRp-PABP1 binding occurs via a different domain than for the host interactors.

# Materials and Methods

## Amplification of CS-PABP2:

Two degenerate primers, RG194 (GC(A/G)CA(A/G)(A/C)G(C/G/T)AA (A/G)GAAGA(A/C/G T/)AG) and RG196 (CAT(C/G/T)CC(A/C/G/T)GT(A/C/G/T) AC(C/T)TT(A/G)GC) were designed according to two conserved regions among PABP-1, 2, and 5 of Arabidopsis (Belostotsky and Meagher, 1993; Hilson et al, 1993), CS-PABP1 (Wang et al, 2000), and PABPs from tobacco (Le and Gillie, 2001) and wheat (Le et al, 1997). Total RNA isolated from cucumber leaf, root and flower was digested with RQ1 DNase (Promega) to eliminate any contaminating DNA. In each reverse transcription (RT) reaction, 2 μg total RNA was used to make first strand cDNA. Typically, 2 μl of the RT reaction and 75 pmol primers were used in a 50 μl PCR reaction with Taq DNA polymerase (Gibco BRL). The amplification was conducted with 5 cycles of 94°C (1 minute), 55 °C (1 minute), 72°C (1 minute), and 30 cycles of 94°C (1 minute), 45 °C (1 minute), 72°C (1 minute). The PCR product was ligated to pGEM-T easy vector (Promega) and transformed to *E.coli* strain XL1-Blue (Stratagen). The cDNA inserts from white colonies were amplified with RG194 and RG196 using Vent DNA polymerase (New England Biolabs), and digested with *HaeIII* and *DpnII*. The cDNA inserts, which gave different digestion patterns from that of CS-PABP1, were deemed as new putative CS-PABP paralogs. The plasmid DNA was isolated from colonies harboring CS-PABP paralog cDNAs and subjected for sequencing. From sequence acquired from these cDNAs, a gene-specific primer RG199 (AAGAATT CATAC GGTCTGTT CCCATG) was designed according to a CS-PABP2 region that is distinct from CS-PABP1. CS-PABP2 was then amplified from the cucumber leaf cDNA library

using RG199 and RG93 and inserted into the yeast two-hybrid vector pADGAL4. To check the expression patterns of *CS-PABP1* and *CS-PABP2*, two pairs of gene specific primers were used: RG199 and RG263(GGGGTTTCCAACAT TCAGA) for *CS-PABP1*, RG241 (AAGAATTCATGCAGCCAATGGCAATG) and RG264 (GGGAGCT TTAT GAACACAAG) for *CS-PABP2*.

**Yeast two-hybrid and *In vitro* protein binding assay**

Yeast two-hybrid assay was performed as described in Wang et al (2000). *In vitro* protein-protein binding assays were performed similarly to the procedure described in Chapter 3 and Wang et al (2000). *In vitro* labeled PCI6 or RdRp (4 μL labeling reaction using Promega's TNT system) was incubated with 1.0 uM BSA (1.4 μg), 1.0 μM RdRp (1.2 μg), or 2.5 μM PCI6 (0.8 μg) at room temperature for 30 minutes in 20 μL protein-protein binding buffer (Wang et al, 2000). Forty μL PABP-poly-A-sepharose (50% slurry, containing about 5 μg PABP) was then added to the incubation along with 10 μL bovine serum and 30 μL binding buffer, and was incubated for 60 minutes at room temperature. The PABP-poly-A-sepharose was washed six times with binding buffer plus 200 mM NaCl. The proteins bound to PABP-poly-A-sepharose were analyzied by SDS-PAGE and auto-radiography.

**PCR-based site-directed mutagenesis:**

The PCR-based site-directed mutagenesis was done following Ho et al (1989) with a few modifications. Two master primers: RG89 (CCGGAATTCAGCAAGCG AGAAAGATG, 5' end primer) and RG88 (AGAGTCGACTTGGAGCATCACAGTGT, 3' end primer), which amplify the full-length RdRp coding sequence (Wang et al, 2000), were used for all mutations. For each mutation, a pair of complementary sense and anti-

sense primers was designed with desired nucleic acid (s) changes. Primer RG89 and an anti-sense primer, primer RG88 and the corresponding sense primer were used to amplify the 5'end and 3' end of the gene with the mutation respectively. Both the 5' and 3' end of the fragments were gel purified. About 100 ng of each fragment were mixed and used as template in the final run PCR using RG89 and RG88. The mutant RdpR was then digested with *EcoRI* and *SalI* and inserted into the pBDGAL4 vector. Each mutant was sequenced to verify the mutation. Primer pairs: RG209 (GTACCAGCCGGCCAAGTTG AACAA) and RG210 (TTGTTCAACTTGGCCGGCTGGTAC), 211 (GCCGAGCAAG GCGAACAAAGAAG) and 212 (CTTCTTTGTTCGCCTTGCTCGGC), 213 (AACAAAGAAGGCTTTAAAAAG) and 214 (CTTTTTAAAGCCTTCTTTGTT), 224 (AGCAAGTTGGCCAAAGAAGCC) and 225 (GGCTTCTTTGGCCAACTTGCT), 226 (GTTGAACAAAGCAGCCTTTAA) and 227 (TTAAAGGCTGCTTTGTTCAAC), 228 (CCGAGCAAGTCGAACAAAGAA) and 229 (TTCTTTGTTCGACTTGCTCGG), 230 (GAACAAAGAATCCTTTAAAAAG) and 231 (CTTTTTAAAGGATTCTTTGTTC), 232 (AACAAAGAAGCCGCTAAAAAGGATTTC) and 233 (GAAATCCTTTTTAGC GGC TTCTTTGTT), 234 (TTTAAAAAGGATGCCTTTAAATACAAT) and 235 (ATTGTATTTAAAGGCATCCTTTTTAAA) were used to make RdRp S62A, N64A, A68G, N65A, E67A, L64S, A68S, F70A, F74A respectively. RG218 (GTACCAGCCG GCCAAGGCGAACAAAGA) and 219 (TTCTTTGTTCGCCTTGGCCGGCTGGTAC) were used to make the double mutants LN624AA, LA628AG, NA648AA, and triple mutant LNA6248AAG. RG251 (CAAAGAAGCCGCTTAAAAGGA) and RG253 (GGC ATCCTTTTTAGCGGCTTCTTTGTT) were used to make the double mutant FF704AA.

# Results and Discussion

## PABPs in cucumber exist as a gene family

The initial screening for host proteins interacting with ZYMV RdRp identified six independent clones of PABP, all of which represented the same PABP gene, CS-PABP1 (Wang et al, 2000). In other species, however, PABPs exist as gene familes. We sought to determine whether there are also multiple PABPs in cucumber, and if so, why only one member of the family was identified based on the yeast two-hybrid interaction with ZYMV RdRp. To amplify other PABP members in cucumber, two degenerate primers were designed according to conserved regions present in PABP2, 3, and 5 of Arabidopsis (Belostotsky and Meagher, 1993; Hilson et al, 1993), PABP3 and 7 of tobacco (Le and Gallie, 2000), PABP1 of cucumber (Wang et al, 2000), and PABP of wheat (Le et al, 1997). Since different PABP members of *Arabidopsis* are expressed in different tissues and different developmental stages (Belostotsky and Meagher, 1993, 1996; Palanivelu et al, 2000a; 2000b), RNA was isolated from cucumber leaf, root, and flower and used for RT-PCR.

Among the 114 cDNAs examined (26 cDNAs originating from leaf mRNA, 54 from root, and 34 from flower), two types of patterns were observed based on digestion with *HaeIII* and *DpnII* (Table 4-1); 21% of the tested cDNAs from leaf, 39% from root and 23% from flower had the same digestion pattern as each other, but different from CS-PABP1, and the remaining clones had the same digestion pattern as CS-PABP1. Sequence analysis of a pair of clones representing the two different restriction patterns from each tissue source showed that the three cDNAs with the digestion pattern of *CS-PABP1* had the same sequences as *CS-PABP1*. The sequences of three cDNAs that

**Table 4-1. Amplification of the paralogs of CS-PABP1.** Each cDNA insert was amplified from colony with PCR and digested with *HaeIII* and *DpnII*. The digestion patterns of each cDNA were compared with those of CS-PABP1. The cDNA inserts with a different digestion pattern from CS-PABP1 were picked as a putative parolog of CS-PABP1.

| | Leaf | Root | Flower |
|---|---|---|---|
| Number of cDNAs tested | 26 | 54 | 34 |
| Number of cDNAs with CS-PABP1 digestion pattern | 20 | 33 | 27 |
| Number of cNDAs with CS-PABP2 digestion pattern* | 6 | 21 | 7 |
| Percentage of the CS-PABP2 cDNA | 21% | 39% | 23% |

* All the cDNAs in this category have the same digestion pattern.

differed from the CS-PABP1 digestion pattern were identical and had 86% identity to *CS-PABP1* (Fig. 4-1). These three cDNAs contained the conserved CTC domain characteristic of PABP, indicating that they represent a second cucumber PABP, *CS-PABP2*.

To determine whether CS-PABP2 was represented in the original leaf cDNA library that had been screened using ZYMV RdRp as bait, a *PABP2* specific primer was designed to amplify the 3' end sequence of CS-PABP2, along with another primer complementary to the pADGAL4 vector. A fragment was amplified from the library and sequence data showed that this fragment includes the coding sequence of the C-terminal 254 amino acids of CS-PABP2 and a 264 bp 3' NTR. The fragment was named CS-PABP2-C254 because it has a similar size to the PABP1 fragment CS-PABP1-C254, which interacted with ZYMV RdRp (Wang et al, 2000). The above results demonstrated that there are at least two members in the cucumber PABP gene family. Since both could be amplified from the leaf two-hybrid cDNA library, the failure to identify CS-PABP2 using RdRp as bait was not due to lack of representation in the library.

Gene specific primers corresponding to the coding sequences and 3'NTRs of CS-PABP1 and CS-PABP2 were designed to distinguish each of the genes and examine expression patterns. Equal amounts of total RNAs from flower, leaf, root and stem were converted to cDNAs that were used as template for PCR. Digestion of the PCR products with *Mlu I* that cuts only *PABP*1, and with *Nde I* that cuts both *PABP*1 and *PABP*2 in the same position, confirmed the specific amplification of *PABP1* and *PABP2* (Fig. 4-2). *PABP1* and *PABP*2 were amplified from flower, leaf, root, and stem with similar intensities, suggesting that both genes are expressed in all cucumber tissues (Fig. 4-2).

A.

GCGCAGAGGAAGGAAGATAGGAGAGCCAGATTGCAGGCTCAGTTCTCTCAA**ATACGGTCTGTTCCCATG**CCT

A Q R K E D R R A R L Q A Q F S Q I R S V P M P

GCTTCAGTTGCCCCACGGATGCCAATATATCCCCCTGGTGGACCAGGTATCGGACAGCAGTTGTTTTATGGT

A S V A P R M P I Y P P G G P G I G Q Q L F Y G

CAAGCTCCTCCTGCAATGATTCCTTCCCAAGGTGGGTTTGGTTATCAGCAGCAGCTTGTTCCTGGTATAAGG

Q A P P A M I P S Q G G F G Y Q Q Q L V P G I R

CCTGGTGGAGGACCAATGCCAAATTTCTTTGTCCCAATGGTTCAGCAGGGTCAGCAAGGCCAGCGCCCTGGG

P G G G P M P N F F V P M V Q Q G Q Q G Q R P G

GGCAGACGTGCAGGAGCTGTCCAGCAAACTCAGCAGCCTGTTCCAATGATGCAACAACAGATGTTGCCTAGA

G R R A G A V Q Q T Q Q P V P M M Q Q Q M L P R

GGAGGACGTGTCTATCGCTATCCCCCTGGAAGAGGATTGCCTGATGTTGCTATGCCTGGTGTTGCTGGAGGC

G G R V Y R Y P P G R G L P D V A M P G V A G G

ATGTTTTCTGTTCCATATGATATGGGAGGTATGCCATTGCGTGATGCGGCACACTCTCAACCTATCCCTATT

M F S V P Y D M G G M P L R D A A H S Q P I P I

GGTGCACTGGCTTCTGCTCTGGCAAATGCTACTCCAGACCAACAGAGAACAATGCTGGGGGAGAATCTTTAC

G A **L A S A L A N A T P D Q Q R T M L G E N L Y**

CCGCTTGTGGAACAGTTAGAGCCTGACAACGCGGCCAAGGTAACTGGAATGCTTCTGGAGATGGACCAGACT

**P L V E Q L E P D N A A K V T G M L L E M D Q T**

GAGGTTTTGCATTTACTAGAGTCCCCGGAAGCCTTGAAGGCGAAAGTAGCTGAAGCTATGGAGGTTTTGAGG

**E V L H L L E S P E A L K A K V A E A M E V L R**

AATGTTGCGGCAGCTCAGCAGCAGCAGCAAGCTGGCAATGCAGCAGATCAGCTAGCTTCTCTGTCGCTGACG

**N V A A A Q Q Q Q Q** A G N A A D Q L A S L S L T

GAGAACCTCGTGTCG**TGA***GTTACTTGTGTTCATAAAGCTCCCTGATTCATGAAATGAAAATGTAGTAGTTGT*

E N L V S

*GTGTGATACAATTGACTGTTTTTTTAATTTGTGGAGACTGCAGTACTGGCATAATTTGTTTGTAGTACTGGC*

*TTTTATCTCTGAAGACTTTGCTGACATTTTCTTTTCTTTTTAGTTTTCGGTTAGCAGTACTCTCTTTTTCCC*

*TAGAGGATGAAGGTTTTTGTTCTTGTTTTTTTTTTTTAAACATTAATCCGACTTAGTGCTCTACTCCAAAAAA*

*AAAAAAAAAAAA*

B.

CS-PABP2: AQRKEDRRARLQAQFSQIRSVPMPASVAPR-MPIYPPGGPGIGQQLFYGQAPPAMIPSQG

**AQRKEDR ARLQAQFSQ++ + M +SVAPR MP+YPPGGPGIGQQ+FYGQAPP +I SQ**

CS-PABP1: AQRKEDRIARLQAQFSQMQPMAMASSVAPRGMPMYPPGGPGIGQQIFYGQAPPTIISSQP

CS-PABP2: GFGYQQQLVPGIRPGGGPMPNFFVPMVQQGQQGQRPGGRRAGAVQQTQQPVPMMQQQMLP

**GFGYQQQL+PG+RPGGGPMPNFFVPMVQQGQQGQR GGRRAGA+QQTQQPVP+MQQQMLP**

CS-PABP1: GFGYQQQLMPGMRPGGGPMPNFFVPMVQQGQQGQRSGGRRAGAIQQTQQPVPLMQQQMLP

CS-PABP2: RGGRVYRYPPGRGLPDVAMPGVAGGMFSVPYDMGGMPLRDAAHSQPIPIGALASALANAT

**R GRVYRYPPGRGLPD+ MPGVAGGMFSVPY+MGGMP RDA H QP+P+GALASALANAT**

CS-PABP1: R-GRVYRYPPGRGLPDLPMPGVAGGMFSVPYEMGGMPPRDAVHPQPVPVGALASALANAT

CS-PABP2: PDQQRTMLGENLYPLVEQLEPDNA*AKVTGM*LLEMDQTEVLHLLESPEALKAKVAEAMEVL

**PDQQRTMLGENLYPLVEQLEPDNAAKVTGMLLEMDQTEVLHLLESPEALKAKVAEAMEVL**

CS-PABP1: PDQQRTMLGENLYPLVEQLEPDNA*AKVTGM*LLEMDQTEVLHLLESPEALKAKVAEAMEVL

CS-PABP2: RNVAAAQQQQQAGNAADQLASLSLTENLVS

**R+VA QQ+GNAADQLASLSLT+NL S**

CS-PABP1: RSVA-----QQSGNAADQLASLSLTDNLDS

**Fig. 4-1. Nucleic acid and amino acid sequences of CS-PABP2.** A. The nuclei acid sequence of the 3' end of *CS-PABP2*. The 3' end of CS-PABP2 was obtained first with RT-PCR and then amplified from cucumber leaf cDNA library with PCR. The location of the degenerate primers is indicated by underline. The CS-PABP2 gene specific primer is indicated by the region bold and underlined, and 3'NTR is italic. Amino acids in bold are the CTC domain. B. Sequence alignment between the C-terminal regions of CS-PABP1 and CS-PABP2. The identity between these two fragments is about 86%. The conserved amino acid sequence in between two sequences are indicated by bold and + represents similar amino acids. The CTC domain is indicated by bold and underline.

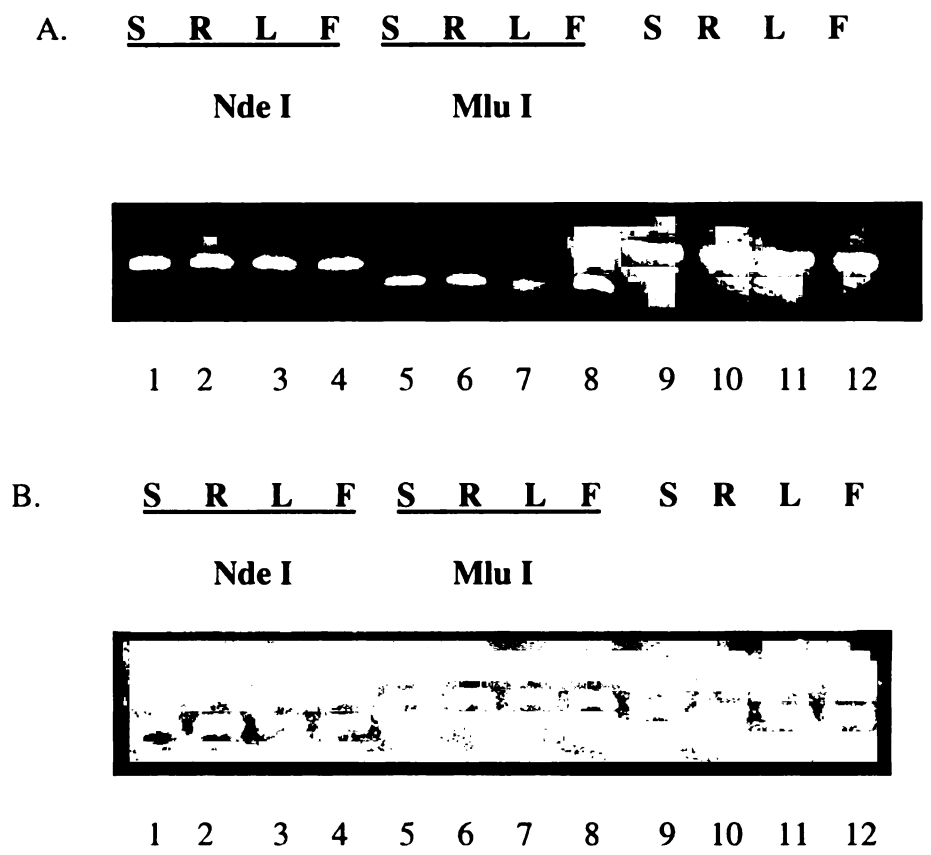



**Fig. 4-2. *CS-PABP1* and CS-PABP2 are constitutively expressed in different tissues tested.** Equal amounts of total RNA from cucumber stem (S), root (R), leaf (L), and flower (F) were converted to cDNA and equal volumes of cDNAs were used as templates for PCR (See Materials and Methods for detail). Primer pairs RG241 and RG263, RG199 and RG264 were used to amplify CS-PABP1 and CS-PABP2 respectively. PCR products were digested with *MluI* and *NdeI* respectively. A. cDNAs amplified using CS-PABP1 specific primers (Lane 9-12) and digested with either *NdeI* (Lane 1-4) or *MluI* (Lane 5-8). B. cDNAs amplified using CS-PABP2 specific primers (Lane 9-12) and digested with *NdeI* (Lane 1-4) or *MluI* (Lane 5-8).

The reduced frequency of CS-PABP2 observed in the original cloning (Table 4-1) may be due to the use of degenerate primers, which could favor the amplification of CS-PABP1.

The inability to amplify more than two members may suggest that only two PABP genes are expressed in cucumber. Interestingly, two members have been identified to date in tobacco (Le and Gallie, 2000). For both cucumber and tobacco, the two PABP parologs share high similarity at the amino acid level (Le and Gallie, 2000). Alternatively, other cucumber PABPs may not have been amplified due to a more divergent sequence, at least at the regions where primers were designed. It may be useful to try lower annealing temperatures in RT-PCR in the future.

**The CTC domain is conserved among plant PABPs**

The CTC domain is conserved among all PABPs from different species and the solution structure of the CTC domain of human PABP1 was recently determined (Kozlov et al, 2001; Deo et al, 2001). The CTC contains five α-helices that are arranged as an arrowhead; Helix 1 is the tip, helices 2 and 4 are located at the side of the arrow with helix 3 crossing in between them, and helix 5 is the shaft of the arrow. Helices 2 and 3 form a hydrophobic pocket that is proposed as the contacting site with its partners. Based on the chemical shift perturbation data upon the addition of human Paip2 protein, 3 amino acids in PABP1, Lys-580, Val-613, and Met-584 (K35, V68, and M39 in CTC domain) were identified as potential residues close to the peptide binding site.

Amino acid sequences of the CTC domain from all available plant PABPs, including Arabidopsis AtPABP2, 3, and 5, cucumber CS-PABP1, and 2, Tobacco

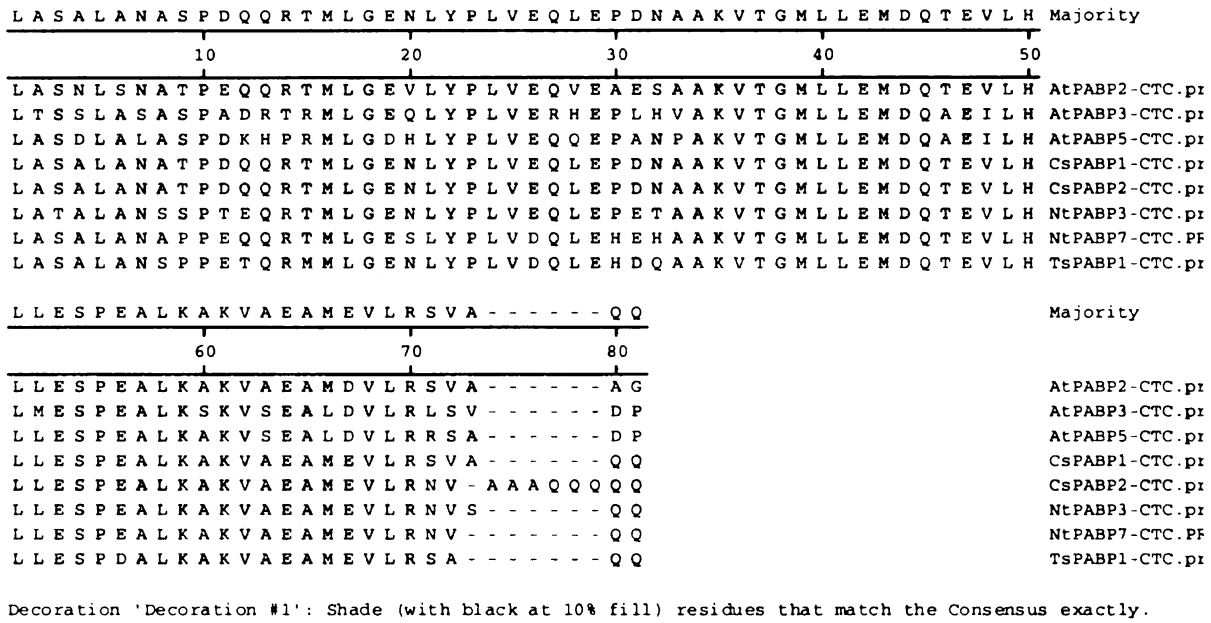


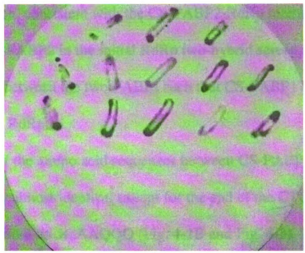

**Fig. 4-3. Alignment of the CTC domain of PABPs from different plants.** Amino acid sequences of the CTC domain including three members from Arabidopsis (AtPABP2, 3, and 5), two from cucumber (CS-PABP1, and 2), two from tobacco (NtPABP3, and 7) and one from wheat (TsPABP1). Helix 1 is from a.a. 1 to 7; helix 2 is from a.a. 21-27; helix 3 from a.a. 35-41; helix 4 from 46-53, and helix 5 from 56-74 (for CS-PABP2 is from 56-80).

NtPABP3, and 7, and wheat TsPABP, were compared using the clustal alignment method (Fig. 4-3). Except AtPABP3 and AtPABP5, the other PABPs are quite similar to AtPABP2, ranging from 77.3% (TsPABP vs. AtPABP2) to 85.5% (CS-PABP1 vs.AtPABP2). AtPABP3 and AtPABP5 have only 68.4% and 63.2% identity to At PABP2 and other PABPs; the similarity drops to 65% and 55% identity to AtPABP2 if full-length sequences are compared. The most divergent regions were the linkers between helices 1 and 2 (a.a.8-20), and 3 and 4 (a.a.28 to 34). Helices 1 (a.a.1-7) and 5 (a.a.56-74) are less conserved, while the most conserved regions are Helics 2 (a.a.21-27) and 3 (a.a.35-41) (Fig. 4-3). Since helices 2 and 3 comprise the partner-interacting hydrophobic pocket, the conservation at these two helices may suggest that these PABPs interact with the same or similar partners. Further, the three amino acids, K35, V68 and M39 that were found in close contact with Paip2 (or other interactors) are identical in all CTC domains.

**CS-PABP2-CT does not interact with ZYMV RdRp in yeast**

To test whether PABP2-CT interacts with ZYMV RdRp, cDNA PABP2-439 was inserted into the yeast two-hybrid vector pADGAL4 and co-transformed into yeast cells with construct pBDRdRp. Yeast cells with both fusion proteins failed to activate either reporter gene; no colonies grew on the medium without histidine and no yeast cells turned X-gal to blue (Fig. 4-4). Thus, PABP2-CT is not able to interact with RdRp, at least in the yeast two-hybrid assay. Possible explanations for the failure to interact include: PABP2-439 could be unstable in yeast, the NT of PABP2 is necessary for the interaction with RdRp, or PABP2 is not able to interact with ZYMV RdRp. Since PABP2-439 interacted with PCI6, PCI243 and ERD15 (see below), failure to interact with ZYMV RdRp is not likely due to the lack of stability in yeast. Although we can not

pADPABP1-C254+pBDRdRp



pADPABP2-C254+pBDRdRp

Fig. 4-4. CS-PABP2 does not interact with RdRp in the yeast two-hybrid assay. Yeast cells were co-transformed either with pADPABP1-C254 and pBDRdRp (top), or with pADPABP2-C254 and pBDRdRp (bottom). The cells were grown for 6 days on medium SD without histidine, transferred to nitrocellulose membrane, broken by liquid nitrogen and incubated with X-gal.

rule out a need for the PABP-NT, if the CS-PABP2-NT is needed, there is a difference between the two PABPs since the NT is not required for PABP1-RdRp interaction. The most likely explanation, consistent with the CS-PABP2-RdRp interaction results and failure to identify CS-PABP2 in the initial RdRp two-hybrid screen, is that there is a functional difference between the two PABPs such that CS-PABP1 but not CS-PABP2 interacts with ZYMV RdRp.

Comparison of the amino acid sequences between CS-PABP1 and CS-PABP2 indicated that they are almost identical except for the end of the CTC domain, where CS-PABP2 has extra 5 amino acids, AAQQQ (Fig. 4-1B and Fig. 4-3). These extra amino acids might affect the CTC conformation and lead to loss of the interaction between CS-PABP2 and ZYMV RdRp. Although expression differences have been observed among members of plant PABP families, and AtPABP2 and AtPABP5 differed in ability to partially restore the coupling between mRNA deadnylation and decapping in Pab1-deficient yeast strain (Belostotsky and Meagher, 1996; Palanivelu et al, 2000a; 2000b), the functional differences among PABP parologs have not been determined yet in planta. Whether the difference of CS-PABP1 and CS-PABP2 in interaction with ZYMV RdRp reflects differences related to viral infection, remains an interesting question.

**PCI6, PCI243 and ERD15 interacted with CS-PABP2-CT**

CS-PABP2 was also tested for ability to interact with the host interactors: PCI6, PCI243 and ERD15 in yeast two-hybrid assays. In contrast to the results obtained with ZYMV RdRp, CS-PABP2 interacted strongly with all three plant proteins. PCI6 and ERD15 interacted with CS-PABP1-CT and CS-PABP2-CT with similar affinities (Table 4-2), but, PCI243 interacted more strongly with CS-PABP2 than with CS-PABP1 (Table

**Table 4-2. Quantitative β-galactosidase assay for protein-protein interactions in the yeast two-hybrid system.**

| Protein fused to BD | Protein fused to AD | β-galactosidase activity |
|---|---|---|
| PABP1-439 | PCI6 | 92±6 |
| PABP2-439 | PCI6 | 93±5 |
| PABP1-439 | PCI243 | 143±17 |
| PABP2-439 | PCI243 | 236±30 |
| PABP1-439 | ERD15 | 187±19 |
| PABP2-439 | ERD15 | 149±14 |

The PCI6 data are the mean of three experiments with three replications per experiment. The PCI243 and ERD15 data are the mean of four experiments with three replications per experiment. The β-galactosidase activity was measured as "Materials and Methods".

4-2). The different affinity of PCI243 to different members of PABPs could be biologically significant and needs to be further addressed.

**RdRp binding to PABP-CT depends on a different motif than the host interactors**

Most of the PABP-CT interactors identified to date share a 12-amino-acid motif (**S**m**L**N**p**n**A**p**l**F**v**p, Fig. 4-5). This motif in PCI6 has been demonstrated to be necessary for the interaction with PABP1 (Wang chapter 3), substitutions at the Leu-16 and Phe-23 abolished the interaction (Wang chapter 3). ZYMV RdRp also possesses a motif which shares partial similarity to that in the host PABP-CT interactors (Fig. 4-5).

Seven amino acids within this motif in RdRp were subjected to point mutations: Ser-62, Leu-64, Asn-65, Glu-67, Ala-68, Phe-70 and Phe-74. A total of 14 mutants were made either with one, two, or three mutations of these amino acids and tested for interaction with PABP1 in the yeast two-hybrid system (Fig. 4-5). Each mutant interacted with CS-PABP1 at a similar level to that of wild type RdRp (data not shown), suggesting that the motif present in the ZYMV RdRp is not responsible for the interaction with the PABP-CT. Structural studies by Kozlov et al (2001) suggested that the Phe amino acid in the 12- amino-acid motif was likely to be critical for the interaction between human PABP1 and Paip2 and this has been confirmed in the CS-PABP1-PCI6 interaction (Wang chapter 3). Although there are two Phe amino acids (F70 and F74) within the region in ZYMV RdRp, they are not located at the same position as Phe in the host interactors. This suggests that there is a significant difference between the motifs presented in ZYMV RdRp and in the host proteins interacting with PABP-CT. In support of this, RdRps from three other potyviruses also interacted with CS-PABP1, bean common mosaic necrosis potyvirus (BCMNV)(data not shown), and tobacco etch potyvirus (TEV) and plum pox

A.

| | |
|---|---|
| CS-PCI 6 * | **S**M**LN**PN**A**PL**F**V**P**MAYRTVEDFSDQWWELIQSSPWFREYWLQERFQ |
| CS-PCI 243 * | **S**K**LN**PN**A**PL**FIP**AAY-QVEDFSPQWWQLVTTSTWYRDYWLSQHQE |
| At ERD15* | **S**T**LN**PD**A**PL**FIP**AAVRQVEDFSPEWWQLVTTSTWYPDYWISQQQQ |
| Le ERD15 | **S**T**LN**PN**A**PL**FVP**SFVRQVEDFSPEWWNLVTTSTWFHDYWMSQNQG |
| Human Paip1* | **S**K**L**SVN**A**PE**FYP** |
| Human Paip 2* | **S**N**LN**PN**A**KE**F**V**P** |
| ZYMV RdRp* | **S**K**LN**KE**A**FKKDF |

B.

ZYMV RdRp $^{62}$**S**K**LN**KE**A***F*KKD*F*

Single mutations: S62A, L64A, L64S, N65A, E67A, A68G, A68S, F70A, F74A.

Double mutations: 62,64AA, 62,68AG, 64, 68AG, 70, 74AA.

Triple mutqtions: 62, 64, 68AAG

**Fig. 4-5. Conserved amino acids among all PABP-CT interacting proteins.** A. The conserved motif among PABP-CT interacting proteins, from cucumber, Arabidopsis and humans. Amino acids in bold are conserved. L and F have been verified to be critical for the interaction between CS-PABP1 and CS-PCI6 (Wang, chapter 3). B. The similar motif present in ZYMV RdRp and mutations made in this motif. The amino acids underlined are those that were mutated.

* The interaction with PABP-CT has been verified.

potyvirus (PPV) (Dr. Saegesser Rudolf, personal communication). All the viral RdRps have a similar 12-amino-acid motif, but like ZYMV, they either do not have Phe in the motif (PPV, Saenz et al, 2000), or the Phe is not in the right position (TEV and PPV, Allison et al, 1985 and Fang et al, 1995). Two other proteins, human PCBP [poly (rC) binding protein] and polioviral $3CD^{pro}$ were found to interact with human PABP1 and the PABP-CT was necessary for the interaction (Herold and Andino, 2001). However, no such motif is present in these two proteins, suggesting they also interact with PABP using a different motif. Difference in the interacting motif may explain why PABP2 binds the host interactors but not ZYMV RdRp. Serial deletions from N- or C-terminus of ZYMV RdRp eliminated the interaction, suggesting conformation of the RdRp could be dramatically changed. Random mutagenesis should be used in the future to define the key motif and amino acids in RdRp that are responsible for the interaction.

**ZYMV RdRp facilitates Binding of PCI6 to PABP**

Both PCI6 and ZYMV RdRp bind to PABP; they both interact with the C-terminal 131 amino acid region, and a 50 amino-acid deletion from the C-terminus of PABP1 abolished the interaction (Wang et al, 2000; Wang, chapter 3). These observations suggest that PCI6 and ZYMV RdRp may bind to the same region in PABP and may compete with each other for binding to PABP.

Both PCI6 and ZYMV RdRp bind to poly- (A) sepharose in the presence, but not absence of PABP (Wang et al, 2000; Wang, chapter 3). Preliminary results of a competition experiment (performed only once) are described below. When unlabeled PCI6 protein was added to a fixed amount of labeled RdRp, less RdRp was retained on the PABP-poly-A-sepharose (Fig. 4-6, Lane 9 vs.Lane 7), suggesting that PCI6 partially

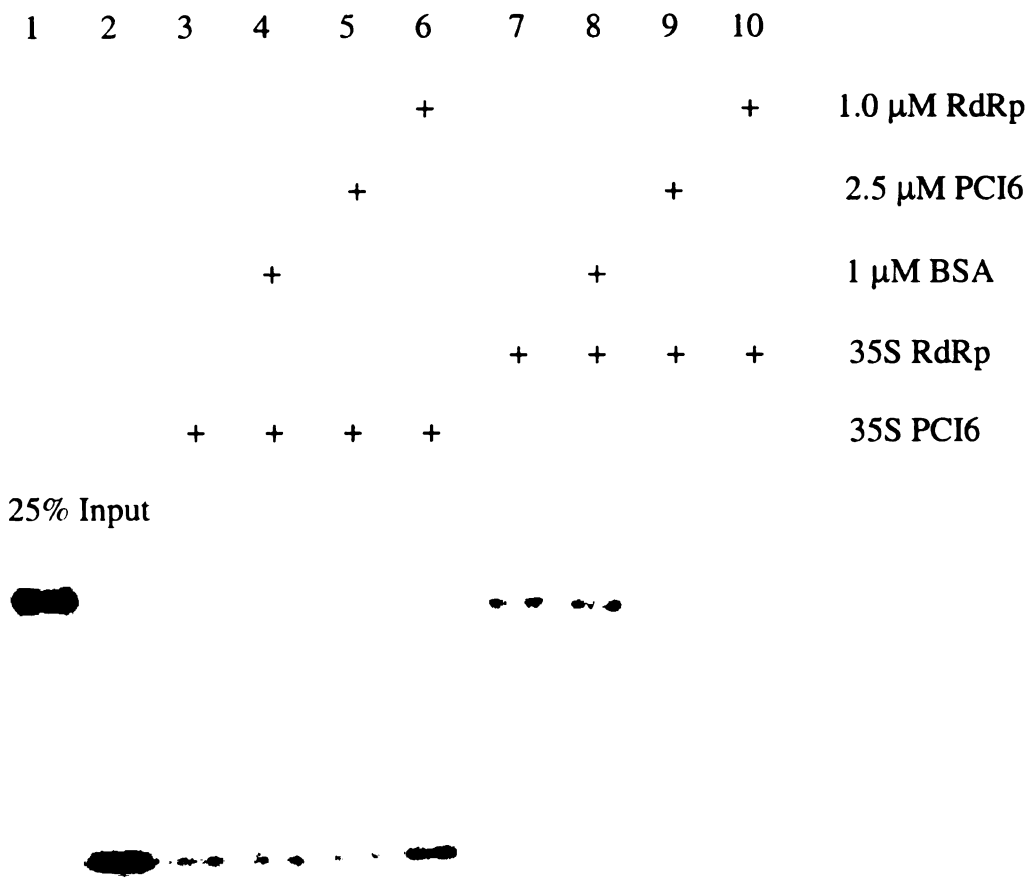



**Fig. 4-6. *In vitro* competition between PCI6 and ZYMV RdRp for binding to CS-PABP1.** *In vitro* labeled PCI6 or RdRp was first incubated with 1.0 μM BSA (1.4 μg, Lane 4, 8), 2.5 μM PCI6 (0.8 μg , Lane 5, 9), or 1.0 μM RdRp (1.2 μg, Lane 6, 10), and then with PABP-poly-A-sepharose. The beads were washed extensively and proteins bound to sepharose were analyzed by SDS-PAGE and auto-radiography. Twenty five percent input of RdRp and PCI6 were loaded as controls in Lane 1 and Lane 2.

replaced RdRp in binding to CS-PABP1. Addition of cold RdRp also reduced the amount of labeled RdRp retained on the resin while BSA did not cause a change, indicating the specificity of the competition (Fig. 4-6, Lane 10 and 8 vs. Lane 7). These results suggest that PCI6 and ZYMV RdRp bind to the same region of PABP, or that binding of PCI6 to PABP could partially change the conformation of PABP leding to reduced binding of ZYMV RdRp to PABP.

As expected, cold PCI6 reduced binding of labeled PCI6 to PABP (Lane 5 vs. Lane 3). Surprisingly, however, cold ZYMV RdRp at 1.0 μM did not compete off PCI6, but helped the binding of PCI6 to PABP (Lane 6 vs. Lane 3). If reproducible, the facilitation of the binding between PCI6 and PABP by ZYMV RdRp might result from the conformational change of PABP by RdRp binding. Although ZYMV RdRp might enhance PCI6 binding to PABP, it is not known whether ZYMV RdRp could enhance the ability of PCI6 to inhibit cellular translation.

In summary, there are at least two members of the PABP family in cucumber. While both CS-PABP1 and CS-PABP2 were expressed at similar levels in the tissues tested, including flower, leaf, root and stem, they exhibited differences in binding with PABP-CT interacting proteins. The differences in binding may reflect functional differences between different PABP members. Mutational analysis of ZYMV RdRp and the lack of the host CTC interacting domain in polioviral $3CD^{pro}$ and human PCBP suggest that binding of the host and potyviral interactors to PABP-CT appears to be mediated by different amino acid motifs. The potyviral RdRp binding motif remains to be further elucidated.

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# Conclusions and future work

Like other viruses, potyviruses rely on host factors to complete their replication and life cycles. In chapter 2, I reported that a host protein, cucumber poly- (A) binding protein, CS-PABP1 was identified as a candidate that might be involved in potyviral replication. CS-PABP1 was first isolated based on its ability to interact with ZYMV RdRp by yeast two-hybrid assay. The interaction was confirmed by *in vitro* binding assay. We proposed that the interaction was related to potyviral replication because potyviruses have a positive sense, single-stranded RNA genome that is polyadenylated at the 3' end where the minus strand synthesis starts. Our results showed that ZYMV RdRp, like many other viral RdRps, did not have poly- (A) bind activity. We propose that PABP recruits RdRp or partially assembled replication complexes onto poly- (A) tail of potyviral RNA genome. Other possible outcomes of the RdRp-PABP interaction include a potential shut down of host translation, which was discussed in chapter 2 and needs to be further examined.

Deletion analysis indicated that the PABP-CT was essential for the interaction between PABP and ZYMV RdRp. Our understanding of functions of the PABP-CT is currently evolving. I reported in chapter 3 that PABP-CT interacted with several host proteins, and that it may play a role in translational regulation along with its partners, such as PCI6. The plant interactors, cucumber PCI6, PCI243 and Arabidopsis ERD15 contain a 12-amino-acid motif, which is also present in human PABP-CT interactors. Deletion and point mutations of this motif in PCI6 demonstrated that it was necessary for the interaction. PCI6 inhibited translation of capped reporter RNA with or without poly- (A) tail in the wheat germ translation system. However, mutant PCI6 that was unable to

interact with PABP did not inhibit translation, indicating that ability to inhibit translation depends on interaction with the PABP-CT. PCI6 also inhibited translation in a mammalian system, suggesting that this protein or related proteins are functionally conserved in mammalian systems. PCI6 expression was induced by wounding treatment, not only in wounded leaves but also in upper, unwounded leaves, suggesting that the induction signal moves systemically. The finding that PCI6 regulated translation is consistent with the recently proposed hypothesis that translation can be regulated through PABP by PABP interactors, such as Paip1 and Paip2 and suggests that translational regulation may be a component of stress responses in plants.

In chapter 4, I further examined the interactions between different cucumber PABPs and PCIs and ZYMV RdRp. The C-terminus of CS-PABP2 (CS-PABP2-439), a second cucumber PABP, was amplified and its expression pattern was studied. CS-PABP2 has 86% identity to CS-PABP1 and is constitutively expressed in all tissues similar to CS-PABP1. CS-PABP2-439 did not interact with ZYMV RdRp in yeast, but interacted with PCIs and ERD15. Point mutation analysis of ZYMV RdRp suggested that ZYMV RdRp interacted with CS-PABP1 via a motif different from the 12-amino acid motif present in host PABP-CT interactors.

The above results suggested a possible role of host PABP in potyviral replication. Several working models could be drawn based on this work and other related works. One is that the interactions between host and potyviral proteins could bridge the 5' and 3' ends of potyviral RNA genome and lead to a circular molecule. At the 5' end, host eIF4E interacted with potyviral VPg (Viral protein, genome linked) which is covalently linked to 5' end of RNA genome (Wittmann et al., 1997; Léonard et al., 2000). At the 3' end,

host PABPs bind to poly- (A) tail of viral genome and interact with eIF4G, which is in the same complex with eIF4E (Gingras, 1999). These interactions could result in a circular molecule which could facilitate viral translation at first (Fig. 5-1). Once enough viral proteins are made or at a certain point that is currently unknown, potyviral RdRp or patially assembled replication complex could be recruited onto poly- (A) by interacting with PABP. The recruitment of RdRp or partially assembled replicaton complex could stop translation and start replication. It was also found that TVMV VPg interacted with RdRp and this interaction will allow RdRp to distinguish eukaryotic mRNA and potyviral RNA genome (Hong et al, 1995).

To test this model, the interaction between PABP and RdRp could be disrupted and examine to see if this disruption would inhibit potyviral replication. My first attempt to identify RdRp mutants unable to interact with CS-PABP1 via site-specific mutagenesis failed. To further address this, random mutagenesis should be used to make all possible mutations. The yeast split-hybrid system could be used to identify full-length mutants that fail to interact with CS-PABP1. These mutants will be verified with *in vitro* polymerase activity assay to further identify mutants still having polymerase activity [an assay has been developed and polymerizaton activity of ZYMV RdRp verified in vitro- (Appendix)]. RdRp mutants satisfying the above two criteria could replace wild type RdRp in the full-length ZYMV cDNA clone and to test the effect on infectivity of those ZYMV mutants in its host. If the interaction between ZYMV RdRp and CS-PABP1 is involved in viral replication, disruption of the interaction would affect ZYMV infection.

Another way to test the model is to eliminate expression of CS-PABP1 in transgenic cucumber plants. If CS-PABP1 is necessary for ZYMV replication, depletion

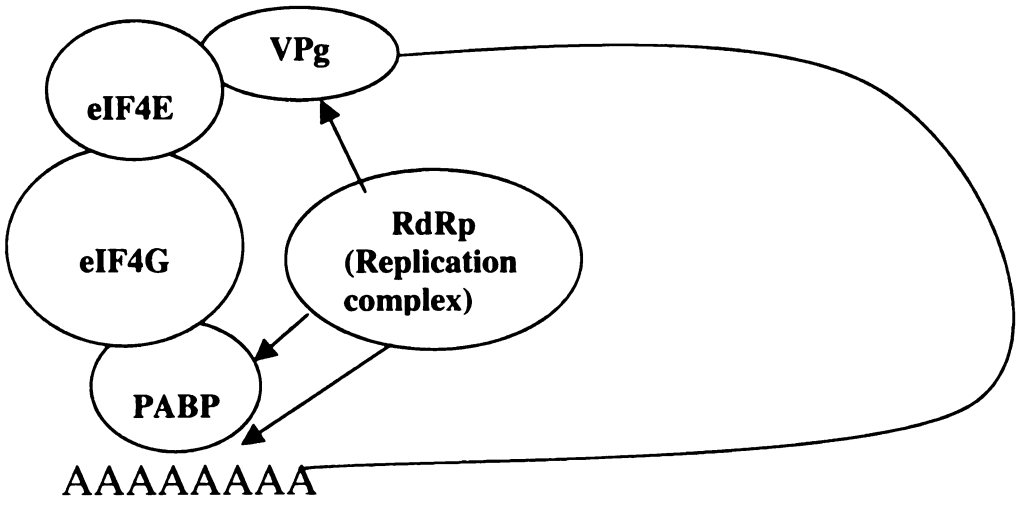


**Fig. 5-1. Proposed model for potyviral replication.** Host initiation factor eIF4E binds to viral protein VPg which is covalently attached to the 5' end of potyviral RNA genome. Host PABP binds to poly- (A) tail of viral RNA. Both PABP and eIF4E bind to eIF4G and these protein-protein interactions bring 5' and 3' ends of potyviral RNA close as a circular molecule. Potyviral RdRp or partially assembled replication complex will be recruited onto 3' end poly- (A) and start synthesis of minus strand. Replication complex will move from 3' end poly- (A) tail directly to 5'NTR after one round of synthesis and re-start next round of synthesis of minus strand. RdRp interacts with VPg and other proteins in replication complex will recognize 3' NTR of potyviral RNA, and these interactions will distinguish potyviral RNA and host mRNAs.

of CS-PABP1 would stop viral infection. Our results indicated that both CS-PABP1 and CS-PABP2 are constitutively expressed in all tissues. Although both share high homology at the coding sequence region (86% identity), the 3' NTRs are quite different with only 28% identity. The 3' NTR sequence of CS-PABP1 could be used to silence CS-PABP1 but not CS-PABP2 in transgenic plants. If CS-PABP1 is essential, no such transgenic will be recovered, otherwise, we could infect these transgenic plants with ZYMV to determine whether the lack of CS-PABP1 protein could inhibit ZYMV infection. We could also study the phenotype that is caused by lack of CS-PABP1 protein and functions.

The second model is that the interaction will inhibit host translation, since RdRp interacted with PABP-CT and other host PABP-CT interactors, such as PCI6 which inhibits translation. RdRp could inhibit host translation directly, or indirectly by facilitating PCI6 binding to PABP-CT and enhance its ability to inhibit host translation. To test this model, we could either add ZYMV RdRp protein directly in wheat germ translation system to see if it is able to inhibit translation. If RdRp inhibits translation in vitro, we could overexpress ZYMV RdRp in cucumber transgenic plants to further examine the effect in planta. Since ZYMV RdRp enhanced PCI6 binding to PABP, we could test if RdRp could facilitate PCI6 to inhibit host translation. This could be tested *in vitro* by adding increasing amount of RdRp and fixed amount of PCI6 to see if translation can be further inhibited by addition of RdRp.

This work also showed that CS-PABP is involved in cellular translational regulation via the interaction with PCI6. Since PCI243 and ERD15 interacted with PABP-CT and share the 12-amino-acid PABP-CT binding motif, we need to test whether

PCI243 and ERD15 could affect translation *in vitro*. If ERD15 inhibits translation *in vitro*, we could overexpress ERD15 in Arabidopsis transgenic plants and examine if over production of ERD15 constitutively could affect plant growth and development. The protein expressions in transgenic and wild type plants could be compared using two-dimensional gel assays.

# Appendix

## *In vitro* RdRp polymerase activity assay

### Method:

The polymerase assay was done following Hong and Hunt (1996) with a few modifications. His-tagged RdRp was overexpressed and purified from *E.coli* (Wang et al, 2000); approximately 1 μg protein was used in the assay. A typical 30 μl reaction contained 25 mM Tris-HCl (pH 8.0), 40 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM DTT, 0.05 mM EDTA, 1 μg poly- (A) (Sigma), 0.15 μg oligo (dT), and 0.5 mM Digoxigenin (Dig)-labeled UTP mix (65% UTP, 35% Digoxigenin-11-uridine-5'-triphosphate, Roche). The reaction was incubated at 30 °C for 90 minutes; extracted once with phenol-chloroform and precipitated with 2 volumes of ethanol. The pellet was resuspended in RNase-free water and dropped onto Nylon membrane (Amersham) and UV cross-linked. Digoxigenin was detected following the manufacturer's protocol (Roche).

### Result:

Point mutations in RdRp may not only lead to the loss of the ability to interact with PABP1, but also the loss of the polymerase activity or the overall conformation of the protein. To select RdRp mutants that lose the ability to interact with PABP1 but still maintain the polymerase activity, an *in vitro* RdRp polymerase activity assay was developed following Hong and Hunt (1996), which was used to measure the polymease activity of tobacco vein mottling potyvirus (TVMV).

With purified ZYMV RdRp from *E.coli*, poly- (A) as template and oligo- (dT) as primer, Dig-UTP was incorporated into oligo- (dT) fragments (Fig. 6, spot 6) as was

detected by Dig antibody. However, without template poly- (A) (spot 1), or Dig-UTP ( spot 3), or RdRp (spot 4), no incorporation of Dig-UTP was detected. This suggested that the system used for assaying TVMV RdRp can be used to measure the activity of ZYMV RdRp. One difference between RdRps of TVMV and ZYMV was the necessity of primer oligo- (dT). As indicated in Fig. 6 (spot 2), Dig was detectable without primer, though in a much lower intensity compared to that with primer. This may suggest that ZYMV RdRp is different from TVMV RdRp in that no primer is necessary. Another possibility is that TdT (Terminal transferase) from *E. coli* was contaminated in the RdRp during protein purification. This can be verified in the future by including a RdRp mutant GGC that lost 90% of its polymerase activity (Hong and Hunt, 1996).

| | | | | | | |
|---|---|---|---|---|---|---|
| poly- (A) | - | + | + | + | + | + |
| oligo dT | + | - | + | + | + | + |
| Dig UTP | + | + | - | + | + | + |
| His-RdRp | + | + | + | - | - | + |
| BSA | - | - | - | - | + | - |

1 2 3 4 5 6

Fig. 6-1 ***In vitro*** **enzymatic activity assay for ZYMV RdRp.** The activity assay was conducted using purified ZYMV RdRp, poly- (A) as template, oligo-(dT) as primer, and Dig-UTP as label. The reaction was progressed at 30 C for 90 minutes and stopped by phenol-chloroform extraction. The incorporation of Dig-UTP was detected following the protocol of manufacturer (Roche). 1. Without template poly- (A). 2. Without primer oligo- (dT). 3. Without label Dig-UTP. 4. Without RdRp. 5. Replace RdRp with BSA in the reaction. 6. With all the components.