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The Interaction Between Eukaryotic Translation Initiation Factor eIF4G and 3’ Cap Independent Translation Element of Barley Yellow Dwarf Virus Is Affected by Multiple Initiation Factors

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THE INTERACTION BETWEEN EUKARYOTIC TRANSLATION INITIATION FACTOR EIF4G AND 3’ CAP INDEPENDENT TRANSLATION ELEMENT OF BARLEY YELLOW DWARF VIRUS IS AFFECTED BY MULTIPLE INITIATION FACTORS

by

PEI ZHAO

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York 2016
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ABSTRACT

THE INTERACTION BETWEEN EUKARYOTIC TRANSLATION INITIATION FACTOR EIF4G AND 3’ CAP INDEPENDENT TRANSLATION ELEMENT OF BARLEY YELLOW DWARF VIRUS IS AFFECTED BY MULTIPLE INITIATION FACTORS

by

Pei Zhao

Adviser: Dixie J. Goss

Barley Yellow Dwarf Virus (BYDV) lacks a 5’ (7-methyl guanosine) cap as well as a 3’ poly A tail. Like many plant viruses, BYDV contains a cap independent translation element (CITE) in the 3’ untranslated region of the viral mRNA. BTE (Barley Yellow Dwarf Virus like cap-independent translation element) is one of the well characterized CITEs. BTE mediated translation primarily depends on eukaryotic initiation factor eIF4G. BTE binds to eIF4G; however, the details of BTE initiated translation are still unclear. Three eIF4G deletion mutants with different domain organization were used to investigate BTE interaction with eIF4G: eIF4G601-1196 is the eIF4G fragment containing amino acid residues from 601 to 1196, including binding domains for eIF4E, central eIF4A, eIF4B and the possible BTE binding region; eIF4G601-1488 is a longer fragment with one additional C-terminal eIF4A binding domain; eIF4G742-1196 is a shorter deletion mutant lacking the eIF4E binding sequence. eIF4G601-1196 binds BTE as efficiently as wild type eIF4G and supports translation. Translation initiation factor eIF4A and eIF4B with ATP (helicase complex) stimulate eIF4G601-1196 binding with BTE but not eIF4G601-1488, suggesting that the helicase complex function relies on the eIF4G central eIF4A binding domain, not the C-terminal eIF4A binding domain. This suggests that, similar to human eIF4G, the wheat eIF4A binding site may serve a regulatory role. eIF4E, upon binding with eIF4G mutants which have the eIF4E binding region,
significantly increases the binding to BTE. This indicates that the smaller eIF4G mutant has a more flexible structure that can be positively influenced by eIF4E.
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CHAPTER 1. INTRODUCTION

1.1 OVERVIEW OF TRANSLATION

RNA translation is the post-transcriptional process that synthesizes protein peptides by ribosomes decoding messenger RNA (mRNA). It is involved in numerous biological processes, including gene expression, cell growth, cell development, cell division and stress responses. Eukaryotic translation is a cyclic process composed of three steps: initiation, elongation and termination, each requiring specific translation factors (1) (Figure 1).

Initiation begins with cellular messenger RNA (mRNA) binding to translation initiation factors eIF4F (multisubunit in the mammalian system, containing eIF4G, eIF4E and eIF4A; but only eIF4G and eIF4E in plants), poly (A) binding protein (PAPB) and eIF4E kinase MNK. At the same time, the 40s ribosome with initiation factor 1(eIF1), eIF1A, eIF3, eIF5, eIF2 and initiator-methionyl-tRNA (Met-tRNAi) together form as 43S pre-initiation complex. Subsequently, the 43S complex is loaded onto the 5′ end of a capped mRNA. In this way, eIF4F directed complex and 43S pre-initiation complex assemble as 48S pre-imitation complex. Then the ribosomal subunit scans the mRNA to locate the AUG start codon. After AUG recognition, facilitated by eIF3, eIF1, and eIF1A, the 60S subunit joins in and with the 40S subunit together becomes as the 80S ribosome (Figure 1).

In the beginning of elongation, the first tRNA (Met-tRNAi) is already docked at the peptidyl site (or P site) of the 80S ribosome. With the assistance of eEF1A•GTP, the second tRNA carrying an amino acid is delivered to the ribosome aminoacyl site (or A site). Ribosomes catalyze the first peptide bond formation. Eukaryotic elongation factor 2 (eEF2) catalyses 80S translocation in which the deacetylated tRNA is transferred to the exit site (E site), positioning the peptidyl-
tRNA in the P site and re-emptying the A site. The polypeptide chain keeps growing until the ribosome reaches the stop codon (Figure 1).

For termination, eukaryotic release factor 1 (eRF1) recognizes the stop codon at the A site, triggering 80S ribosome arrest and polypeptide release. Subsequently, eRF1 is released by eRF3. The post-termination ribosome is dissociated into the 60S and the 40S ribosomal subunits. At the same time, mRNA and tRNAs are released from the ribosome (1). Translation factors and ribosomal subunits are recycled for the next round of protein synthesis (2).
Figure 1. Three steps of translation (Source: Walsh E and Mohr I, 2011)(3). The process of translation includes three phases: initiation, elongation and termination. Each stage requires specific translation factors. A. Initiation. A set of initiation factors assists 40S ribosomal small
subunit to load on mRNA. The assembled 48S complex scans the mRNA until the AUG start
codon is reached. After AUG recognition, 60S subunit joining triggers initiation factor release.

B. Elongation. Each charged tRNA is delivered to the 80S ribosome A site. Ribosome catalyses
peptide bond formation. Eukaryotic elongation factor 2 (eEF2) catalyses 80S translocation. It
also transfers the deacetylated tRNA to the E site, positioning the peptidyl-tRNA in the P site
and re-exposing the A site. C. Termination. Eukaryotic release factor 1 (eRF1) recognizes the
stop codon in the A site and triggers 80S arrest with polypeptide release. eRF3 releases eRF1 and
several initiation factors from the ribosome, dismantling the complex. Thus ribosome subunits
are recycled.

1.2 EUKARYOTIC CANONICAL TRANSLATION INITIATION MECHANISMS—THE
CAP-DEPENDENT TRANSLATION INITIATION

Recruitment of translation factors and ribosomes is crucial for initiation (4), which serves as the
rate-limiting step for the whole translation process. Most eukaryotic mRNAs have a cap
(m⁷GpppX) structure in the 5′ untranslated region (5′UTR) and polyadenylated sequence (poly
A tail) in the 3′ untranslated region (3′UTR) (5, 6). Canonical eukaryotic cellular mRNA
translation initiation starts with the 5′ cap of mRNA recognition by cap-binding protein eIF4E
(Figure 2), one subunit of eIF4F. Another subunit of eIF4F, eIF4G, can increase eIF4E binding
to mRNA (7). eIF4G also acts as a scaffold protein, bound with other initiation factors such as
eIF4A, eIF4B and poly (A) binding protein (PABP) (8, 9). The poly A tail is recognized by
PAPB (10, 11), which associates with eIF4G bound to the 5′ end of mRNA. The eIF4G directed
initiation factor complex circularizes the cellular mRNA linking 5′ and 3′ ends (12). This
“closed-loop” conformation of cellular mRNA is assumed as the efficient translation initiation model for canonical cap-dependent translation (13, 14) (Figure 2).

eIF4G/eIF4F directed cap-recognized complex, mRNA and 43 pre-initiation complex, which contains 40S ribosomal subunits, eIF3, eIF2, eIF1A and Met-tRNAi, form as the 48S pre-initiation complex. This complex scans mRNA 5’UTR to the start codon, where the 60S ribosomal subunit joins and assembles as the 80S ribosome. The binding of 60S ribosomal subunit to 40S subunit triggers the release of initiation factors such as eIF2, eIF3 and eIF1A.

Each translation initiation factor (eIF) has its specific function during initiation. For example eIF4A, eIF4B and eIF4F with ATP together possess a helicase function, which assists 40S ribosomal subunit binding to mRNA. When scanning, the movement of the 40S ribosomal subunit on mRNA can be hindered by the secondary structures in the 5’ UTR. eIF4A directed ATP-dependent hydrolysis helicase complex unwinds the secondary structures of 5’ UTR and facilitates the ribosomal subunit movement along the mRNA (15, 16). Translation initiation of most cellular mRNA employs a cap-dependent mechanism. Both eIF4F assembly and eIF4E binding to mRNA are rate-limiting steps for initiation, which are regulated by several signal pathways to control the translation on-off (17).
Figure 2. Eukaryotic translation initiation (Source: Klann E and Dever TE, 2004)(18) A complex of eukaryotic translation initiation factor 2 (eIF2), GTP bounded methionyl-transfer RNA (Met–tRNAiMet), the 40S ribosomal subunit and additional factors, such as eIF3 and
eIF1A (1A) form as a 43S pre-initiation complex. In the cap-binding complex, eIF4E (4E), eIF4G and eIF4A (4A), bind to the 7-methyl-GTP (m7GTP) cap structure at the 5’ end of a messenger RNA (mRNA). At the same time, eIF4G also binds to the poly(A)-binding protein (PABP), thereby bridging the 5’ and 3’ ends of the mRNA. The cap-binding complex and the 43S pre-initiation complex associate as a 48S pre-initiation complex, in which ribosomal subunits scan the 5’UTR of mRNA till the AUG start codon. GTP is hydrolysed by eIF2, which triggers the dissociation of factors from the 48S complex. The 60S ribosomal subunit joins in with the 40S ribosomal subunit forming as the 80S ribosome.

1.3 THE CAP-INDEPENDENT TRANSLATION INITIATION

Although the canonical cap-dependent mechanism accounts for most eukaryotic mRNA translation, many viral mRNAs and some cellular mRNAs take a non-canonical cap-independent pathway for efficient translation (19). Many virus RNAs lack the cap structure in the 5’ UTR or poly A tail in the 3’UTR, but they possess special translation regulating elements, located in their mRNA untranslated regions to mediate translation. Viral RNAs fully rely on the host cell translation machinery to generate the viral protein polypeptides, which are essential for viral replication. After infection, viruses not only recruit host cell translation factors and ribosomes to viral mRNA, but also seize the control of cellular translation apparatus to impair the host cell gene expression (3, 20, 21).

Viruses evolve different ways to utilize host plant initiation factors for the synthesis of viral proteins (22-25). They subvert host cellular mRNA translation by manipulating host initiation
factors and/or ribosome in the initiation step. There are two major translation mechanisms for uncapped viral mRNA. One is internal ribosome entry site (IRES) directed translation; the other is a 3’ cap-independent translation element (CITE) mediated mechanism.

1.3.1 IRES translation

It has been reported that at least 39 viral RNAs adopt IRES translation (26), such as hepatitis A virus (HAV) (27), hepatitis C virus (HCV) (24), foot-and-mouth-disease virus (FMDV) (28), and human immunodeficiency virus (HIV) (29). Most IRES viral RNAs do not harbor the 5’cap (m7GpppX) structure, but contain a long and highly structured sequence in the 5’UTR, including many AUG codons upstream of the start codon of the main open reading frame (ORF). During IRES viral RNA translation initiation, the ribosome skips scanning the 5’UTR and directly binds at the internal ribosomal entry site (IRES), which is just upstream of the start codon(25). Although IRES mediated cap-independent translation does not require the 5’cap–eIF4E recognition, many of them still need the host cellular initiation factors. For example, viruses like Hepatitis C Virus (HCV) (24), Classical Swine Fever Virus (CSFV)(30) and Porcine Teschovirus1 (PTV-1)(31) bind to the 40 ribosomal subunit and also require the host cell initiation factors and methionyl-tRNAi for viral mRNA translation initiation.

1.3.2 CITE translation

Different from IRES viruses, many plant viruses employ the 3’ cap-independent translation enhancer element (CITE) to direct viral mRNA translation(32). 3’CITEs are usually located within the 3’UTR of viral genomes. The 3’CITEs from different plant viruses show no apparent similarity in sequence or structure to each other (32). Based on their distinctive secondary structures, 3’CITE viruses can be divided into at least six major classes (33).
The first 3’CITE was discovered in *Satellite Tobacco Necrosis Virus* (STNV) with a translation enhancer domain (TED) (34) as its translation regulated element. The STNV TED consists of a 93 nt long sequence and folds as an extended stem-loop structure.

The second class of 3’CITE is the BYDV-like translation element (BTE)(35), which exists in *luteoviruses* (36), two genera of *Tombusviridae: Necrovirus* (37) and *Dianthovirus* (38), as well as *umbraviruses* (39). All of these BTEs contain a highly conserved 17nt long sequence in their stem loop I. The *luteovirus barley yellow dwarf virus* (BYDV) BTE has a cloverleaf shape secondary structure.

The 3’CITE of the *Panicovirus, Panicum mosaic virus* (PMV) is the PMV-like translation element (PTE)(40). PTEs have been identified in some *carmoviruses*, including *Saguaro cactus virus* (SCV)(41), and in some *aureusviruses*, such as *Cucumber leaf spot virus* and *Pothos latent virus*. PTE has a T-shaped secondary structure with a G-rich sequence bulge in the main stem and a C-rich sequence at the three-helix junction (42).

Another type of 3’CITE, which also has a T-shape structure (TSS) was found in the *carmovirus Turnip crinkle virus* (TCV) (43). This TSS 3’CITE is a 140 nt long sequence, mediated by two pseudoknots, which resembles a tRNA.

The Y-shape 3’CITE was discovered in *Tomato bushy stunt virus* (TBSV) (44, 45) and *CarnationItalian ringspot virus* (CIRV) (46), composed of three major helices.

*Maize necrotic streak virus* (MNeSV) and *Cucumber Bulgarian virus* (CBV) contain I-shaped 3’CITEs (47, 48). The I-shape class of 3’CITE has also been identified in *aureusviruses*, and *carmovirus Melon necrotic spot virus* (MNSV) (49, 50). Among all the 3’CITEs, the I-shaped is the smallest, with a 64nt functional sequence containing a consensus internal loop motif (48).
Despite the differences in sequences and secondary structures, 3’CITEs still share some general mechanisms in translation initiation. First, 3’CITEs recruit initiation factors via 3’CITE binding; second, communication between the 3’CITE and the viral 5’UTR helps the delivery of the translation machinery from the 3’ end of viral mRNA to 5’ upstream of mRNA ORF.

1.4 BTE MEDIATED CAP-INDEPENDENT TRANSLATION

1.4.1 BTE is the cap-independent translation element for BYDV

The barley yellow dwarf virus (BYDV)-like cap-independent translation element (BTE) is one of the well characterized CITEs. The genome of BYDV is an uncapped, nonpolyadenylated positive-sense RNA with 5677 nucleotides. BYDV BTE (105nt) spans at 3’ end of BYDV genome nucleotides 4814-4918 (19, 51)(Figure 3).

BTE (105nt) serves as the minimal in vitro functional translation regulated sequence of BYDV. For stimulating in vivo translation, an additional 869 nt sequence in the 3’UTR of BYDV mRNA is necessary for full translation activity (51, 52). BTE mediated translation eliminates the need for an efficient 5’ cap dependent translation. BTE mediated in vitro translation in wheat germ extract (WGE) can be replaced by a 5’cap, but cannot be replaced by a poly (A) tail. In addition, BTE stabilizes mRNA to the same extent as a 5’cap in vivo. 3’BTE also functions when located at the 5’end of mRNA. All of these suggest BTE is an equivalent translation regulated element as the 5’ cap (51). Adding free m\(^7\)G RNAs (Wang and Miller, 1995) or free BTE in trans inhibited BTE activity. The inhibition by m\(^7\)G and free BTE in trans can be relieved by exogenous eIF4F(52).
Figure 3. BYDV genome organization and the secondary structure of 3’BTE and 5’BCL

(Source: Rakotondrafara et al, 2006) Boxes represent 6 ORF of BYDV mRNA. BYDV 5’UTR genomic RNA has the stem loops: A, B, C and D. BTE is located in the 3’UTR of mRNA, between ORF5 and ORF6 (19).

1.4.2 The secondary structure of BTE and the long-distance RNA-RNA kissing loop of BTE

tertiary structure

BTE forms a cruciform secondary structure with 3 major stem-loops (SL-I, SL-II, SL-III) and flanked by stem IV (Figure 3)(51, 53). Five nucleotides (UGUAC) in the stem loop III of BTE base pair to the nucleotides (UGACA) in BYDV 5’ UTR stem loop D. SL-III and SL-D form a long distance RNA-RNA “kissing” loop. This 3’-5’ communicating kissing loop facilitates the delivery of the translation machinery, which is recruited by 3’BTE, to the 5’ UTR of viral mRNA (45, 51). A single point mutation within the five bases of the stem loop III that disrupted base–pairing of this kissing loop destroyed translation both in cells and in wheat germ extract; while compensatory double mutations that restored base-pairing rescue translation (51).
Figure 4. The predicted secondary structure of 3’BTE and its mutants 3’BTE structure is predicated by SHAPE analysis and mfold calculation. The highlighted nucleotides are the positions where eIF4G binds to BTE. Red characters represent the nucleotides which are in the stem loop III and 5’UTR stem loop D, base pared complementary to each other. BTEBF is the mutant, which has 4 nucleotides (GAUC) inserted in the stem loop I. SL-II-M1 mutant has 2 nucleotides changing from UC to AA in stem loop II. SL-II-R mutant has double mutation but still maintains the stem structure. SL-III-3 nucleotides changed in the kissing loop which are responsible for base paring with long-distance stem loop D in BYDV 5’UTR. SL-III-SWAP is the mutant whose entire wild type stem loop III is replaced by stem loop II of PAV-129 strain (51).
1.4.3 Each of the BTE stem loops is required in translation

BTE mediated translation depends on BTE recruiting initiation factors eIF4G/eIF4F and the 40S ribosomal subunit (19, 51). Each of the BTE structured stem loops is necessary in translation. Deletion of any stem loop abolished BTE translation activity.

The 17-nt sequence (GGAUCCUGGGAAACAGG) composed BTE stem loop I (SL-I), is highly conserved among BTEs from different viruses(54). This conserved sequence is essential for translation. BTEBF is one of the BTE mutants with 4 bases, GAUC, inserted into the SL-I 17nt conserved sequence. Translation was abolished by this insertion (51). In addition, toe-print experiments revealed SL-I is where the 40S ribosomal subunit bound to BTE (55). The sequence GAUCCU4838-4843 in the SL-I 17-nt conserved region can potentially base pair to the AGGAUC sequence, which is the anti-Shine–Dalgarno sequence in prokaryotic ribosome, located in the 3’ end of 18S rRNA (55).

Maintaining the structure of stem loop II (SL-II) is required for BTE mediated translation. The single mutation SL-II-m1, which disrupted the stem structure of SL-II (Figure 4), lost translation ability. The double mutation SL-II-R (Figure 4) which restored the stem structure, regained the full translational function of the BTE. Thus the secondary structure, but not the primary sequence of SL-II is required for translation (51).

Like SL-II, stem loop III (SL-III) tolerates sequence changes as long as the secondary structure is maintained. Replacement of BYDV wild type PAV126 strain SL-III by PAV 129 SL-III, which has a larger stem loop III with 11-nt insertions, reduced translation to 50% of wild type (Figure 4). Mutation SL-III-mL3, which has the sequence AGCGACC substituted for SL-III sequence CUGUCA4883-4910, disrupted base paring between SL-III and 5’end SL-D. This mutant
knocked out the cap-independent translation when it was placed in the 3’UTR but not in the 5’UTR (51). It suggests stem Loop III has little or no role in actual recruitment of the translation apparatus, but is involved primarily in 3’-5’ communication.

1.4.4 BTE mediated BYDV translation via interacting with eIF4G.
Previous studies showed that 3’BTE interacts specifically with eIF4G or eIF4F in a wheat germ extract (56, 57). BTE mediated translation is primarily eIF4G dependent (52). When eIF4F was depleted from wheat germ extract, there was a low translation for BTE. The reduced translation can be rescued by eIF4F or recombinant eIF4F. eIF4G alone also can rescue BTE directed translation as efficiently as eIF4F. However eIF4E had no ability to restore the translation (52). Similarly, when wheat germ extract was inhibited by BTE in trans, eIF4G alone restored translation nearly as much as eIF4F did; while eIF4E alone failed to rescue translation. Both filter binding assay and fluorescence anisotropy measurements showed BTE bound to eIF4G and eIF4F with high affinities, but very weekly to eIF4E (52, 56, 57).

1.4.5 BTE binds with eIF4G at stem loop I.
SHAPE experiments (55, 56) showed in the presence of eIF4G, the protein protected region of BTE was around the conserved SL-I and an internal bulge downstream of the SL-III. The diverse BTEs from luteoviruses fold in a similar way to expose their SL-I surface for eIF4G recognition (56).

1.5 EUKARYOTIC INITIATION FACTORS

1.5.1 The domain organization of eIF4G
The eukaryotic translation initiation factor eIF4G serves as a scaffold protein which recruits several initiation factors: eIF4E, eIF4A, eIF4B, eIF3 and the poly (A) binding protein (PAPB). eIF4G is a 165kd protein with 1489 amino acid residues (58), whose domain organization has been investigated (Figure.5)(59). The N-terminal of eIF4G has eIF4B and PAPB binding sites. eIF4B and PAPB competitively bind with the N-terminal eIF4G1-203, as well as the central part of eIF4G from amino acid 1100 to 1196 (59). Two HEAT domains which are located in the middle and C-terminal region of eIF4G, overlap with eIF4A binding regions and span from amino acid residues 883 to 1196 and from 1300 to 1489. eIF4E only needs a short region of eIF4G between amino acids 710 and 721 for binding (59).

1.5.2 The middle domains of eIF4G are the possible BTE binding region.

Plants express two eIF4G isoforms: eIF4G and isoelIF4G (60), which are highly different in sequence and size. Plant viral RNAs preferentially use eIF4G or isoelIF4G for their translation. For BYDV, eIFiso4F is less efficient at facilitating translation. BTE directed translation primarily depends on eIF4G. Miller’s group found that (56)one truncated protein of eIF4G--p86 (including amino acid residues from 766 to 1488) was able to bind to BTE; while another truncated mutant p70 (amino acid residues 863-1488) lost the ability of binding to BTE. The region between eIF4G amino acid residues 766 and 883 is important for BTE binding.

1.5.3 eIF4E has minor effect on BTE mediated translation.

eIF4E, the cap binding protein, binds to most eukaryotic mRNA 5’ cap structure of m7GpppN. eIF4E has been shown to play multiple roles during viral infection (16). In IRES virus – Rhinovirus translation, eIF4E increases Rhinovirus 2A protease cleavage of eIF4G. After cleavage, the N-terminal region of eIF4G, which contains the eIF4E binding site, is separated from the middle and C-terminal two-thirds. Thus it disrupts eIF4F assembly and impairs host
cellular eIF4F dependent mRNA translation; while *Picornaviruses* mRNA is able to use the C-terminal two-thirds eIF4G to start viral mRNA translation (61).

For BYDV, it is possible that BTE mediated translation is a cooperative result from multiple initiation factors. Wheat eIF4E has been shown to have a minor effect on BTE mediated translation (52). When eIF4E with eIF4G assembles as eIF4F, it exhibits 20–30% higher activity than eIF4G in promoting BTE translation *in vitro* (52). SHAPE experiments also showed adding eIF4E enhanced the protection of eIF4G-BTE binding (56). All of these data indicate that eIF4E may play a role in BTE-mediated translation. However the details of how eIF4E affects eIF4G interaction with BTE are still unknown.

**1.5.4 eIF4F-eIF4A -eIF4B-ATP, the helicase complex, increases BTE binding to the 40S ribosomal subunit.**

Wheat eIF4A, a 45 KD protein, exhibits ATPase hydrolysis activity and RNA helicase activity when together with eIF4B and ATP (15, 28, 62-64). Mammalian eIF4A is a part of eIF4F, but in plants eIF4A exists as an individual protein. eIF4A is the most abundant cellular initiation factor in wheat germ. It shares 9 highly conserved regions with DEAD box family and participates in multiple cellular processes, such as translation, RNA degradation, RNA splicing, and ribosome biogenesis (65). eIF4A undergoes a cycle of conformational changes during ligand binding and is used by DEAD box proteins to transduce the energy derived from ATP hydrolysis into physical work (66, 67). How these changes result in RNA unwinding is not clear yet.

Wheat eIF4B, a 59 KD protein, is necessary for accelerating eIF4A-ATP dependent helicase activity (67-69). eIF4B also interacts with translation initiation factor eIF3, which along with eIF4G is responsible for recruiting the 43S pre-initiation complex to mRNA (70, 71).
Our previous study showed that eIF4A or eIF4B alone did not increase eIF4F binding with BTE (57), however eIF4A-eIF4B-ATP helicase complex increases eIF4F and BTE binding to the 40S ribosomal small subunit (55). The increased binding is probably caused by the helicase complex unwinding RNA secondary structure to make it more accessible for ribosome recognition. The mechanism of action of eIF4A in plant viruses is still poorly understood. Whether eIF4A and eIF4B can further affect the translation efficiency of BTE is also unknown.

1.6 Hypothesis and Specific Aims

The current model for BTE initiating translation is that BTE recruits eIF4G and the 40S ribosomal subunit at the 3’UTR of viral mRNA. Meanwhile eIF4G recruits other initiation factors such as eIF4E, eIF4A, eIF4B and eIF3. BTE utilizes its long distance 3’-5’ kissing loop to deliver the initiation machinery to 5’ UTR of mRNA, where the translation pre-initiation complex forms. However, the details of BTE binding to eIF4G and how other initiation factors affect BTE binding and translation are still unclear. Understanding the mechanism of BYDV utilizing initiation factors provides an opportunity to target virus without damaging the host plants. To provide a clear picture of the mechanism of BTE protein synthesis initiation, two hypotheses are proposed in this study.

Hypothesis 1

*BTE only needs the middle region of eIF4G for binding and initiating translation. The C-terminal eIF4G is not required for BTE interaction.*

To test this hypothesis, we investigated the specific aims as below:
(I) Identification of the core domain of eIF4G required for binding to BTE

Here we aimed to identify the “core domains” of wheat eIF4G for binding to BTE. We performed biochemical and biophysical assays to profile the binding affinities of three eIF4G deletion mutants (eIF4G601-1196, eIF4G601-742-1196 and eIF4G601-1488) to BTE. eIF4G601-1196 is the fragment with eIF4G amino acid residues from 601 to 1196 (59), which has the BTE possible binding site, eIF4E binding site, one binding site for eIF4A and for eIF4B. eIF4G601-1488 is a longer fragment with one extra eIF4A binding site in the C-terminal sequence. The shortest fragment is eIF4G742-1196, which lacks the eIF4E binding site (Figure 5). (N-terminal wheat eIF4G is extremely unstable and the C-terminal eIF4G is as functional as full-length protein. Different research groups (56, 59) in previous studies used the C-terminal two-thirds of eIF4G as full-length eIF4G.)

(II) Examining the abilities of eIF4G deletion mutants to rescue of BTE-mediated translation

We investigated the translational activities of three eIF4G deletion mutants in rescue of BTE-mediated translation in 4F-depleted wheat germ extract. We aimed to find out whether the three eIF4G mutants have biological activities and whether their binding affinities are correlated to their translation abilities.
Figure 5. Wheat eIF4G deletion mutants Protein factor binding sites are highlighted by light grey shaded boxes; the possible BTE interacting region is represented by dark grey box.

Hypothesis 2

*BTE not only uses eIF4G to direct viral RNA translation, but also utilizes different eIF4G domains to coordinate with eIF4A, eIF4B and eIF4E to maximize viral protein synthesis.*

To test this hypothesis, studies were conducted according to the specific aims below:

(III) Investigate the effects of helicase complex (eIF4A-eIF4B-ATP) on binding of eIF4G deletion mutants to BTE
The helicase complex is able to increase the binding between BTE and the 40S ribosomal subunit, but whether it influences eIF4G binding with BTE is still unclear. Here we examined the binding affinities between eIF4G deletion mutants and BTE in the presence of helicase complex (eIF4A-eIF4B-ATP).

(IV) Investigate the effects of translation Initiation factors eIF4A and eIF4B on BTE mediated translation

To determine whether BTE mediated translation is only eIF4G dependent, or affected by other translation initiation factors, the rescue ability of eIF4G mutants in BTE mediated translation were measured when supplemented with eIF4A and eIF4B.

(V) Determine the function of eIF4E, the cap-binding protein, in binding of eIF4G deletion mutants to BTE

In order to determine whether eIF4E, the cap-binding protein, has function in BTE cap-independent translation, we measured the binding affinities of BTE with eIF4G deletion mutants and eIF4E. In addition, a thermodynamic study was used to examine whether the binding between eIF4E, eIF4G601-1196 and BTE is entropically and/or enthalpically favorable.

(VI) Investigate eIF4E function in eIF4G rescue of BTE mediated translation

eIF4E was also supplied with eIF4G mutants together in translation assays. We aimed to determine whether eIF4E can stimulate eIF4G mutants’ rescue of BTE mediated translation or not.
CHAPTER 2. EXPERIMENTAL PROCEDURES

2.1 PLASMID CONSTRUCTION

2.1.1 Constructs for expressing eIF4G deletion mutants

eIF4G601-1196 recombinant protein expression construct was a generous gift from Dr. D.R. Gallie (University of California, Riverside). The construct harbored the eIF4G cDNA sequence for coding amino acid residues 601 to 1196 in expression vector pGex-2TK. GST tag was fused to the N-terminal of eIF4G601-1196 (59). Plasmid PGEX-2TK was also used for expression of eIF4G601-1488 and eIF4G742-1196. The DNA for coding protein fragments were generated by PCR from eIF4G full length cDNA template (a generous gift from Dr. Karen Browning, University of Texas, Austin). The forward primer included a BamHI site in the 5’ end, followed by eIF4G ORF (forward primer for eIF4G601-1488: TTAAGGGATCCAGAAGAAACGGAAGG; forward Primer for eIF4G742-1196: GAAGGATCCTTTCAAAGATTTGGCAGG). The reverse primer contained another BamHI site at the 5’ end, followed by a stop codon and eIF4G ORF (reverse Primer for eIF4G601-1488: GCTGGATCCCTATTAAAGTCAACATG AAG; reverse primer for eIF4G742-1196: CCTGGATCCTCAAAGGGGAACAGTTC). PCR products were digested with BamHI and inserted into plasmid PGEX-2TK which also had been digested with the same enzyme. Clones with the correct eIF4G ORF direction were selected by sequencing (Genewiz) and then were chosen for expressing GST-eIF4G-fusion-protein expression.

2.1.2 Site directed mutagenesis for generating Bluc-SLII-m1 reporter construct

BlucB is a reporter construct which contains the BYDV 5’UTR upstream of a luciferase reporter gene and the 3’BTE flanks the 3’ downstream of the firefly luciferase gene (a generous gift from
Dr. W. Allen Miller, Iowa State University, Ames). The reporter construct Bluc-SLII-m1 was generated from the BlucB template using a site mutagenesis kit (NEB). The mutagenesis Primers TTCG GAAC ATA AGCTCGGGTAGGCTG (forward primer) and GTTCTGCCT GTTTCCCAGGATCCG (reverse primer) were used to amplify the construct. The PCR products containing the mutated nucleotides were ligated back and treated with the restriction enzyme DpnI to remove the BlucB template. After transformation, clones with the desired mutations were selected and confirmed via sequencing (Genewiz).

2.2 PROTEIN EXPRESSION AND PURIFICATION

2.2.1 Protein expression and purification for eIF4G deletion mutants

All the GST-eIF4G deletion mutant constructs were harbored in PGEX-2TK plasmids and expressed in BL21 (DE3) E. coli cells (NEB). Single colonies of cloned cells were grown overnight at 37°C in LB medium, then transferred to fresh LB medium at a ratio of 1:100, cultured another 3 hours until a cellular concentration of OD_{600} = 0.7 was obtained. The cultured cells were induced with 0.5 mM IPTG at 30°C for 2.5 hours. Bacterial cells were harvested and lysed by sonication (Sonication Buffer: 50mM Tris-Cl, 500mM NaCl 10% Glycerol, protease inhibitors tablets (Roche)). The cell debris was removed by centrifugation at 4°C. The supernatants, which contained recombinant eIF4G mutant proteins, were loaded onto a GST-Trap affinity exchange column (GE Healthcare). Biotin-conjugated thrombin (Merck Millipore) was used for cleavage of the GST-tag. Thrombin was removed by affinity separation using Streptavidin agarose. The cleaved GST tag was removed using GST affinity sepharose resin. The purity of the protein was examined by 8% SDS-PAGE gel electrophoresis where the
proteins on the gel were visualized using Coomassie blue staining. The protein concentration was determined by Bradford assay (72) (Thermo Scientific).

2.2.2 Protein expression and purification for eIF4F and eIF4E

The eIF4F expression construct (a gift from Dr. K.S Browning, University of Texas at Austin) is a discistronic construct with the eIF4G and eIF4E coding genes. eIF4G and eIF4E are co-expressed and assemble as the complex, eIF4F, after expression (73). BL21(DE3) E.coli cells were used for expressing eIF4F. A single colony was grown in an overnight culture and then transferred to large volume (1L) LB culture in a 1:50 ratio. The culture was incubated at 30 °C to an OD_{600} of approximately 0.8. 0.5 mM IPTG (final concentration) was used to induce eIF4F expression. Cells were harvested after an induction of 3 hours. Cells were re-suspended in Buffer B-150 (20 mM HEPES, 10% glycerol, 1 mM EDTA, 1 mM DTT, KCl 150 mM). Sonication was used to lyse the cells and centrifugation to separate the cell debris. The supernatant was diluted to 100 mM KCl and loaded onto a 10 mL phosphocellulose column pre-equilibrated with Buffer B-100 (20 mM HEPES, 10% glycerol, 1 mM EDTA, 1 mM DTT, KCl 100 mM). Bound eIF4F was eluted with Buffer B-300 (20 mM HEPES, 10% glycerol, 1 mM EDTA, 1 mM DTT, KCl 300 mM) to get the fractions containing the highest concentration. The eluted proteins were diluted to 100 mM KCl by additional Buffer B-0 (20 mM HEPES, 10% glycerol, 1 mM EDTA, 1 mM DTT) and loaded onto a 1-mL m^{7}GTP Sepharose column (Jena bioscience). eIF4F, which bound to m^{7}GTP affinity exchange column, was eluted with Buffer B-100 containing 30 mM GTP. Overnight dialysis in buffer B-100 buffer removed excess GTP. The purity of eIF4F was confirmed by 10% SDS-PAGE and the yield was determined using Bradford assay (72) (Thermo Scientific).
eIF4E was also expressed in BL21(DE3) *E.coli* cells. A single colony was cultured overnight and transferred to a large volume (1L) LB culture. After incubation at 37°C to OD\_\text{\lambda600} \approx 0.9, the eIF4E expression was induced with 0.5 mM IPTG (final concentration) for another 2 hours. The cells were re-suspended in Buffer B-50 (20 mM HEPES, 10% glycerol, 1 mM EDTA, 1 mM DTT, KCl 50 mM). The supernatant obtained after sonication and centrifugation was applied directly to a 4-ml m\text{\textsuperscript{7}}GTP Sepharose column and eluted with buffer B-100 containing 30 mM GTP. Overnight dialysis in B-100 buffer removed excess GTP (73).

### 2.2.3 Protein expression and purification for eIF4A and eIF4B

eIF4A cDNA was harbored in pET23d vector (a generous gift from Dr. D.R. Gallie, University of California, Riverside, CA) and used for expression of the protein. BL21 (DE3) pLysS *E.coli* was used to express His- tagged eIF4A protein (Table 1). A single colony was grown overnight at 37°C in fresh LB with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. Then the culture was transferred to 1L of LB. When the OD\_\text{\lambda600} was 0.6, protein expression was induced with 0.5 mM IPTG for another 3 hours at 37°C. The cells were harvested by centrifugation at 6000 rpm for 15 minutes and suspended into binding buffer (50mM sodium phosphate, 300mM sodium chloride, pH 7.4) containing soybean trypsin inhibitor and one Roche complete EDTA free Protease Inhibitor Cocktail Tablet. Sonication was used to lyse the cells. The lysate was centrifuged to separate the cell debris. The supernatant was loaded onto a 1 ml HisTrap HP affinity column (GE Healthcare). The column manufacturer’s protocol for protein purification was followed. eIF4A was eluted with PBS buffer (pH 7.4) containing 300 mM imidazole. An overnight dialysis in PBS buffer was performed to remove excess imidazole at 4°C. 8% polyacrylamide denaturing gel electrophoresis and Bradford assay (Thermo Scientific) were used to examine protein quality and yield, respectively (72).
GST fused eIF4B construct was also from Dr. D.R. Gallie, (University of California, Riverside, CA). Protein expression was performed in *E. coli* BL21 (DE3) pLysS. A 1 L bacterial cells culture was grown at 37 °C to an OD$_{600}$ of 0.5. 1 mM IPTG was used to induce protein expression for 3 hours at 30 °C. Harvested cells lysed and loaded on a 1 ml GST-trap column (GE Healthcare) which had been equilibrated with PBS Binding buffer (PBS, pH 7.4 along with 140 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, pH 7.4). Purification was done according to the column manufacturer’s specifications. 10 mM L-reduce glutathione was used to elute the protein. Purified protein was dialyzed overnight in PBS buffer. Quality and yield were determined before performing the next experiments (74, 75).
Table 1. Expression of wheat translation initiation factors in *E. coli*.

<table>
<thead>
<tr>
<th>Initiation factors</th>
<th><em>E. coli</em> strain</th>
<th>Antibiotic</th>
<th>OD_{600}</th>
<th>Culture/Inducing Tmp(°C)</th>
<th>Inducing Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>eIF4G601-1196</td>
<td>BL21(DE3)</td>
<td>amp</td>
<td>0.7</td>
<td>37/30</td>
<td>2.5h</td>
</tr>
<tr>
<td>eIF4G601-1488</td>
<td>BL21(DE3)</td>
<td>amp</td>
<td>0.7</td>
<td>37/30</td>
<td>2.5h</td>
</tr>
<tr>
<td>eIF4G742-1196</td>
<td>BL21(DE3)</td>
<td>amp</td>
<td>0.7</td>
<td>37/30</td>
<td>2.5h</td>
</tr>
<tr>
<td>eIF4F</td>
<td>BL21(DE3)</td>
<td>amp</td>
<td>0.8</td>
<td>30/30</td>
<td>3h</td>
</tr>
<tr>
<td>eIF4E</td>
<td>BL21(DE3)</td>
<td>amp</td>
<td>0.9</td>
<td>37/37</td>
<td>2h</td>
</tr>
<tr>
<td>eIF4A</td>
<td>BLR(DE3)</td>
<td>amp,chlor</td>
<td>0.5</td>
<td>37/37</td>
<td>3h</td>
</tr>
<tr>
<td>eIF4B</td>
<td>BLR(DE3)</td>
<td>amp,chlor</td>
<td>0.5</td>
<td>37/30</td>
<td>3h</td>
</tr>
</tbody>
</table>

2.3 RNA OLIGONUCLEOTIDES SYNTHESIS AND 5’END FLUORESCIN LABELING

The RNA oligomers (BTE and BTE mutants: BTEBF, SL-II-m1, SL-III-3, and SL-III-SWAP) were transcribed by T7 RNA polymerase from double stranded DNA templates (purchased from Integrated DNA Technologies Inc). The procedure was obtained from Megashortscript transcript
T7 kit standard protocol (Ambion). 5 µg DNA template, reaction buffer, 0.15 nM ATP, GTP, UTP, CTP and T7 RNA polymerase enzyme were mixed together. The volume was brought up to the desired transcription volume using Nuclease free water. The reaction was incubated at 37°C overnight. DNase was used to remove the DNA template. The in vitro transcription products were purified by phenol-chloroform (volume 1:1) extraction and ethanol precipitation. Nucaway Spin Columns from Ambion were used to remove free nucleotides. RNA concentrations were determined by nano-drop UV/Vis spectrometer. The purity of RNA was confirmed by 8% poly-acrylamide denaturing gel electrophoresis.

BTE and BTE mutant RNAs were labeled with fluorescein at the 5’end using Vector labs’ 5’end tag labeling kit and then purified according to manufacturer’s recommended protocol. Up to 0.6 nMole RNA was treated with alkaline phosphatase to remove the 5’ phosphate group. The reaction mix was incubated with T4 polynucleotide kinase and ATPγS to add the thiol–phosphate group. Fluorescein maleimide dye (dissolved in DMSO) was incubated with the reaction mix for 30 minutes at 65°C. Phenol-chloroform extraction and ethanol precipitation were carried out to purify 5’end fluorescein labeled RNA. Nucaway Spin Columns from Ambion were used to remove free dye and excess ATPγS. The purity of RNA was confirmed by 8% poly-acrylamide denaturing gel electrophoresis.

2.4 ELECTROPHORETIC MOBILITY SHIFT ASSAY

Electrophoretic mobility shift assays were used to determine whether eIF4G601-1196 and BTE bind or not. Before incubating BTE with eIF4G protein, RNA was refolded and re-natured to establish the secondary structure. BTE was heated to 95°C for 3 min in binding buffer (20 mM
Tris-HCl, pH 7.8, 140 mM KCl), slowly cooled to 37°C and kept at 37°C for 15 min. 10 mM MgCl₂ was added to the RNA solution and incubated for another 15 min and then cooled to room temperature. Refolded 200 ng BTE was incubated with eIF4G601-1196 at different mole ratios: 1:1, 1:2, and 1:5 for 20 min. The RNA and protein mix were applied to a native 6% polyacrylamide gel, which had been pre-run at 30 mA for 30 min. Electrophoresis was performed at 50 mA for 3 h at 4°C. SBRY green (Thermo Scientific) dye was used to stain the gel.

The gel was incubated in the 1X stain with continuous, gentle agitation for 20 minutes with protection from light. Gel was washed twice in 150 mL of dH₂O for 10 seconds to remove excess stain. Imaging by Typhoon Molecular Dynamics imager 9410 was used for analysis.

### 2.5 Fluorescence Anisotropy Measurements

In this study, fluorescence techniques were used to provide the equilibrium dissociation constants for the bindings between RNA and Proteins. The fluorescence anisotropy measurements can give the true binding affinity in solution compared with other methods such as filtering-binding assay, Electrophoretic Mobility Shift Assay (EMSA ) and traditional Isothermal Titration Calorimetry (ITC) (76). Fluorescence anisotropy changes detect fluorophore molecular motion changes or environmental changes. The fluorophore labeled samples are excited with a vertically polarized light (Ivv) (Figure 6). The electric vector of the excitation beam is along the “Z” axis (Figure 6). When polarized light hits a fluorophore, the emission will be polarized too. The amount of emitted polarized light varies according to the size and movement of sample molecules. Polarized emission which is parallel to the excitation electric field is represented as...
I_{VV}; the emission which is perpendicular to the excitation field, the intensity is called I_{VH}. The equation giving the definition of anisotropy (r) is as below (i) (77).

\[ r = \frac{(I_{VV} - I_{VH})}{(I_{VV} + 2I_{VH})} \] .............................. (i).

FIGURE 6. GEOMETRY OF FLUORESCENCE ANISOTROPY EXPERIMENT.

(Source : BASIC PHOTOPHYSICS Visser. Antonie J and Rolinski. Olaf) (78) A fluorophore labeled with sample molecule in the cuvette is excited with vertically polarized light (z) causing photoselection. A polarizer in the fluorescence channel (x) can be rotated from the vertical to the horizontal position. The emission intensity of vertically polarized fluorescence I_{VV} is measured
and also the horizontal polarized fluorescence $I_{vh}$ is recorded.

If a fluorophore rotates slower in solution than the fluorescent life time, the anisotropy will be large because $I_{vv}$ value is larger than $I_{vh}$ value. If a fluorophore rotates rapidly relative to its lifetime; the anisotropy will be close to zero because $I_{vv}$ value is equal to $I_{vh}$ value. When the binding of protein to a fluorophore labeled RNA, the increase of mass is sufficient to decrease the rotation rate of the fluorophore and change the anisotropy (76). Therefore, it allows us to measure the fraction RNA bound and calculate the equilibrium dissociation constant between RNAs and proteins (77).

Our fluorescence anisotropy measurements were performed with a Horiba JobinYvon Fluorolog-3 FluorEssenceTM spectro-fluorimeter equipped with excitation and emission polarizers and an L-format detection configuration. Vertically polarized light was used for excitation (slit width 4 nm) and the emission (slit width 5 nm) was measured in both the horizontal and vertical directions. Direct fluorescence anisotropy titration was employed to study protein-RNA interactions. The titration temperature was 25°C for all the experiments except where otherwise indicated (temperature dependent study). 50nM of 5’fluorescein labeled BTE or BTE mutants were incubated with increasing concentrations of protein or protein complex in titration buffer (20 mM HEPES Buffer, pH 7.4, 100 mM KCl, 1mMDTT). The helicase complex (eIF4A, eIF4B and ATP) and eIFs complex were pre-incubated before adding into titration mix (28, 55). The anisotropy of each sample was measured by excitation at 494 nm (4 nm slit), and the emission was measured at 520 nm (5 nm slit). The anisotropy data were fitted to equation (ii) to determine the dissociation equilibrium constant (57, 74).
The $r_{\text{obs}}$ is the observed anisotropy value for any point in the titration curve; $r_{\text{min}}$ is the minimal anisotropy value in the absence of protein or protein complex; $r_{\text{max}}$ is final saturated anisotropy value. $b = K_D + [F^\text{BTE/mutants}] + [\text{eIFs}]$. $[F^\text{BTE/mutants}]$ and $[\text{eIFs}]$ are the concentration of BTE or BTE mutants and initiation factor concentration, respectively. $K_D$ is the equilibrium dissociation constant for protein one site binding with RNA (79, 80). Titration data were nonlinear least squares fitted by Kaleida Graph (Abelbeck Software). Equilibrium values were determined from at least 3 independent individual experiments.

### 2.6 Thermodynamic Analyses of eIF4G601-1196, eIF4E Interaction with BTE

Temperature dependence of the association equilibrium constant was used to determine the thermodynamic parameters for eIF4G601-1196 or eIF4G601-1196•eIF4E complex binding to BTE. Enthalpy ($\Delta H$), entropy ($\Delta S$), and free energy ($\Delta G$) were calculated by Van’t Hoff plots of $-\ln K_{\text{eq}}$ versus $1/T$, according to the following equations (iii) and (iv)

$$-RT \ln K_{\text{eq}} = \Delta H - T \Delta S \quad \text{(iii)}$$

$$\Delta G = -RT \ln K_{\text{eq}} \quad \text{(iv)}$$

Where $R$ is the universal gas constant and $T$ is the absolute temperature. $K_{\text{eq}}$, the association equilibrium constant, was determined at different temperatures. $\Delta H$ and $\Delta S$ were obtained from the slope and intercept, respectively, of the plot of $\ln K_{\text{eq}}$ vs $1/T$. The titration reactions were performed at temperatures of 5 °C, 15 °C, 20 °C, 25 °C, and 30 °C ±0.5 °C as described above. A
thermocouple was used inside the cuvette to monitor temperature. The temperature dependence experiments for binding between eIF4G601-1196•eIF4E complex and BTE were done in the range 20-30°C because the tight binding constants for lower temperature make measurements impractical. ∆G was calculated for 25°C.

2.7 CD Measurements

An AVIV 200 CD spectrometer equipped with Peltier thermal controller with 1mm optical length and 1nm bandwidth was used for recording CD signals. Spectra were acquired from 200 to 260 nm at 25 °C. 10 µM eIF4E, eIF4G and eIF4G601-1196•eIF4E complex were measured in 10 mM phosphate buffer (pH 7.0). Each spectrum is an average of 5 scans. eIF4G601-1196 and eIF4E were pre-incubated to form the protein complex before scanning. Spectra were corrected for buffer background signal. Protein peptide α helix content is calculated with mean residue ellipticity of 222nm, based on equation (v) (81).

\[
\alpha\text{-helix (\%)} = \left( \frac{-\text{MRE}_{222-2340}}{30300 \times 100} \right) (v)
\]

2.8 RNA SYNTHESIS FOR BlucB, BlucBF, Bluc-SLII-m1 AND Bluc-SLIII-Swap mRNA

BlucB is the reporter plasmid (22, 51, 55), in which the 5’UTR of BYDV is inserted 5’ upstream of firefly luciferase gene and the 3’BTE flanked to 3’ downstream of the luciferase reporter gene. All these sequences were harbored in a pUC MINUS MSC plasmid (51). BlucBF is the report construct which has 4 nucleotides inserted into the stem loop I of 3’BTE. The stem loop I structure was disrupted and resulted in BlucBF losing translation function. Bluc-SLII-m1 and
Bluc-SLIII-SWAP are two mutated reporter constructs. In Bluc-SLII-m1, BTE mutants—SLII-m1 replaces BTE in the 3’ UTR downstream of luciferase gene. In Bluc-SLIII-SWAP (Generating by Genscript), SLIII-SWAP replaces BTE. All the reporter constructs plasmids were linearized with the restriction enzyme SmaI and then used as templates for transcription of the mRNA. To generate mRNA under the T7 promoter, Megscript T7 kit was used according to the manufacturing protocol (Ambion). The transcription mix was incubated at 37 °C for 4 hours. RNA products were purified by phenol-chloroform extraction and ethanol precipitation. Nucaway Spin Columns from Ambion were used to remove free nucleotides. The purity of RNA was confirmed by 8% poly-acrylamide gel electrophoresis. RNA concentrations were determined by nano-drop UV/Vis spectrometer.

2.9 In Vitro Translation in Wheat Germ Extract

2.9.1 Generating 4F-depleted wheat germ extract

Wheat germ extract was purchased from Promega. The depleted extract was prepared with m7GTP sepharose (Jena bioscience). 200 μl of wheat germ extract was added to 300 μl of m7GTP-sepharose and incubated with rotation at 4 °C for 1 hour. The lysate was collected by centrifugation (500 ×g for 3 min) through a spin column (Promega) and then used immediately or stored at -80 °C (58).

2.9.2 Western Blot assay to determine the depletion extent of initiation factors in 4F-depleted wheat germ extract.

In order to determine protein deletion levels, Western blot assays were performed. Proteins from wheat germ extract and 4F-depleted wheat germ lysates were resolved with 8% SDS-
polyacrylamid gel electrophoresis. The proteins were transferred to 0.45 µM nitrocellulose membrane by immersion electro-blotting. 5% milk in TBST buffer (20 mM Tris pH 7.4, 150 mM NaCl, 0.1% Tween 20) was used to block the nitrocellulose membrane. The primary antibody (generous gifts from Dr. Karen Browning, University of Texas, Austin) were diluted 1:2000 in TBST buffer with 1% BSA and overnight incubated with the blot membrane at 4 °C. The blots were washed by TBST three times and then incubated with 800CW infrared dye conjugated goat anti-rabbit antibodies (LI-COR) 1:5000 diluted for 1 hour. The blots were washed three times with TBST buffer, washed with TBS once, and washed with distilled deionized water once. The Odyssey infrared imager (LI-COR) was used to detect the signals.

2.9.3 in vitro translation

The BlucB, BlucBF, Bluc-SLII-m1 and Bluc-SLIII-SWAP in vitro translation reactions were performed using the Promega standard protocol. eIF4F, eIF4Gs, and eIF4E were supplied in a final concentration of 60 nM. In wheat germ lysate, eIF4A is present in a 30-fold molar excess relative to eIF4G (58). The final concentrations for eIF4A and eIF4B supplied in depleted WGE were 1.8 µM and 600 nM, respectively. 20 nM mRNA was mixed with 25µl 4F-depleted wheat germ extract, amino acid mix, potassium acetate, ribonuclease inhibitor and supplemented proteins at 25 °C for 1.5 hour. The final volume of each reaction mix was 50µl.

Luciferase assays were performed after the translation reaction. 3µl of the translation mix was added to 50µl luciferase assay reagent (Promega) and measured immediately using a Glomax-96 microplate illuminometer. The fluorescence intensity represents the firefly luciferase reporter gene expressing level. Each mRNA construct was translated in triplicate, and the mean ± S.D. for each construct is reported.
CHAPTER 3 IDENTIFICATION OF THE eIF4G CORE DOMAIN FOR INTERACTION WITH BTE

Plant viruses usually evolve efficient ways to synthesize viral proteins for replication. They occupy host cellular translation machinery to sequester the plant protein synthesis components. BTE controls its viral mRNA translation by targeting eIF4G. To understand how BTE utilizes the different eIF4G domains, the binding affinities of three eIF4G mutants to BTE and translational activities were examined. We identified the core domain of eIF4G for binding to BTE.

3.1 Results

3.1.1 Three eIF4G deletion mutants bind to BTE with different binding affinities

eIF4G601-1196 containing amino acid residues from 601 to 1196, includes the eIF4E binding site, one eIF4A binding site and one eIF4B binding site (Figure 5). The Equilibrium dissociation constant (K_D) was determined from the anisotropy changes during the labeled BTE titration with eIF4G deletion mutant proteins. The results showed that eIF4G601-1196 bound to 3’BTE with K_D = 40±4 nM; whereas eIF4G601-1488, with one additional eIF4A binding site in the C-terminal sequence had K_D = 68±5 nM. The binding between eIF4G601-1196 and BTE was tighter than eIF4G601-1488 to BTE (Table 2, Figure 7), but eIF4G742-1196, the protein fragment lacking the eIF4E binding region, had a reduced binding affinity to BTE (K_D 91±12nM) compared with the other two eIF4G deletion mutants (Figure 7, Table 2).
Table 2. Equilibrium binding affinity constants (K\textsubscript{D}) of BTE to eIF4G deletion mutants

The dissociation constants were measured at 25 °C. \textsuperscript{fi}BTE was titrated with eIF4G mutants.

<table>
<thead>
<tr>
<th></th>
<th>K\textsubscript{D} (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTE •eIF4G601-1196</td>
<td>40±4</td>
</tr>
<tr>
<td>BTE •eIF4G601-1488</td>
<td>68±5</td>
</tr>
<tr>
<td>BTE •eIF4G742-1196</td>
<td>91±12</td>
</tr>
</tbody>
</table>
**Figure 7. Equilibrium binding of BTE to eIF4G deletion mutants**  
BTE was labeled with fluorescein at the 5’end. The $^{[4]}$BTE (50nM in titration Buffer) was titrated by eIF4G mutants (○ eIF4G601-1196, Δ eIF4G601-1488, □ eIF4G742-1196) at 25 °C. The excitation and emission wave lengths were 494 nm and 520nm, respectively. The dissociation constant ($K_D$) was obtained from the fit curve as described in Chapter 2 Experimental Procedure 2.5 fluorescence anisotropy measurement. Equilibrium values were averaged from at least 3 independent individual experiments. Error bars represent the standard deviation.
Electrophoretic mobility shift assay (EMSA) was used to confirm eIF4G601-1196 binding to BTE. In EMSA, RNA bound to protein shows a shift during electrophoresis as compared to unbound RNA. The result showed that after incubation with eIF4G601-1196, BTE showed two distinct bands on the gel; while BTE RNA alone only had one band. The band on the bottom of gel represented the unbound RNA (Figure 8); whereas the top band represented the BTE bound to eIF4G601-1196. With increasing amount of eIF4G601-1196, the intensity of top band increased, indicating more RNA binding to protein. This result further confirmed that the middle region of eIF4G was sufficient to bind with BTE.

![Figure 8. electrophoretic mobility shift assay of BTE and eIF4G601-1196](image)

200 nanogram BTE was pre-incubated with eIF4G601-1196 in 15 µl binding buffer and was subjected to
electrophoresis in 6% polyacrylamide non-denaturing gel. Lane 1, 2 and 3 are BTE with eIF4G601-1196 in molar ratio of 1:1, 1:2 and 1:5 respectively. Lane 4 is BTE alone without any protein.

### 3.1.2 eIF4G601-1196 binds tighter than the other two mutants and than full length eIF4G

eIF4G601-1196 showed the tightest binding among three eIF4G deletion mutants. The equilibrium dissociation constant ($K_D$) for BTE binding to eIF4G601-1196 (40±4 nM) was significantly smaller (tighter binding) than the $K_D$ reported for binding of full-length eIF4G to BTE (177±10 nM) (56). Our results suggested that the N-terminal and C-terminal regions of eIF4G are not necessary for binding to BTE. In addition, the results demonstrate eIF4G601-1196 is the core domain required for binding with BTE.

### 3.1.3 Three eIF4G mutants were able to rescue BTE mediated translation in 4F-depleted wheat germ extract

After profiling the binding abilities of different eIF4G deletion mutants to BTE, we probed the translational function of the three eIF4G mutants. Investigations were performed to find out whether or not a short region of eIF4G was able to initiate translation.

We used BlucB as the reporter construct which contained 5’UTR of BYDV upstream of firefly luciferase reporter gene with 3’BTE flanking in the 3’ downstream region (51, 52). After *in vitro* translation, the measured luciferase activities were used to represent the BTE-mediated translation abilities. eIF4F-depleted wheat germ extract was used as the *in vitro* translational system (58). Western blot assays showed that both eIF4G and isoeIF4G were almost absent in
the 4F-depleted wheat germ extract (Figure 9). Due to binding to eIF4F, eIF4A and eIF4B levels were also partially reduced in 4F-depleted wheat germ extracts (Figure 9).

BlucB showed significantly reduced expression in 4F-depleted wheat germ extract as compare to the untreated wheat germ extract. Recombinant eIF4F was able to rescue BlucB translation in 4F-depleted wheat germ extract (Figure 10). For all the in vitro translation assays, the rescue ability of 60 nM eIF4F for 20 nM BlucB translation in 4F-depleted WGE was used as 100%. The same molar amounts of the eIF4G deletion mutants were used to compare their translation abilities with eIF4F.

Figure 9. 4F-depleted wheat germ extract had low level of eIF4F. Western blot analysis was performed to determine the extent of depletion of eIF4G, isoelF4G, eIF4A and eIF4B. Equal amounts of protein (8µg) were loaded in each lane. Lane 1 represents the untreated wheat germ extract; Lane 2 is the m7GTP sepharose treated 4F-depleted wheat germ extract.
Figure 10. Recombinant eIF4F rescued BlucB translation in 4F-depleted wheat germ extract. BlucB had a low level expression in 4F-depleted WGE, which was less than 5% of its expression as untreated WGE. Recombinant eIF4F was able to rescue the reporter gene expression. We set the translation ability of 60 nM eIF4F rescue of 20 nM BlucB as 100%. All the other translation assay readings were normalized to it.

eIF4G601-1196 rescued 66±5% of BlucB translation as compared to eIF4F (Figure 11, Table 3). eIF4G601-1488, which includes one additional eIF4A binding site in the C-terminal region, had slightly stronger translation ability, and was able to restore BlucB translation to 72±4% of the
eIF4F level. The mutant without the eIF4E binding site, eIF4G742-1196 showed 63±10% rescue ability (Figure 11, Table 3). eIF4G742-1196 restored BlucB translation in 4F-depleted WGE (Figure 11), suggesting that the eIF4E interacting region on eIF4G is not necessary for translation. Previous results have shown that eIF4G alone can facilitate the translation of BlucB to 75% of same amount as eIF4F (56). Our data showed that all three eIF4G deletion mutants were competent for rescuing BTE-mediated translation. eIF4G601-1196, the central domain of eIF4G, which is considered as the eIF4G core domain for binding to BTE, was able to facilitate the translation almost as efficiently as the full length eIF4G, showing that the N-terminal and C-terminal of eIF4G are not necessary for BTE-mediated translation.
Figure 11. eIF4G deletion mutants were able to rescue BlucB translation in 4F-depleted wheat germ extract. 60nM eIF4F, eIF4G601-1196, eIF4G601-1488 and eIF4G742-1196 were tested separately in 25 µl 4F-depleted wheat germ extract for 20 nM BlucB translation. The reactions were incubated at 25 °C for 1.5 hours. The relative luciferase intensity units (RLU) were obtained to represent translation activity.
Table 3. The translation ability of eIF4G deletion mutants for rescue of BTE-mediated translation in 4F-depleted wheat germ extract

<table>
<thead>
<tr>
<th></th>
<th>BlucB translation (RLU%)</th>
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<tbody>
<tr>
<td>eIF4F</td>
<td>100±1</td>
</tr>
<tr>
<td>eIF4G601-1196</td>
<td>66±5</td>
</tr>
<tr>
<td>eIF4G601-1488</td>
<td>72±4</td>
</tr>
<tr>
<td>eIF4G742-1196</td>
<td>63±10</td>
</tr>
</tbody>
</table>

Bluc-SLII-m1 is another reporter construct, in which the 5’UTR of BYDV was inserted into upstream of firefly luciferase reporter gene and mutant SL-II-m1 instead of BTE flanked downstream of the firefly luciferase gene. Bluc-SLII-m1 only had 30% of the translation ability of BlucB in 4F-deplete wheat germ extract (Figure 12, Table 4). Three-fold (180 nM) eIF4F was able to increase the translation of Bluc-SLII-m1 to the level of BlucB. Additional eIF4G601-1196 also increased the Bluc-SLII-m1 translation as eIF4F did. At the high concentration,
eIF4G601-1196 and eIF4E together have similar translation ability (> 80%) as eIF4F (Figure 12, Table 4), further suggesting that eIF4G601-1196 possesses the core function in translation.

Figure 12. eIF4G601-1196 increased Bluc-SLII-m1 translation in a concentration dependent manner similar to eIF4F. eIF4F and eIF4G601-1196 at different concentrations were supplied to 25 µl 4F-depleted wheat germ extract for 20 nM Bluc-SL-II-m1 translation. The translation ability of 60 nM eIF4F with 20 nM BlucB was set as 100%. Bluc-SL-II-m1 translation activities were normalized to it.
Table 4. Rescue ability of eIF4G601-1196 for Bluc-SLII-m1 translation in 4F-depleted wheat germ extract

<table>
<thead>
<tr>
<th>mRNA Concentration (nM)</th>
<th>Bluc-SLII-m1 translation (RLU%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+eIF4F</td>
</tr>
<tr>
<td>60</td>
<td>32±2</td>
</tr>
<tr>
<td>120</td>
<td>85±4</td>
</tr>
<tr>
<td>180</td>
<td>93±5</td>
</tr>
<tr>
<td>240</td>
<td>89±3</td>
</tr>
</tbody>
</table>

3.2 DISCUSSION

Human eIF4G central domain acts as an active “ribosome recruitment core” and is implicated, along with eIF4A, as a critical binding partner to drive mRNA translation in living cells (82). The central domain of human eIF4G also preserved the activating effect on the translation of uncapped mRNAs (83). Human eIF4G496-935 has been shown to exert a dominant negative effect on the translation of capped mRNA, but stimulated the translation of uncapped mRNA in vitro. The C-terminal third of human eIF4G was confirmed dispensable and serves as a regulatory domain in translation (84).
In this study we find that the middle domain of wheat eIF4G possessed the core function in binding RNA and initiating translation. Our data show that even without the N-terminal and C-terminal, only a middle middle region of wheat eIF4G (eIF4G601-1196) was sufficient for binding to BTE and rescue BTE directed cap-independent translation. Compared with previous studies, which showed that the C-terminal two-thirds length of wheat eIF4G (eIF4G766-1488) initiate BTE-mediated translation in wheat germ extract as efficiently as the full length eIF4G (56), for the first time we showed that only the middle one-third of eIF4G is required by BTE; the N-terminal and the C-terminal of eIF4G are not necessary.

Our data showed that the binding affinities between three eIF4G deletion mutants are not correlated to their translation initiating abilities very well. eIF4G601-1196, the central domain of eIF4G, showed the tightest binding to BTE among these three mutants, but showed slightly weaker translation activity than eIF4G601-1488, which has a longer C-terminal sequence, not contributing to tighter binding. Considering eIF4G601-1488 has strongest translation activity among the three mutants, the C-terminal of eIF4G may interact with other initiation factors to promote translation.

The inconsistency between the eIF4G mutants translation abilities and the binding affinities to BTE is probably may be due to the kinetics. It is possible that the binding starts with the interaction between the central domains of eIF4G and BTE. This interaction may trigger eIF4G full-length protein conformational change. The shifted new conformation of eIF4G may result in a decreased binding affinity between BTE and full length eIF4G. This is consistent with the fact that eIF4G601-1196 showed almost 4-fold more binding affinity with BTE as compared to full length eIF4G. Our previous kinetic studies also support this, which have shown that eIF4F
(eIF4G•eIF4E) had a two-step binding to BTE, with a fast first step and slow, concentration independent second step, with presumably a conformation change (57).
CHAPTER 4 HELICASE COMPLEX (eIF4A-eIF4B-ATP) INCREASES BINDING OF eIF4G MUTANTS TO BTE AND STIMULATES BTE MEDIATED TRANSLATION.

Previous results (55) showed that a combination of eIF4A-4B-ATP (helicase complex) and eIF4F enhanced the binding of the 40S ribosomal subunit to BTE nearly three-fold. To further explore the helicase complex function in plant virus cap-independent translation, we examined the helicase complex effects on eIF4G mutants binding to BTE and the effects on stimulating BTE mediated translation.

4.1 RESULTS

4.1.1 Helicase complex increases binding of eIF4G mutants to BTE

The helicase complex (eIF4A-eIF4B-ATP) in the presence of eIF4G unwinds double stranded RNA (17, 64, 69). This complex increased the binding affinity between BTE and eIF4G601-1196 about three-fold (K_D changed from 40±4 nM to 13±3nM), significantly tightening the binding (Figure 13, Table 5).

The helicase complex affected BTE binding to eIF4G742-1196 as well, which has the central eIF4A binding domain but without the eIF4E binding region. The binding was significantly tighter in the presence of helicase complex (K_D changed from 91±12 nM to 59±5 nM) (Figure 14, Table 5). The increase in binding affinities was observed for two eIF4G mutants, both possessing one central eIF4A binding domain.
Figure 13. Helicase complex significantly increases eIF4G601-1196 binding to BTE

BTE was labeled with fluorescein. Fluorescence anisotropy was measured at 25 °C when the

\[^{\text{f}}\text{BTE (50nM in titration Buffer)}\] was titrated by \(\circ\text{eIF4G601-1196}\) and \(\Delta\text{eIF4G601-1196-eIF4A-eIF4B-ATP}\). The dissociation constant \((K_D)\) was obtained from the fit curve as described in

Chapter 2 Experimental Procedure. Equilibrium values were averaged from at least 3

independent individual experiments. Error bars represent the standard deviation.
Figure 14. Helicase complex increases eIF4G742-1196 binding to BTE. BTE was labeled with fluorescein. Fluorescence anisotropy was measured at 25 °C when the BTE (50nM in titration Buffer) was titrated by eIF4G742-1196 and ΔeIF4G742-1196-eIF4A-eIF4B-ATP. The dissociation constant (K_D) was obtained from the fit curve as described in Chapter 2 Experimental Procedure. Equilibrium values were averaged from at least 3 independent individual experiments. Error bars represent the standard deviation.
Figure 15. Helicase complex does not affect eIF4G601-1488 binding to BTE. BTE was labeled with fluorescein. Fluorescence anisotropy was measured at 25 °C when the $^{3}$BTE (50nM in titration Buffer) was titrated by ◦ eIF4G601-1488 and Δ eIF4G601-1488-eIF4A-eIF4B-ATP. The dissociation constant ($K_D$) was obtained from the fit curve as described in Chapter 2 Experimental Procedure. Equilibrium values were averaged from at least 3 independent individual experiments. Error bars represent the standard deviation.
Table 5. Helicase complex affected binding of eIF4G deletion mutants to BTE

The dissociation constants were obtained at 25 °C. ^BTE was titrated by eIF4G mutants alone and eIF4G mutants in complex with other initiation factors. The mole amount ratio of the eIF4G mutant, eIF4A and eIF4B was 1:30:10. Protein complexes were pre-incubated before titration into ^BTE.

<table>
<thead>
<tr>
<th>K_D (nM)</th>
<th>+ 4A 4B ATP</th>
<th>+ 4A 4B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-hydrolyzable ATP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BTE •eIF4G601-1196</td>
<td>40±4</td>
<td>13±3</td>
</tr>
<tr>
<td>BTE •eIF4G742-1196</td>
<td>91±12</td>
<td>59±5</td>
</tr>
<tr>
<td>BTE •eIF4G601-1488</td>
<td>68±5</td>
<td>58±1</td>
</tr>
</tbody>
</table>

4.1.2 Helicase complex effects on binding to BTE were different for eIF4G mutants.

In contrast, eIF4G601-1488, when incubated with helicase complex, did not show a similar increase in binding affinity as the other two mutants, suggesting that the second eIF4A binding site on eIF4G counteracted the helicase complex effect (Figure 15, Table 5). eIF4G mutants with
different number of eIF4A binding domains, showed distinctive responses in presence of helicase complex.

4.1.3 eIF4A and eIF4B affected eIF4G binding to BTE via protein-protein interactions in addition to RNA unwinding effects.

When non-hydrolyzable ATP was used instead of ATP with the helicase complex, the equilibrium dissociation constant was slightly reduced for binding between eIF4G601-1196 and BTE (reduced from 40±4 nM to 29±1nM) (Table 5), and eIF4G742-1196 binding to BTE (reduced from 91±12 nM to 76±2 nM). These results indicated that the binding of eIF4G mutants to BTE were affected not only by the helicase complex, but also by the protein-protein interactions between eIF4A, eIF4B and eIF4G.

4.1.4 eIF4A and eIF4B stimulated eIF4G deletion mutant dependent BlucB translation
eIF4A and eIF4B levels were slightly reduced in the 4F-depleted wheat germ extract (Figure 9), due to binding to eIF4F and consequent removal by the m^7GTP sepharose beads. When only recombinant proteins eIF4A and eIF4B were added to 4F-depleted wheat germ extract, they failed to support BlucB translation (Figure 16, Table 6).
Figure 16. BTE mediated translation is eIF4F dependent. 60 nM eIF4F, eIF4E, eIF4A and eIF4B were tested in 25 μl 4F-depleted wheat germ extract for 20 nM BlucB translation. The in vivo translation experiments were performed as described in Chapter 2 Experimental Procedure 2.9.3. The translation ability of 60 nM eIF4F with 20 nM BlucB was set as 100%. The readings of all other translation assays were normalized to it.
Table 6. The translation ability of initiation factors for BTE-mediated translation.

20 nM BlucB translation was measured in 25 μl 4F-depleted wheat germ extract when supplement with 60 nM eIF4F, eIF4E, eIF4A and eIF4B.

<table>
<thead>
<tr>
<th></th>
<th>+eIF4F</th>
<th>+eIF4E</th>
<th>+eIF4A•eIF4B</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BlucB translation</strong></td>
<td>100±1</td>
<td>15±2</td>
<td>18±2</td>
</tr>
<tr>
<td>(RLU%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Supplementation of additional eIF4A and eIF4B with eIF4G601-1196 resulted in an increase of BlucB translation levels. eIF4G601-1196 dependent BlucB translation showed an increase of 3.84±0.16 fold, whereas for eIF4G601-1488 and for eIF4G742-1196 the increase was 3.2±0.05 fold and 3.56±0.09 fold, respectively (Figure 17, Table 7). When eIF4F together with eIF4A and eIF4B were added into the 4F-depleted wheat germ extract, BlucB translation increased to 2.92±0.14 fold as compared with added eIF4F alone (Figure 17, Table 7). These results showed that eIF4A and eIF4B increased the abilities of both eIF4F and eIF4G deletion mutants to stimulate translation of BlucB in 4F-depleted wheat germ extract.
Figure 17. eIF4A and eIF4B stimulated eIF4F and eIF4G deletion mutants’ rescue of BlucB translation in 4F-depleted wheat germ extract. 60 nM eIF4F or eIF4G mutant were added to 25 μl 4F-depleted wheat germ extract for 20 nM BlucB translation. 1.8μM eIF4A and 600 nM eIF4B were pre-incubated with eIF4F or eIF4G to form the protein complex and then added to 4F-depleted WGE. The in vivo translation experiments were performed as described in Chapter 2. The fluorescence intensities were obtained for calculation for relative translation activity. The translation ability of 60 nM eIF4F with 20 nM BlucB was set as 100%. The readings of all other translation assays were normalized to it. White columns represent translation with eIF4G.
deletion mutants alone. Grey shade columns are BlucB translation with eIF4F or eIF4G mutants when eIF4A and eIF4B were added.

Table 7. Effects of eIF4A and eIF4B on eIF4G dependent BlucB translation

<table>
<thead>
<tr>
<th></th>
<th>BlucB translation</th>
<th>(RLU%)</th>
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<tbody>
<tr>
<td></td>
<td>eIF4F</td>
<td>100±1</td>
</tr>
<tr>
<td></td>
<td>+ eIF4A eIF4B</td>
<td>292±11</td>
</tr>
<tr>
<td>eIF4G601-1196</td>
<td>66±5</td>
<td>249±9</td>
</tr>
<tr>
<td>eIF4G601-1488</td>
<td>72±4</td>
<td>231±8</td>
</tr>
<tr>
<td>eIF4G742-1196</td>
<td>63±10</td>
<td>230±12</td>
</tr>
</tbody>
</table>
4.1.5 Effects of eIF4A and eIF4B on BTE-mediated translation were different for eIF4G mutants with only central eIF4A binding domain from the mutant with two eIF4A binding domains.

The effects of eIF4A and eIF4B on stimulation eIF4G mutants rescue BlucB translation were different for eIF4G mutants with one or with two eIF4A binding domains. eIF4G601-1488 mutant had two eIF4A binding sites, but additional eIF4A and eIF4B did not increase eIF4G601-1488 translation efficiency as compare to eIF4G601-1196 or eIF4G742-1196, which have only one eIF4A binding domain. The same phenomenon was observed on another reporter construct Bluc-SLII-m1, but with larger differences. Reporter construct Bluc-SLII-m1, which has only 30% translation activity compared to BlucB translation, was used to test the effects of eIF4A and eIF4B. eIF4A and eIF4B stimulated eIF4G601-1196 Bluc-SLII-m1 translation 3.68±0.26 fold, for eIF4G742-1196 3.46±0.46 fold; while for eIF4G601-1488 the stimulation was only 2.62±0.23 fold (Figure 18, Table 8). Due to slight leftover of eIF4E in 4F-depleted wheat germ extract (Western blot, data not shown), eIF4G601-1196, which has eIF4E-binding site, had a slightly higher translation stimulation than the mutant eIF4G742-1196 without eIF4E binding site.
Figure 18. Effects of eIF4A and eIF4B on eIF4F dependent translation of Bluc-SLII-m1.

Translation of 20 nM Bluc-SL-II-m1 mRNA was used to examine the effects of eIF4A and eIF4B on eIF4G mutants. The translation ability of 60 nM eIF4F to rescue 20 nM BlucB was set as 100%. All the Bluc-SL-II-m1 mRNA translation activities were normalized to it.
Table 8. Effects of eIF4A and eIF4B on eIF4G deletion mutant dependent translation of Bluc-SLII-M1

The translation ability of 60 nM eIF4F to rescue 20 nM BlucB was set as 100%. All the eIF4G mutants translation activities were normalized to it.

<table>
<thead>
<tr>
<th>Bluc-SLII-M1</th>
<th>translation (RLU%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ eIF4A eIF4B</td>
</tr>
<tr>
<td>eIF4F</td>
<td>32±2</td>
</tr>
<tr>
<td>eIF4G601-1196</td>
<td>27±3</td>
</tr>
<tr>
<td>eIF4G601-1488</td>
<td>25±3</td>
</tr>
<tr>
<td>eIF4G742-1196</td>
<td>22±4</td>
</tr>
</tbody>
</table>
4.2 Discussion

In addition to eIF4G, translation initiation factors eIF4A and eIF4B were also involved in BTE mediated translation. Our results show that eIF4G was required by BTE regulated translation. eIF4A and eIF4B had profound effects on BTE interaction with eIF4G. eIF4A directed helicase complex significantly increased the binding between eIF4G mutants (eIF4G601-1196 and eIF4G742-1196) to BTE. Once eIF4G central domain upon binds to eIF4A, the helicase complex melts the double stranded region of BTE and makes it more accessible for tighter binding by proteins. Apart from the ATP dependent helicase activity, eIF4A and eIF4B also increased binding of eIF4G mutants to BTE by protein-protein interactions. eIF4A and eIF4B stimulated eIF4G deletion mutants rescue of BTE-mediated translation to more than three fold. All of these data indicated our hypothesis is correct that eIF4G BTE not only uses eIF4G to direct viral RNA translation, but also utilizes eIF4G to coordinate with eIF4A and eIF4B to maximize viral protein synthesis.

The central eIF4A binding domain on human eIF4G stimulates ATP-hydrolytic activity, but the C-terminal eIF4A binding domain did not stimulate additional ATP-hydrolytic efficiency (85). Human eIF4G middle domain eIF4G557-1137, which includes the eIF4E binding site and only one eIF4A binding site, had a faster eIF4A directed helicase RNA unwinding rate than full length eIF4G or than longer eIF4G557-1600 (with two eIF4A binding domains)(86). The second eIF4A binding domain on mammalian eIF4G possessed an anti-cooperative function for binding RNA toward the first eIF4A binding domain on the central region of eIF4G (84). In a number of fungal species, the C-terminal eIF4A binding domain of eIF4G has been lost during the evolution (2).
Like mammalian eIF4G, eIF4A directed helicase complex effects on wheat eIF4G binding to BTE are primarily dependent on the central eIF4A binding domain of eIF4G. The helicase complex had stronger effects on eIF4G mutants with one central eIF4A binding domain than the mutant with two eIF4A domains for binding to BTE. The second C-terminal eIF4A binding domain of eIF4G did not contribute to the tight binding between eIF4G and BTE in the presence of helicase complex. Therefore the central eIF4A binding domain on wheat eIF4G is required by the helicase complex; while the C-terminal eIF4A binding domain of eIF4G counteracts the helicase complex effects.
CHAPTER 5. eIF4E, THE CAP BINDING PROTEIN, PLAYS A ROLE IN eIF4G BINDING TO BTE

eIF4E is the cap-binding protein; while BTE employs a cap-independent translation mechanism. Previous evidence indicated (52, 56) that eIF4G dependent BTE-mediated translation shows a slight increase in the presence of eIF4E. It was also reported that eIF4E enhanced the protection if BTE when eIF4G bound. To explore the possible function of eIF4E in BTE-mediated translation, we investigated the effects of eIF4E on BTE binding to eIF4G deletion mutants and on BTE directed translation.

5.1 RESULTS

5.1.1 eIF4E increased eIF4G601-1196 and eIF4G601-1488 binding to BTE, but not eIF4G742-1196
eIF4E increased the binding between BTE and eIF4G601-1196 almost six-fold (Figure 19, Table 9). The $K_D$ of protein complex eIF4G601-1196•eIF4E binding to BTE, was $6.8\pm 1.5$ nM, compared to eIF4G601-1196 binding ($K_D$ $40\pm 4$ nM). A similar, but smaller effect was observed when eIF4E was added to eIF4G601-1488. $K_D$ values decreased from $68\pm 5$ nM to $28\pm 6$ nM (Figure 20, Table 9). To test the extent to which the eIF4E-eIF4G protein interaction contributed to enhanced binding, we tested the binding of BTE, eIF4E and eIF4G742-1196, the mutant without the eIF4E binding region. There was no increased binding between eIF4G742-1196 and BTE (Figure 21, Table 9).
Figure 19. eIF4E increased eIF4G601-1196 binding to BTE. BTE was labeled with fluorescein. Fluorescence anisotropy was measured at 25 °C when the BTE (50nM in titration Buffer) was titrated by eIF4G601-1196 and eIF4E. The dissociation constant ($K_D$) was obtained from the fit curve as described in Chapter 2 Experimental Procedure. Equilibrium values were averaged from at least 3 independent individual experiments. Error bars represent the standard deviation.
Figure 20. eIF4E increased eIF4G601-1488 binding to BTE. BTE was labeled with fluorescein. Fluorescence anisotropy was measured at 25 °C when the BTE (50nM in titration Buffer) was titrated by eIF4G601-1488 and Δ eIF4G601-1488•eIF4E. The dissociation constant ($K_D$) was obtained from the fit curve as described in Chapter 2 Experimental Procedure. Equilibrium values were averaged from at least 3 independent individual experiments. Error bars represent the standard deviation.
Figure 21. eIF4E failed to increase eIF4G mutant binding to BTE when eIF4E binding region was deleted. BTE was labeled with fluorescein. Fluorescence anisotropy was measured at 25 °C when the BTE (50nM in titration Buffer) was titrated by eIF4G742-1196 and Δ eIF4G742-1496. The dissociation constant (K_D) was obtained from the fit curve as described in Chapter 2 Experimental Procedure. Equilibrium values were averaged from at least 3 independent individual experiments. Error bars represent the standard deviation.
Table 9. eIF4E effects on binding of eIF4G deletion mutants to BTE

<table>
<thead>
<tr>
<th></th>
<th>K_D (nM)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-eIF4E</td>
<td>+eIF4E</td>
<td></td>
</tr>
<tr>
<td>eIF4G601-1196*BTE</td>
<td>40±4</td>
<td>6.8±1.5</td>
<td></td>
</tr>
<tr>
<td>eIF4G601-1488*BTE</td>
<td>68±5</td>
<td>28±6</td>
<td></td>
</tr>
<tr>
<td>eIF4G742-1196*BTE</td>
<td>91±12</td>
<td>101±2</td>
<td></td>
</tr>
</tbody>
</table>

5.1.2 eIF4E increased the binding between BTE mutants and eIF4G mutants

The three eIF4G deletion mutants were also tested for binding with different BTE mutants, which we have reported (57). These BTE mutants vary in translation efficiency and binding affinity to eIF4F. BTEBF mutant has 4 base -GAUC insertion in BTE to SL-I containing the 17nt conserved sequence. BTEBF is translationally inactive mutant, but has been reported to bind with eIF4F with similar affinity as BTE (51, 57). SL-II-m1 mutant has a mutation in stem loop II in which GUUC was changed to GAAC, disrupting the base pairing in the stem. In SL-III-swap mutant is the PAV6 wild type stem loop III replaced by PAV-19 stem loop III, which has an 11-base insertion. Both mutant SL-II-m1 and SL-III-swap have shown weaker binding to eIF4F than BTE and with significantly decreased translation efficiency. In SL-III-3 mutant, 5
nucleotides of stem loop III, which are responsible for forming long distance interaction with 5’ UTR SL-D, were changed from UGUCA to UCAGA. SL-III-3 has been reported to show tighter binding with eIF4F than BTE with increased translation when it was placed in the 5’ UTR of a reporter gene (51, 57). Adding eIF4E increased the binding of eIF4G601-1196 or eIF4G601-1488 to all BTE mutants with the exception of the SL-III-3 interaction with eIF4G601-1196; While eIF4G742-1196 without the eIF4E binding site showed no increased affinity upon addition of eIF4E with any of the BTE mutants (Table 10), further suggesting eIF4E effects on eIF4G deletion mutants were not RNA dependent but dependent on the eIF4E binding region of eIF4G.
Table 10. eIF4E strengthens BTE mutants interaction with eIF4G mutants.

The dissociation constants were obtained at 25 °C. eIF4E and eIF4G mutants were pre-incubated before titrations with \[^{35}\text{S}]\text{BTE}

<table>
<thead>
<tr>
<th>Mutants</th>
<th>eIF4G601-1196</th>
<th>eIF4G601-1488</th>
<th>eIF4G742-1196</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTE</td>
<td>-eIF4E</td>
<td>+eIF4E</td>
<td>-eIF4E</td>
</tr>
<tr>
<td></td>
<td>40±4</td>
<td>6.8±1.5</td>
<td>68±5</td>
</tr>
<tr>
<td>BTEBF</td>
<td>59±6</td>
<td>41±3</td>
<td>67±6</td>
</tr>
<tr>
<td>SL-II-m1</td>
<td>79±2</td>
<td>42±9</td>
<td>75±3</td>
</tr>
<tr>
<td>SL-III</td>
<td>127±11</td>
<td>33±7</td>
<td>139±9</td>
</tr>
<tr>
<td>SWAP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SL-III-3</td>
<td>13±2</td>
<td>14±2</td>
<td>19±2</td>
</tr>
</tbody>
</table>

$K_D$ (nM)
5.1.3 eIF4E induces eIF4G601-1196 conformation change

Our data showed that eIF4E increased the binding affinities of eIF4G mutants to BTE or BTE mutants, and these changes were protein-protein interaction dependent. It further suggested the possibility that eIF4E binding changes eIF4G conformation. CD measurements were used to analyze the changes in secondary structure of eIF4G601-1196 and eIF4E, when they formed a protein complex. The eIF4G601-1196•eIF4E protein complex showed a reduced signal between 200-240nm compared with the sum of spectra of these two individual proteins (Figure 22). This suggested that there was a reduced alpha helix content when the protein complex is formed.

After eIF4G601-1196 interacted with eIF4E, the α-helix content decreased to 37±1% (The α-helix content was calculated from the mean residue ellipticity of each protein spectrum at 222 nm). It has been reported that human eIF4E can induce a short binding motif of eIF4G (12 amino acid residues peptide) folding (87, 88). A yeast 98-amino acid fragment eIF4GI has been reported to undergo a more α-helix conformational shift upon interaction with eIF4E (89).
Figure 22. CD spectra of eIF4E, eIF4G and the eIF4G•eIF4E complex. The concentration of each protein is 10µM. The spectra of eIF4E (■), eIF4G601-1196 (●), eIF4G601-1196•eIF4E complex (▲) are as shown. The sum of isolated eIF4E spectra and eIF4G601-1196 spectra is represented as ◆.

5.1.4 The binding between eIF4G601-1196 •eIF4E and BTE is both entropically and enthalpically favorable.

The equilibrium dissociation constants were measured at different temperatures (Table 11) and thermodynamic analysis was performed. Enthalpy and entropy were obtained from Van’t Hoff
plots (Figure 23). Free energy (ΔG) was calculated at 25°C. The binding between BTE and eIF4G601-1196 was both entropically and enthalpically favorable (Figure 23, Table 12). Enthalpy contributed a large part of the ΔG (81.9±1.6%). Compared to eIF4G601-1196 alone, adding eIF4E increased the enthalpy contribution to 88.4±1.8% and slightly reduced the entropic contribution. eIF4G601-1196 has a higher enthalpic contribution to ΔG than the eIF4F interaction with BTE (around 53% enthalpic contribution). In addition, eIF4G601-1196 binding with BTE is also more enthalpically favorable than eIF4F binding to m^7GTP (90) (no enthalpic contribution) and to PK1 of 5’UTR on TEV (around 40.7% enthalpic contribution) (74)(Table 12). The higher enthalpic value suggests increased hydrogen bonds between RNA and the proteins.

Table 11. Equilibrium binding affinity constants (K_D) for the interaction of ^nBTE and eIF4G 601-1196 and eIF4E at different temperature

<table>
<thead>
<tr>
<th>Complex</th>
<th>5°C</th>
<th>10°C</th>
<th>15°C</th>
<th>20°C</th>
<th>25°C</th>
<th>30°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTE• eIF4G601-1196</td>
<td>7.5±1.2</td>
<td>15±2</td>
<td>23±1</td>
<td>24±2</td>
<td>40.0±4</td>
<td>---</td>
</tr>
<tr>
<td>BTE •eIF4G601-1196</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>5.2±0.8</td>
<td>6.8±1.5</td>
</tr>
<tr>
<td>•eIF4E</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>
Figure 23. Van’t Hoff plots for the interaction of BTE with eIF4G601-1196 and complex eIF4E•eIF4G601-1196 complex  Entropy ($\Delta S$) and enthalpy ($\Delta H$) of the binding (● BTE &eIF4G601-1196, ▲ BTE•eIF4G601-1196•eIF4E) were calculated from the intercept and slope, respectively. Equilibrium values were averaged from at least 3 independent individual experiments. Error bars represent the standard deviation.
Table 12. Thermodynamic parameters for the interaction of $^6$BTE and eIF4G601-1196 and eIF4E

Entropy (ΔS) and enthalpy (ΔH) were determined from Van’t Hoff plot. ΔG was calculated at 25 °C using the equation $ΔG = -RT\ln K_{eq}$

<table>
<thead>
<tr>
<th></th>
<th>BTE•eIF4G601-1196</th>
<th>BTE•eIF4G601-1196</th>
<th>BTE•eIF4F</th>
<th>Pk1•eIF4F</th>
<th>m$^7$GTP•eIF4F</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔH (kJ mol$^{-1}$)</td>
<td>-34.6±1.2</td>
<td>-41.2±0.9</td>
<td>-22.3±2.5</td>
<td>-15.5±1.5</td>
<td>28.7±0.7</td>
</tr>
<tr>
<td>ΔS (J mol$^{-1}$ K$^{-1}$)</td>
<td>25.7±1.8</td>
<td>18.2±2.8</td>
<td>69.2±8.8</td>
<td>76.0±3.6</td>
<td>199±5.0</td>
</tr>
<tr>
<td>ΔG (kJ mol$^{-1}$)</td>
<td>-42.2±0.5</td>
<td>-46.6±0.1</td>
<td>-43.0±0.1</td>
<td>-38.2±0.2</td>
<td>-30.6±0.8</td>
</tr>
<tr>
<td>-TΔS/ΔG</td>
<td>18.1±1.6</td>
<td>11.6±1.8</td>
<td>47.9±6.1</td>
<td>59.3±2.8</td>
<td>--**</td>
</tr>
<tr>
<td>Percentage</td>
<td>18.1±1.6</td>
<td>11.6±1.8</td>
<td>47.9±6.1</td>
<td>59.3±2.8</td>
<td>--**</td>
</tr>
</tbody>
</table>

*The thermodynamic data of the binding between Pk1•eIF4F and the binding between m$^7$GTP•eIF4F were measured in previous studies (74, 90).

**In the binding of m$^7$GTP to eIF4F, the percentage of -TΔS contributing to free energy was more than 100%.
5.1.5 Addition of eIF4E slightly increased eIF4G dependent BlucB translation

Our results showed that eIF4E increased the abilities of two eIF4G deletion mutants eIF4G610-1196 and eIF4G601-1488 to support BlucB translation by 19±10% (Figure 24, Table 13), which was consistent with previous reports (52, 56). In the case of eIF4G742-1196 mutant, which lacks the eIF4E binding site, adding eIF4E did not enhance translation (Figure 24, Table 13). The similar but smaller effects of eIF4E on eIF4G mutants rescue of translation (Figure 25, Table 14) were also observed in the translation of another reporter construct Bluc-SLIII-SWAP, in which mutant SLIII-SWAP replaces BTE in the downstream 3’UTR of luciferase gene. Bluc-SLIII-SWAP has 50% translation ability of BlucB. Therefore, as with the binding affinity, the eIF4E effect on BlucB translation is specifically dependent on the eIF4E binding region of eIF4G.
Figure 24. eIF4E slightly increased BTE-mediated translation in 4F-depleted wheat germ extract. 60nM eIF4F, eIF4G mutant or eIF4G mutant-eIF4E complex were added into 25 μl 4F-depleted wheat germ extract for 20 nM BlucB translation. White columns represent BlucB translation with added eIF4G mutants. Grey shade columns are BlucB translation in presence of eIF4E and eIF4G mutants. Statistical significance testing was analyzed by unpaired t-test. The translation of BlucB when supplement with eIF4E for eIF4G601-1196 or eIF4G601-1488 has statistically significant difference from the translation without eIF4E. ** denotes statistically significant with P value < 0.01; * denotes P value < 0.05.
Table 13. The effect of eIF4E on eIF4G deletion mutants dependent BlucB translation.

The translation ability of 60 nM eIF4F rescue of 20 nM BlucB was set as 100%. All the eIF4G mutants translation activities were normalized to it.

<table>
<thead>
<tr>
<th></th>
<th>BlucB translation (RLU%)</th>
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<tbody>
<tr>
<td></td>
<td>-eIF4E</td>
</tr>
<tr>
<td>eIF4G601-1196</td>
<td>66±5</td>
</tr>
<tr>
<td>eIF4G601-1488</td>
<td>72±4</td>
</tr>
<tr>
<td>eIF4G742-1196</td>
<td>63±10</td>
</tr>
</tbody>
</table>
Figure 25. The effect of eIF4E on IF4G mutants’ rescue of translation was also observed on Bluc-SLIII-SWAP. 60 nM eIF4E was incubated with 60 nM eIF4G mutant and their translation ability of 20 nM Bluc-SLIII-SWAP in 4F-depleted wheat germ extract was measured. Statistical significance testing was analyzed by unpaired t-test. The translation of Bluc-SLIII-SWAP when supplement with eIF4E for eIF4G601-1196 or eIF4G601-1488 has statistically significant different from the translation without eIF4E. ** denotes statistically significant with P value < 0.01.
Table 14. The effect eIF4E on eIF4G deletion mutants translation of Bluc-SLII-m1

The translation ability of 60 nM eIF4F rescue of 20 nM BlucB was set as 100%. All the eIF4G mutants translation activities were normalized to it.

<table>
<thead>
<tr>
<th>Bluc-SLIII-SWAP translation (RLU%)</th>
<th>-4E</th>
<th>+ 4E</th>
</tr>
</thead>
<tbody>
<tr>
<td>eIF4G601-1196</td>
<td>48±1</td>
<td>55±2</td>
</tr>
<tr>
<td>eIF4G601-1488</td>
<td>50±2</td>
<td>56±1</td>
</tr>
<tr>
<td>eIF4G742-1196</td>
<td>44±4</td>
<td>45±2</td>
</tr>
</tbody>
</table>

5.1.6 eIF4E had cooperative function with eIF4A and eIF4B to promote BTE-mediated translation.

Adding eIF4E slightly increased eIF4A-eIF4B-eIF4G601-1196 complex stimulation of BlucB translation from 249±9% to 261±7%. Similarly, supplementation with eIF4E slightly increased eIF4A-eIF4B-eIF4G601-1488 mediated BlucB translation; while no increase of BlucB translation was observed for eIF4G742-1196 (Figure 26, Table 15). Feoktistova et al (86) reported that human eIF4E possesses a function for stimulating eIF4A helicase activity.
independent of its cap-binding function. From our results, wheat eIF4E had a minor effect on enhancing BTE mediated translation in the presence of helicase proteins eIF4A, eIF4B and eIF4G, suggesting that eIF4E may have the cooperative function with eIF4A and eIF4G in cap-independent translation.
Figure 26. eIF4E assisted eIF4A and eIF4B in eIF4G deletion mutants’ rescue of BlucB translation. eIF4G deletion mutants were incubated with eIF4E prior to adding eIF4A and eIF4B. All the eIFs factors were incubated to form the complex before adding to 4F-depleted wheat germ extract. 60nM recombinant eIF4F or eIF4G deletion mutant or protein complex were added in to the 4F-depleted WGE with 20nM BlucB. The data are averaged of at least 3 experiments, error bars represent standard error. White columns represent rescue of BlucB translation by eIF4G deletion mutants alone. Green columns show translation of BlucB in presence of eIF4G mutants along with eIF4E; Blue columns represent restoration of BlucB translation activity in presence of eIF4G with eIF4A-eIF4B. Red columns represent the BlucB
translation activity when 4F-depleted WGE was supplied with eIF4G-4E-4A-4B protein complex. Statistical significance testing was analyzed by unpaired t-test. The translation of BlucB when supplement with eIF4E, eIF4G601-1488, eIF4A and eIF4B has statistically significant difference from the translation when supplement with eIF4G601-1488, eIF4A and eIF4B but no eIF4E. * denotes statistically significant with P value < 0.05.

Table 15. The abilities of eIF4G deletion mutants with other initiation factors to stimulate BTE-mediated translation in 4F-depleted wheat germ extract

The translation ability of 60 nM eIF4F rescue 20 nM BlucB was set as 100%. All the eIF4G mutants translation activities were normalized to it.

<table>
<thead>
<tr>
<th></th>
<th>+4E</th>
<th>+4A 4B</th>
<th>+4E 4A 4B</th>
</tr>
</thead>
<tbody>
<tr>
<td>eIF4G601-1196</td>
<td>66±5</td>
<td>86±3</td>
<td>249±9</td>
</tr>
<tr>
<td>eIF4G601-1488</td>
<td>72±4</td>
<td>88±9</td>
<td>231±8</td>
</tr>
<tr>
<td>eIF4G742-1196</td>
<td>63±10</td>
<td>60±2</td>
<td>230±12</td>
</tr>
</tbody>
</table>
5.2 Discussion

Most human mRNAs have a structured m\(^7\)G cap in the 5’ end, which is active for interacting with eIF4E (91, 92). The m\(^7\)G cap of mRNA binds to eIF4E, which associates with eIF4G. The binding of eIF4E to m\(^7\)G cap can be increased by the presence of eIF4G, which serves as the connector recruiting other initiation factors such as eIF4A, eIF4B, PAPB and eIF3h (93). The cap-dependent translation is the primary way for most mammalian cellular mRNA translation.

It has been reported (16) mammalian viruses utilize eIF4E in their cap-independent translation. For example, Rhinovirus employs an internal ribosomal entry site translation mechanism. It is reported (61) that human eIF4E induced an eIF4G conformation change and increased its sensitivity to Rhinovirus protease cleavage. Rhinovirus protease cleaved eIF4G at R641/G642 to separate the N-terminal with eIF4E binding domain and the C-terminal with eIF4A and eIF3 binding domain. The cleavage impaired the formation of eIF4G/eIF4F directed complex in the translation initiation step, which also made eIF4G fail in recruiting eIF4E, eIF4A, eIF4B and eIF3 simultaneously (61). Cleavage resulted in a shut-off of host cellular cap-dependent translation; while the cleft C-terminal eIF4G fragment was sufficient for viral RNA IRES-mediated translation (61, 89, 94).

However there is no report of BYDV viral protease cleavage of eIF4G. BYDV takes a different mechanism to utilize host plant eIF4E for viral mRNA translation. Our results reveal that wheat eIF4E, the cap-binding protein, is involved in the cap-independent translation of BYDV mRNA. eIF4E significantly increased the binding of eIF4G mutants to BTE and slightly elevated BTE mediated cap-independent translation (around 20%).
The eIF4E effect of increasing the binding between eIF4G and BTE depended on the eIF4E binding region of eIF4G, not the interaction between eIF4E and RNAs. A number of BTE mutants, with varying secondary structure, showed an increase in binding to eIF4G mutants, which contain the eIF4E binding site. There is no report that eIF4E directly interacts with BTE. It may act as a molecular chaperon for its partner eIF4G to influence it binding to BTE. We observed eIF4E induced a change in secondary structure of eIF4G601-1196 by CD measurements. The new conformation of eIF4G601-1196 has less helix content and possibly exposes more regions to RNA for tighter binding.

In addition, the thermodynamic data showed an increased enthalpical contribution (88.4±1.8% compared to 81.9± 1.6%) to free energy of the binding between eIF4G601-1196 •eIF4E and BTE. The higher enthalpical value suggests more hydrogen bonds.

Feoktistova et al (86) found that human eIF4E stimulated eIF4A helicase complex activity and increased its RNA unwinding rate (86). They proposed that eIF4E binding site on human eIF4G functions as an auto-inhibitory domain. In the absence of eIF4E, eIF4E binding domain maintains a conformation of eIF4G with low eIF4A helicase stimulating activity; the binding of eIF4E to the inhibitory domain counteracts the auto-inhibition function and enable eIF4G to stimulate eIF4A helicase activity.

Our results show that wheat eIF4E also has cooperative function with eIF4A and eIF4B in stimulating eIF4G mutants rescue of BTE mediated translation. Protein eIF4A-eIF4B-eIF4G mutant-eIF4E complex had stronger translation restoring ability for BlucB than the protein complex eIF4A-eIF4B-eIF4G mutants without eIF4E. This suggests that plant eIF4E assists eIF4A in stimulating eIF4G dependent BTE mediated translation.
CHAPTER 6. CONCLUSION

Viruses evolve different mechanisms to sequester host cellular apparatus and start viral protein synthesis. The most common strategy is manipulation of the key translation factors or ribosomes during the initiation step. Like many plant viruses, BYDV lacks the 5’ end m\(^7\)G cap and a poly A tail in the 3’ UTR, but possesses a special 3’cap-independent translation element–BTE to regulate viral mRNA translation.

Host cellular initiation factor eIF4G has been utilized by BTE as the target to gain control of the translational machinery. Previous study (52, 57) reported that BTE mediated translation is eIF4G dependent and BTE specifically interact with eIF4G in wheat germ extract. In this study, we made investigations on how BTE utilize wheat eIF4G different domains to interact and to regulate translation.

First, we found BTE only needs a short region of middle parts of eIF4G for binding. This short fragment eIF4G601-1196 sufficiently rescues BTE-mediated translation in 4F-depleted wheat germ extract. In other words, the N-terminal and C-terminal of eIF4G are not required for interaction with BTE. The binding between BTE and eIF4G601-1196•eIF4E complex is much tighter than the binding between 5’ m\(^7\)G cap and eIF4E/eIF4F. The tight binding to the initiation factor probably makes BTE occupy host plant translation machinery.

eIF4A and eIF4B binding to the central domain on eIF4G bring benefits for the interaction of eIF4G to BTE. eIF4A directed helicase complex increases eIF4G binding to BTE and stimulates eIF4G dependent BTE mediated; while the second eIF4A binding domain in C-terminal of eIF4G does not assist the binding between eIF4G and BTE. Its binding to eIF4A may have other functions during translation.
eIF4E, the cap binding protein, when interacting with its binding domain on eIF4G, contributes to the binding of eIF4G and BTE as well. eIF4E significantly tightens the binding between eIF4G mutant and BTE by inducing eIF4G conformational transition. It slightly enhances eIF4G initiating BTE regulated translation. The reason why eIF4E does not stimulate translation as strongly as eIF4A and eIF4B is that eIF4E has no influence on ribosome binding; while the helicase complex (eIF4A-eIF4B-ATP) significantly increases the binding between BTE, eIF4G and 40S ribosomal subunit (55).

BTE recruits translation initiation factor eIF4G and ribosomal small subunit at the 3’ end of BYDV viral mRNA. eIF4G, the scaffold protein, serves as a bridge for several translation initiation factors such as eIF4A, eIF4B and eIF4E and utilizes its different binding domains to recruit them. Through the long distance 3’-5’ RNA-RNA base paring loop, the translation machinery is delivered to 5’ end of viral mRNA at where the pre-initiation complex start ribosome scanning. Our results revealed that BTE chooses eIF4G as the strategy for initiating viral mRNA translation because it can take advantage of the connector function of eIF4G (Figure 27). In this scenario, when BTE interacts with eIF4G, it also benefits from the indirect interactions from eIF4A, eIF4B and eIF4E. The cooperative effects from multiple initiation factors amplify the viral protein synthesis.
Figure 27. The model of BTE mediated Cap-independent translation  BTE tightly binds to eIF4G, which is the bridge for several initiation factors such as eIF4A, eIF4B and eIF4E. The binding between BTE and eIF4G can be tightened by eIF4A directed helicase complex and eIF4E. The helicase complex also increases the binding between BTE and the 40S ribosomal subunit. Except influences on binding, eIF4G dependent BTE mediated translation is stimulated by these initiation factors. BYDV viral mRNA translation is a cooperative result from cooperative multiple initiation.

In this dissertation, we provide more details for the mechanism of how BTE utilizes eIF4G different domains to coordinate with other initiation factors to maximize viral protein synthesis. The data presented here are the first to address eIF4A directed helicase complex stimulation of
BTE mediated translation and to identify the eIF4A binding domains required for helicase complex function on wheat eIF4G.

The data present in this dissertation also provide insights for developing approaches in applied science. First, the binding affinities between BTE and eIF4G different fragments were measured. These data may contribute to screen ligands based on binding affinities. Compared with the binding affinities we provided, it is possible to find some chemical compound ligands or peptide ligands which can bind to BTE but not to host plant eIF4G. It may help in developing the method for inhibiting BYDV viral proliferation without damage to the host plant. Second, in recent studies, some viral RNA translation control elements are used to enhance expression of aimed gene. For example, viral vectors which contain tobacco mosaic virus (TMV) and potato virus X (PVX) viral RNA elements are used for in vivo heterologous expression the antibody for Ebola virus (95). In future studies, we may investigate the possible whether BTE can be used as a translation enhancer in heterologous expression system. We also can investigate the possibility of whether or not the co-expression of initiation factors with BTE controlled target gene brings a stronger translation.
APPENDIX

1.PCR protocols

1.1 PCR protocols for amplifying template for GST-eIF4G601-1196 and GST-eIF4G601-1488 protein express constructs

Template: eIF4F cDNA expression construct

eIF4G742-1196 forward primer: GAAGGATCCTTCAAAGATTTGGCAGG

eIF4G742-1196 reverse primer: CCTGGATCCTCAAAGGGGAACAGTTC

eIF4G601-1488 forward primer: TTAAGGGATCCAAGAAGAAACGGAAGG

eIF4G601-1488 reverse primer: GCTGGATCCCTATTAAGTCAACATGAAG

PCR reaction system

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<tr>
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<tr>
<td>10 mM dNTP</td>
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<tr>
<td>Forward Primer (5 µM)</td>
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</tr>
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<td>DMSO</td>
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<td>Enzyme Phusion</td>
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<td>H₂O</td>
<td>X µL</td>
</tr>
<tr>
<td></td>
<td>20 µL</td>
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PCR cycling conditions for amplifying eIF4G742-1196

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<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
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<tr>
<td>Denaturation</td>
<td>98°C</td>
<td>1 min</td>
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<tr>
<td>Denaturation</td>
<td>98°C</td>
<td>5s</td>
<td>34</td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C</td>
<td>15s</td>
<td>34</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1min30s</td>
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</tr>
<tr>
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<td>10°C</td>
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PCR cycling conditions for amplifying eIF4G601-1488

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</tr>
<tr>
<td></td>
<td>10°C</td>
<td>Hold</td>
<td>1</td>
</tr>
</tbody>
</table>

1.2 Site directed mutagenesis PCR protocol for generating Bluc-SLII-m1 report construct

Template: BlucB report construct

Forward Primers TTCGGAACATAAGCTCGGGTGGCT

Reverse Primer GTTCGCTGCTTTCCCCAGGATCG
25 µL rxn

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<td>Forward Primer (10µM)</td>
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<tr>
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</tr>
<tr>
<td>Template DNA</td>
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<tr>
<td>H2O</td>
<td>X µL</td>
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<td></td>
<td>25 µL</td>
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PCR cycling conditions for amplifying Bluc-SLII-m1

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<td>30s</td>
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<tr>
<td><strong>Denaturation</strong></td>
<td>98°C</td>
<td>10s</td>
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<tr>
<td><strong>Denaturation</strong></td>
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<td>2min30s</td>
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<tr>
<td><strong>Final Extension</strong></td>
<td>72°C</td>
<td>10min</td>
</tr>
<tr>
<td></td>
<td>10°C</td>
<td>Hold</td>
</tr>
</tbody>
</table>

2. Protein culture and Purification protocols for GST-eIF4G601-1488 and GST-eIF4G742-1196
elf4G742-1196/ elf4G601-1488 protein express construct plasmid is transferred into BL21(D3) Cells. Single clone is picked to overnight culture at 37°C. 1:100 diluted into large culture (1.5Litter) till OD$_{600}$ till 0.7.

The culture is induced by 0.5mM IPTG at 30°C for 2.5 hours. Harvest Cells by centrifuge at 6000rpm for 15min.

Resuspend cells in sonification buffer (50mM Tris-Cl, 500mM NaCl 10% Glycerol) with Roche protease inhibitor tablet. Keep on ice and sonicate at AMPL 30%, 1 second pulse on and 3 seconds pulse off for 15 minutes.

After sonication, centrifuge cell lysis for 1.5 hours at 13000rpm. Collect the supernatant.

Loading the supernatant onto a 1ml GST-Trap affinity exchange column, which has already been pre-equilibrium by PBS (Nacl 140mM, KCl 2.7mM, Na$_2$HPO$_4$ 10mM, KH$_2$PO$_4$ 1.8mM, 10mM DTT, PH 7.4.).

After binding the expressed protein in to the GST affinity column. 20mL PBS is used to wash the column.

5 ml Elution Buffer (50mM Tris-HCl, 10mM reduced glutathione PH8.0) is used to elute the GST-elf4G fusion protein.

Dialyse the elution collection again 2Litter PBS overnight and examine the purified protein by 8% SDS-PAGE gel electrophoresis.
FIGURE 28. Purified-Protein GST-eIF4G742-1196

Lane 1 is protein pre-stain ladder. Lane 2 and Lane 3 are the Purified protein GST-eIF4G742-1196

Figure 29. Purified-Protein GST-eIF4G601-1488

Lane 1 is protein pre-stain ladder. Lane 2 is the Purified protein GST-eIF4G601-1488
3. RNA transcription protocol

3.1 BTE or BTE mutant RNA oligos in vitro transcription

<table>
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<tr>
<th>Component</th>
<th>Amount</th>
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<tbody>
<tr>
<td>T7 10X Reaction Buffer</td>
<td>2 µL</td>
</tr>
<tr>
<td>T7 ATP solution (75mM)</td>
<td>2 µL</td>
</tr>
<tr>
<td>T7 GTP solution (75mM)</td>
<td>2 µL</td>
</tr>
<tr>
<td>T7 CTP solution (75mM)</td>
<td>2 µL</td>
</tr>
<tr>
<td>T7 UTP solution (75mM)</td>
<td>2 µL</td>
</tr>
<tr>
<td>Template dsDNA</td>
<td>&lt; 8 µL  (100 nM)</td>
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<tr>
<td>T7 Enzyme Mix</td>
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<tr>
<td>H₂O</td>
<td>X µL</td>
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</table>

20 µL

Reaction incubates at 37°C overnight. TURBO DNase 1 µL is added into the reaction for incubating at 37°C for 15 min.

3.2 BlucB and Bluc-SLI1-m1 mRNA in vitro transcription

<table>
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<th>Component</th>
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<tbody>
<tr>
<td>T7 10X Reaction Buffer</td>
<td>2 µL</td>
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<tr>
<td>T7 ATP solution (75mM)</td>
<td>2 µL</td>
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<tr>
<td>T7 GTP solution (75mM)</td>
<td>2 µL</td>
</tr>
<tr>
<td>T7 CTP solution (75mM)</td>
<td>2 µL</td>
</tr>
<tr>
<td>T7 UTP solution (75mM)</td>
<td>2 µL</td>
</tr>
<tr>
<td>Linear Template dsDNA</td>
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<td>T7 Enzyme Mix</td>
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<tr>
<td>H₂O</td>
<td>X µL</td>
</tr>
</tbody>
</table>

20 µL

Reaction incubates at 37°C overnight, TURBO DNase 1 µL is added into the reaction for incubating at 37°C for 15 min.
4. RNA Phenol-Chloroform purification protocol

Add 100 µL nuclease-free water and then an equal volume of phenol/chloroform (120 µL).
Centrifuge 15 min at 13000 rpm

Take the upper aqueous phase (upper layer) and transfer to a new tube.

Precipitate the RNA by adding 2 volumes of ethanol and 10% volume 3M sodium acetate, mixing well. Chill the at −20°C for 2 hours.

Centrifuge for 30 minutes at 13000 rpm, decant the supernatant.

70% ethanol rinse the pellet and then centrifuge at 13000 rpm for 15 minutes.

Decant the supernatant and dry the pellet. dissolve the RNA in nuclease free water.

5. Depleted eIF4F from Wheat germ extract by M^7GTP Beads

200 µL M^7GTP Beads are washed with 1ml 1× PBS (containing 0.5% Tween 20) three times by centrifuge at 500g for 3 minutes

200ul wheat germ extract is incubated with pre-washed m^7GTP beads at 4 °C for 1 hour.

Spin the incubation mixture with 500g for 5 minutes and collect the supernatant.

The supernatant is aliquot to 25 µL for immediate use or stored at -70°C.
**6. *in vitro* translation**

<table>
<thead>
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<th>Component</th>
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<tr>
<td>Wheat germ extract or 4F-depleted wheat germ extract</td>
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</tr>
<tr>
<td>Amino Acid Mixture Minus Methionine (1mM)</td>
<td>2μL</td>
</tr>
<tr>
<td>Amino Acid Mixture Minus leucine (1mM)</td>
<td>2μL</td>
</tr>
<tr>
<td>Potassium Acetate (1M)</td>
<td>3μL</td>
</tr>
<tr>
<td>RNasin Ribonuclease Inhibitor</td>
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</tr>
<tr>
<td>RNA template</td>
<td>Final concentration 20nM</td>
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<td>Nuclease-free H₂O</td>
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<tr>
<td>Recombinant eIF4F</td>
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<tr>
<td>Recombinant eIF4G mutant</td>
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<tr>
<td>Recombinant eIF4E</td>
<td>-/ Final concentration 60nM</td>
</tr>
<tr>
<td>Recombinant eIF4A</td>
<td>-/ Final concentration 1800nM</td>
</tr>
<tr>
<td>Recombinant eIF4B</td>
<td>-/ Final concentration 600nM</td>
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</table>

Reaction is incubated at 25°C for 1.5 hours. Luciferase subtract reagent is pre-warm at room temperature for 30 minutes. 3 μL of reaction is mixed with 50 μL luciferase subtract reagent. A Glomax-96 microplate illuminometer is used to examine the luciferase expression intensity. The illuminometer program is performed with a 2-second measurement delay followed by a 10-second measurement read.
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