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Caractérisation de l'interaction entre la protéine virale VPg et la protéine de l'hôte eIF4E conduisant à l'infection du virus de la mosaïque du navet.

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Liste des abréviations

4E-BP :	"4E binding protein" (protéine de liaison au facteur 4E)
6K :	protéine de 6 kDa
aa :	acide aminé
ARN :	acide ribonucléique
ARNm :	ARN messager
ARNt :	ARN de transfert
BMV :	"brome mosaic virus" (virus de la mosaïque du brome)
CBP :	cap binding protein (protéine de liaison à la coiffe)
CI :	cylindrical inclusion (corps d'inclusion cylindriques)
CMV :	cucumber mosaic virus (virus de la mosaïque du concombre)
CP :	capside
D.O. :	densité optique
eIF :	facteur eucaryotique d'initiation de la traduction
ELISA :	"enzyme-linked-immunosorbent assay"
EMCV :	" encephalomyocarditis virus" (virus de l'encéphalomyocardite)
GAPDH	glycéraldehyde-3-phosphate déhydrogenase)
HC :	"helper component" (protéine auxiliaire)
IRES :	"internal ribosome entry site" (site d'entrée interne du ribosome)
kb :	kilobase
K_d :	constante de dissociation
kDa :	kilo Dalton
LCMV :	"lymphocytic choriomeningitis virus"
MAPK :	"mitogen activated protein kinase"
MNK1 :	"MAP kinase interacting kinase 1"
NIa :	corps d'inclusion nucléaire a
NIb :	corps d'inclusion nucléaire b
nm :	nanomètre
nt :	nucléotide
NTR :	séquence non traduites
P1 :	protéine N-terminale
PABP :	"poly(A)-binding protein"
Pb :	paire de base
PCR :	"polymerase chain reaction"
PKC :	protéine kinase C
PPV :	"Plum pox virus" (virus variolique de la prune)
Pro :	protéase
PSbMV :	"pea seed-borned mosaic virus" (virus de la mosaïque de l'embryon du pois)
RdRp :	"RNA dependant RNA polymerase" (ARN dépendante ARN polymérase)
SDS-PAGE :	"SDS-polyacrylamide gel electrophoresis"
Ser :	sérine
TEV :	"tobacco etch virus" (virus de la rayure du tabac)
Thio :	thioredoxin
TMV :	"tobacco mosaic virus" (virus de la mosaïque du tabac)

ToRSV:	"Tomato ringspot virus"
Trp :	tryptophane
TuMV :	"turnip mosaic virus" (virus de mosaïque du navet)
Tyr :	tyrosine
VPg :	"viral protein genome-linked" (protéine virale liée de façon covalente à l'ARN)
VPgPro :	protéine VPg à activité protéolytique
ZYMV :	"zucchini yellow mosaic virus" (virus de mosaïque jaunissante de la courgette)

Sommaire

Le virus de la mosaïque du navet (TuMV) appartient au potyvirus qui est le plus important groupe de virus infectant les plantes. Cependant, les mécanismes moléculaires conduisant à une infection virale sont très peu connus chez les potyvirus. Dans ce présent ouvrage, le processus de réplication/traduction conduisant à l'infection du virus a été particulièrement approfondi en utilisant comme modèle le TuMV.

Nous savons qu'une protéine du TuMV, la VPg, interagit avec le facteur initiant la traduction cellulaire 4E chez les plantes. Nous ignorons cependant les conséquences et l'implication de cette interaction. Un de nos objectifs fut donc de caractériser le domaine d'interaction de la VPg pour l'eIF4E. Des travaux de délétions en double-hybride ont permis de cibler chez la VPg une région centrale d'une trentaine d'acides aminés responsables de la liaison avec l'eIF4E. Des études de mutagénèses dirigées dans ce domaine ont également identifié l'acide aspartique en position 77 de la VPg comme étant nécessaire pour l'interaction avec l'eIF4E.

L'eIF4E reconnaît la coiffe des ARNm cellulaires pour ensuite enclencher le processus de traduction cellulaire. Nous nous sommes interrogés à savoir si la VPg, située à l'extrémité 5' de l'ARN viral, ne pourrait pas compétitionner avec la coiffe située à l'extrémité 5'de tous les ARNm cellulaires pour la liaison de l'eIF4E. Nous avons montré *in vitro* à l'aide d'un test de type ELISA que l'ajout de m^7GTP (un analogue de la coiffe) diminue grandement l'interaction entre l'eIF4E et la VPgPro.

De plus, nous avons fait acquisition d'un plasmide infectieux du TuMV , un outil indispensable pour étudier l'étude de l'expression et de la réplication des virus à ARN(+). Nous avons muté, dans ce plasmide, l'acide aspartique en position 77 de la VPg nécessaire pour l'interaction avec l'eIF4E. Nous avons procédé ensuite à l'infection de plantes sensibles au TuMV par la technique du bombardement particulaire. Les plantes infectées par ce clone ne présentaient aucun symptôme d'une infection virale suggérant une corrélation entre l'interaction VPg-eIF4E et la virulence du virus.

La réplication du potyvirus s'assemble dans le cytosol de la cellule sur la membrane du réticulum endoplasmique. Nous avons montré que la 6KVPgPro et l'eIF4E sont co-localisées aux membranes du réticulum endoplasmique. Ce résultat suggère que l'eIF4E et la VPgPro sont toutes deux impliqués lors de la réplication du virus.

De nombreuses études démontrent que la traduction cellulaire s'effectue selon un mécanisme circulaire. La queue de poly(A) présente à l'extrémité 3' des ARNm cellulaire interagit avec les protéines initiant la traduction cellulaire (eIF) par l'intermédiaire de la *poly(A) binding protein* (PABP). Plusieurs virus d'animaux utilisent aussi une stratégie similaire (la circularisation) pour traduire leur ARN et maximiser la production de protéines virales. Bien peu de données sont toutefois connues pour ce qui est des potyvirus. Nous avons donc effectué des expériences *in vitro* en utilisant des protéines virales, comme la VPgPro et la Polymérase, afin d'établir des liens avec les protéines nécessaires à la traduction cellulaire comme l'eIF4E, eIF4G et la PABP. Avec l'aide d'un test de type

ELISA, nous avons montré une nouvelle interaction entre la VPgPro et la PABP. Nos résultats ont indiqué que le domaine Pro est requis pour l'interaction avec la PABP. Nous avons également vérifié l'effet de l'ajout de la polymérase virale sur la formation du complexe VPgPro-PABP. Nos résultats démontrent que l'addition de la polymérase n'affecte pas l'interaction entre la PABP et la VPgPro. Cependant, à l'inverse, l'addition de PABP empêche la polymérase de lier la VPgPro, suggérant que la PABP compétitionne avec la polymérase pour la liaison à la VPgPro. Ces résultats nous permettent d'établir un modèle de réplication/traduction du TuMV .

Les VPgPro de potyvirus et les VPgPro d'autres virus n'appartenant pas à ce groupe sont très variables lorsqu'on compare leur taille ou leur séquence en acides aminés. Ceci est notamment le cas entre la VPgPro du TuMV et celle du tomato ringspot virus (ToRSV) du genre népovirus. Nos travaux ont indiqué que la VPgPro du népovirus interagissait avec l'eIF(iso)4E de blé. Ces résultats ont été montrés avec l'aide d'un test ELISA et ont été confirmés par des collaborateurs avec la technique du Far-Western. Le domaine d'interaction a été ciblé à la partie Pro. En ELISA, cette interaction est sensible à l'ajout de M^7GTP, ce qui suggère que la VPgPro du népovirus peut compétionner avec la coiffe des ARNm pour la liaison avec l'eIF4E. Ces résultats suggèrent également que l'interaction VPgPro-eIF4E est un mécanisme universel, à tout le moins pour les virus phytopathogènes.

Introduction

Le virus de la mosaïque du navet infecte au moins 299 espèces végétales appartenant à 147 genres dans 39 familles. La famille des crucifères est la plus touchée par le TuMV (Edwardson et Christie, 1986). Le TuMV possède un spectre d'hôtes extrêmement large et s'attaque à des espèces ayant une importance économique comme le colza, le rutabaga, le chou-fleur, le brocoli, la laitue, etc. Les symptômes causés par le TuMV sont variés : lésions locales, infection systémique avec apparition de mosaïques, arrêt de croissance, déformation des feuilles et formation de corps d'inclusion.

La structure génomique des potyvirus est particulière : contrairement à tous les ARNm cellulaires et à la plupart des ARNm viraux, l'extrémité 5' des potyvirus n'est pas liée par une coiffe, mais plutôt par une VPg (viral protein genome linked). En plus de jouer un rôle protecteur contre les nucléases, la VPg pourrait être requise pour la réplication du virus et a été retrouvée comme étant le déterminant pathogénique de certains potyvirus (Johansen et al., 2001 ; Borgstrom et Johansen , 2001 ; Keller et al., 1998 ; Nicolas et al., 1997).

Il a récemment été démontré que la VPg du virus de la mosaïque du navet (TuMV) interagit avec le facteur eucaryotique d'initiation de la traduction 4E (eIF4E) (cap binding protein) d'*Arabidopsis thaliana* (Wittmann et al., 1997). Cette interaction a été montré non seulement dans la levure à l'aide du système double-hybride, mais également *in vitro* à l'aide d'un test de type ELISA. L' eIF4E est une protéine cellulaire essentielle. Présente en quantité restreinte dans la cellule, elle joue un rôle clé et limitant pour la traduction des ARNm cellulaires (Hershey, 1991).

Nous ne connaissons pas les conséquences de l'association VPg-eIF4E. Cependant, plusieurs travaux laissent croire que cette interaction serait importante pour l'établissement de l'infection par le TuMV. En effet, certains virus animaux ont élaboré des stratégies permettant la traduction préférentielle de leurs ARNm, bloquant ainsi la traduction des ARNm cellulaires.

Dans bien des cas, les facteurs initiant la traduction cellulaire sont des cibles de choix pour ces virus. Par analogie à ce que l'on retrouve chez le virus de l'influenza, le virus de l'encéphalomyocardite et l'adénovirus, nous croyons que la VPg agit sur l'eIF4E de façon à modifier l'activité de ce facteur d'initiation de la traduction. Ainsi, la traduction des messagers cellulaires serait bloquée mais non celle du virus. De plus, une autre protéine d'un virus humain, le Lymphocytic choriomeningitis virus (LCMV), interagit avec le facteur eIF4E et inhibe sélectivement la synthèse protéique (Campbell Dwyer et al., 2000). Cette protéine du LCMV nommé "RING Z protein" supprime la transformation oncogénique en réduisant l'affinité de l'eIF4E pour l'ARNm (Cohen et al., 2001). Par ailleurs, d'autres virus utilisent les facteurs initiant la traduction pour circulariser leur ARNm. Des modèles circulaires de réplication/traduction ont même été esquissés pour ces virus.

Le processus par lequel un virus génère une infection virale chez les plantes est moins bien connu que pour les virus de mammifères. Nos travaux sur le TuMV nous permettrons de mieux comprendre le mode de réplication des potyvirus.

Les résultats obtenus sont présentés sous forme d'articles scientifiques. Deux de ces articles ont déjà été publiés et un autre a été soumis. Ces articles remplacent la section matériel et méthode ainsi que la section résultat retrouvées dans une thèse conventionnelle. Cette thèse est divisée en quatre chapitres. Le premier est

une revue bibliographique sur le sujet de mon projet de doctorat. Je traiterai donc des potyvirus, plus particulièrement du virus de la mosaique du navet et de la protéine VPg. Nous verrons ensuite les mécanismes d'initiation de la traduction cellulaire en mettant l'accent sur le rôle de la protéine eIF4E. Finalement, nous pourrons voir comment certains virus mobilisent les facteurs initiant la traduction cellulaire pour mettre à profit la traduction virale. Le deuxième chapitre traite de l'étude de l'interaction entre la VPg du virus de la mosaïque du navet (TuMV) et le facteur eucaryotique inititiant la traduction cellulaire 4E. Le troisième chapitre est l'étude du mécanisme d'infection du potyvirus : l'effet sur l'expression des isomères d'eIF4E et interaction entre la VPgPro et la PABP "poly(A) binding protein". Le quatrième chapitre est l'étude de l'intéraction entre la VPgPro du tomato ringspot virus du genre népovirus et l'eIF(iso)4E de blé.

Finalement, la thèse se terminera par une discussion et par une conclusion générale.

Chapitre 1:

Revue bibliographique

1.Potyvirus

1.1 Généralités

Le groupe des potyvirus est le plus grand des 34 groupes de virus plantes connues à ce jour (Ward et Shukla, 1991). Ce groupe contient plus de 180 membres (30 % de tous les virus connus de plantes) et cause des dommages significatifs, que ce soit dans le domaine de l'agriculture ou de l'horticulture (Ward et Shukla, 1991). Le potyvirus est très similaire en termes de structure génomique et de stratégie d'expression aux comovirus, aux népovirus et aux picornavirus. Il a donc été proposé que les como, népo et potyvirus soient associés au supergroupe des picorna-like (Goldbach, 1987; Goldbach, 1992).

Le potyvirus est un virus non-enveloppé (Langenberg et Zhang, 1997) et se présente sous forme de particules filamenteuses de 680 à 900 nm de long et 11 à 15 nm de large (Carrington et Dougherty, 1988; Riechmann, et al., 1992). Il produit dans les cellules infectées, des corps d'inclusion au niveau cytoplasmiques et nucléaires. Le génome viral est composé d'une molécule d'ARN simple brin de polarité positive d'environ 10 000 nucléotides. Environ 2000 copies de la protéine de la capside (CP) sont assemblées autour de l'ARN viral.

Nos travaux ont été effectués sur un potyvirus, soit le virus de la mosaïque du navet (TuMV). Il sera décrit dans la prochaine section.

1.2 Virus de la mosaïque du navet.

1.2.1 Structure génomique

Le génome du virus de la mosaïque du navet contient une séquence 5' non traduite (NTR) de 129 nucléotides (nt), un cadre de lecture ouvert de 9489 nt, une séquence 3' NTR de 209 nt suivie d'une queue poly(A) (voir figure 1) (Nicolas et Laliberté, 1992). Le TuMV est également caractérisé par la présence d'une VPg liée à l'extrémité 5' de l'ARN génomique

1.2.2 Polyprotéine

Le génome du TuMV possède un seul cadre de lecture ouvert et est traduit en une unique polyprotéine de 3863 acides aminées (aa) ayant une masse moléculaire de 358 kDa (figure 1). Cette polyprotéine est ensuite clivée, libérant ainsi des protéines structurales ou non-structurales. L'activité protéolytique est générée par trois protéases virales qui agissent en cis et/ou en trans sur des sites spécifiques situés sur la polyprotéine. Les protéases virales sont la protéine N-terminale (P1), la protéine auxiliaire (HC :helper component) et la NIa (ou VPgPro) (Riechmann et al., 1992). Les autres protéines relâchées sont la P3, la CI, la RdRp (pol), la CP ainsi que deux autres petites protéines 6K1 et 6K2 (Riechmann et al., 1992 ; Langenberg et Zhang, 1997). Les fonctions possibles attribuées aux protéines des potyvirus sont résumées dans le tableau I.

Parmi ces protéines, la VPgPro a été l'object d'étude approfondie par notre laboratoire. Dans les prochaines sections, nous décrirons différents aspects de cette protéine.

Figure 1 : Organisation du génome et de la polyprotéine des potyvirus. Le génome viral d'une longueur de 9830 nucléotides est traduit en une seule polyprotéine de 348 kDa.

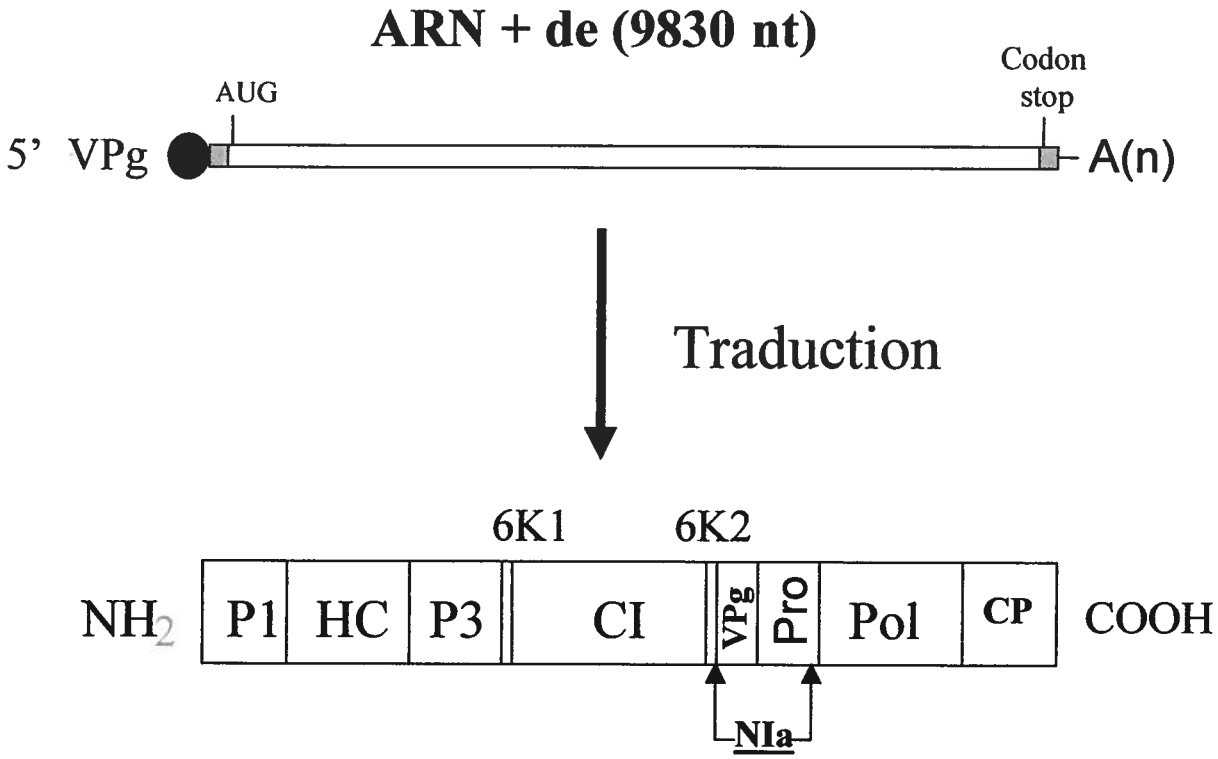

Tableau I : Résumé des fonctions possibles attribuées aux protéines des potyvirus.

Protéines	Fonctions
P1 Protéine N-terminale 1	Protéase Liaison aux acides nucléiques Transport de cellule - cellule
HC Helper component	Protéase Propagation par les insectes Mouvement sur longue distance du virus Aide à la réplication du virus Suppresseur du "gene silencing" posttranscriptionnelle
P3	Aucune connue
6K1, 6K2	Facteurs impliqués dans la réplication
CI Cylindrical inclusion	Hélicase
VPg Viral protein genome-linked	Liée à l'extrémité 5' de l'ARN Déterminant pathogénique Facteur initiant la réplication
Pro Protéase	Protéase
Pol (NIb) RNA dépendant RNA polymérase	Réplication de l'ARN viral
CP Capsid protein	Mouvement cellule-cellule du virus Mouvement sur longue distance Enrobage de l'ARN génomique

1.2.3 Protéine VPg

1.2.3.1 Propriétés physiques

Lors de la traduction virale, la VPg est libérée suite à l'autoprotéolyse de son précurseur de 49 kDa, la protéine NIa (nuclear inclusion protein a1). La VPg constitue la partie N-terminale de la protéine NIa. Une protéase pro (trypsine like sérine protéase), du côté carboxyle, est l'autre composante de la NIa. La VPg du virus de la mosaïque du navet est constitué d'une séquence de 196 acides aminés. Elle est similaire aux autres VPg de potyvirus lorsqu'on compare leurs séquences en acides aminés (voir tableau II). Ce tableau nous indique que 19 % des acides aminés sont identiques chez les 10 VPg de potyvirus étudiées. De plus, ce chiffre augmente à 38 % lorsqu'on compare leurs caractères hydrophobique-hydrophile (Plochocka et al., 1996). Les VPg de potyvirus ont également en commun un site de localisation nucléaire situé à l'extrémité C-terminale de la protéine. Pour la plupart des potyvirus la VPg est co-localisée avec la RdRp sous forme de corps d'inclusion dans le noyau des cellules infectées. Cependant des études plus détaillées ont indiqué que cette localisation peut varier selon les potyvirus (Riedel et al., 1998).

La VPg se retrouve également fixée à l'extrémité 5' de l'ARN viral par un lien covalent; un lien phosphodiester entre le groupement phosphate de l'ARN et le groupement hydroxyle d'un acide aminé de la VPg. Des études ont montré que le résidu tyrosine en position 1860 (Tyr 1860) de la polyprotéine est responsable du lien entre la VPg et l'ARN viral (Murphy et al., 1996). Cette tyrosine est également conservée chez tous les autres potyvirus (voir tableau II).

Le rôle que joue la VPg n'est pas clairement établi. Cependant de nombreuses fonctions ont été attribuées à la VPg.

Figure 2 : Précurseur de la VPg ; NIa protéine de 49 kDa.

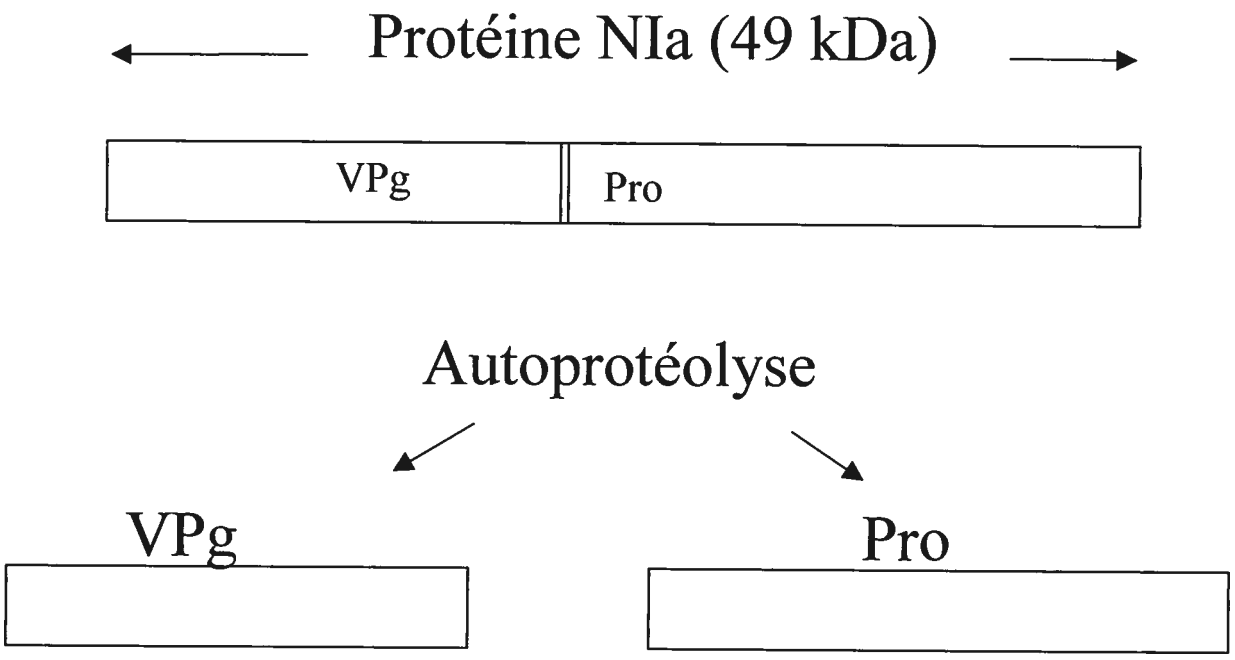

Tableau II : Comparaison en acides aminés de 10 VPg de potyvirus. Les acides aminés identiques sont représentés en caractère gras.

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PVY15    GKNKSKRIQALKFRHARDKRAGFEIDNNDDTIEEFFGSAYRKKGKGKG..
PPVD     .GFNRRQRQKLKFRQARDNRMAREVYGDDSTMEDYFGSAYSKKGKSKG..
TUMV     ...GKKQRQKLKFRNARDNKMGREVYGDDDTIEHFFGDAYTKKGKSKG..
PRV      .GFSARQRQKLRFKSAANAKLGREVYGDDGTIEHYFGEAYTKKGNKKG..
TVMV     ....GKSRRRLQFRKARDDKMGYIMHGEGDTIEHFFGAAYTKKGKSKG..
SBMV     ..GKKRQIQKLKFRDAFDRKVGREVYADDYTMEHTFGEAYTKKGKQKGST
PEMV     GRSKTKRIQALKFRKARDKRAGFEIDNNEDTIEEYFGSAYTKKGKGKG..
TEV      ..GKKNQKHKLKMREARGARGQYEVAAEPEALEHYFGSAYNNKGKRKG..
OMV      ..................................................
PSBMV    ...GKSKAKTLRFRQARDNNAKYEVFADEDTKRHYFGEAYTKKGKKSG..

PVY15    TTVGMGKSSRRFINMYGFDPTEYSFIQFVDPLTGAQIEENVYADIRDIQE
PPVD     KTRGMGTKTRKFVNMYGYDPTDYNFVRFVDPLTGHTLDEDPLMDINLVQE
TUMV     RTRGIGHKNRKFINMYGFDPEDFSAVRFVDPLTGATLDDNPFTDITLVQK
PRV      KMHGMGVKTRKFVATYGFKPEDYSYVRYLDPLTGETLDESPQTDISMVQD
TVMV     KTHGAGTKAHKFVNMYGVSPDEYSYVRYLDPVTGATLDESPMTDLNIVQE
SBMV     RTKGMGRKSRNFIHLYGVEPENYSMIRVVDPLTGHTMDEHPRVDIRMVQQ
PEMV     TTVGMGRTNRRFINMYGFEPGQFSYIKFVDPLTGAQMEENVYADIVDVQE
TEV      TTRGMGAKSRKFINMYGFDPTDFSYIRFVDPLTGHTIDESTNAPIDLVQH
OMV      ....................EYTIVRYVDPLTGATQDENPLMAIDLVQE
PSBMV    KARGMGVKTKKFVNVYGFDPCEYSLVRFVDPLTGLTYDRHPMEHMMDVQE

PVY15    RFSEVRKKMVENDDIEMQALGSNTTIHAYFRKDWSDKALKIDLMPHNPLK
PPVD     HFSQIRNDYIGDDKITMQHIMSNPGIVAYYIKDATQKALKVDLTPHNPLR
TUMV     HFGDIRMDLLGEDELDSNEIRMNKTIQAYYMNNKTGKALKVDLTPHIPLK
PRV      HFSDIRRKYMDSDSFDRQALIANNTIKAYYVRNSAKAALEVDLTPHNPLK
TVMV     HFGEIRREAILADAMSPQQ..RNKGIQAYFVRNSTMPILKVDLTPHIPLK
SBMV     EFEEIRKDMIGEGELDRQRVYHNPGLQAYFIGKNTEEALKVDLTPHRPTL
PEMV     KFGDIRRQMILDDELDRRQTDVHNTIHAYLIKDWSNKALKVDLTPHNPLR
TEV      EFGKVRTRMLIDDEIEPQSLSTHTTIHAYLVNSGTKKVLKVDLTPHSSLR
OMV      YFAKIRSQLVSEEKLETQNIIANPGIQAYYMKNRGDAALKVDLTPHNPLL
PSBMV    TIGDDRREAMWNDELDKQLFVTRPTIEAYYIKDKTTPALKIDLNPHNPMR

PVY15    VCDKTNGIAKFPEREELELRQTGPAVEVDVKDIPAQEVEHE.........
PPVD     VCDKTATIAGFPERERFELRQTGHPIFVEPNAIPKINEEGDEEVDHE...
TUMV     VCDLHATIAGFPERENELRQTGKAQPINIDEVPRANNE.LVPVDHESNS
PRV      VCDNKLTIAGFPDREAELRQTGPPRTIQVDQVPPPSK....SVHHE...
TVMV     VCESN.NIAGFPEREGELRRTGPTETLPFDALPPEKQEVAFE.......
SBMV     LCQNSNAIAGFPERDDLRQTGLPQVVSKSDVPRAKERVEME.......
PEMV     VSDKASAIMKFPEREGELRQTGQAVEVDVCDIPKEVVKH..........
TEV      ASEKSTAIMGFPERENELRQTGMAVPVAYDQLPPKNEDLTFE.......
OMV      V.TKTGTIAGFPENEFILRQTGKAVNVKMSEVPVENELEEVEHEG....
PSBMV    VCDKAETIAGFPEREFELRQSGSATLVPYSEVPVQNEKQEFDEEHWRTE
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PVY15, potato virus Y, Brtitish isolate ; PPVD, plum poxvirus, strain D ; TuMV, turnip mosaic virus ; PRV, papaya ringspot virus, isolate HA ; TVMV, tobacco vein mottling virus ; SBMV, soybean mosaic virus, strain G-6, isolate K-1 ; PEMV, pepper mottle virus, Califournia strain ; TEV, tobacco etch virus ; OMV, ornithogalum mosaic virus ; PSBMV, pea seed-borne mosaic virus, isolate DP1.

(Tiré de Plochocka et al., PNAS 1996)

1.2.3.3 Fonctions

1.2.3.3.1 Réplication virale

Le complexe de réplication du potyvirus se trouve dans le cytoplasme associé à une membrane, probablement celle du réticulum endoplasmique (Martin et Garcia, 1991; Schaad et al., 1997). L'encrage du complexe de réplication est dû à une protéine virale de 6 kDa, la protéine 6K2 (Restrepo-Hartwig et Carrington, 1994 ; Schaad et al., 1997). Cette protéine est située en N terminal de la NIa dans la polyprotéine. Des chercheurs ont suggéré que la protéine VPg pourrait être associée à la 6K2 lors de la réplication virale.

De nombreuses études démontrent que le domaine VPg de la NIa joue une fonction importante lors de la réplication du génome viral. L'association de la VPg à l'extrémité 5' de l'ARN est essentielle pour la réplication virale. Des études ont montré que la substitution du résidu tyrosine liant la VPg à l'ARN viral abolie la réplication du virus dans les protoplastes (Murphy et al., 1996). Il existe également une corrélation entre le taux d'infectivité de l'ARN viral et le taux de dégradation de la protéine VPg (Hari, 1981 ; Riechmann et al., 1989).

D'autres travaux démontrent que la VPg de potyvirus est membre du complexe de réplication de ces virus. Chez différents potyvirus, la VPg interagit avec l'ARN dépendante ARN polymérase (RdRp) (Hong et al., 1995 ; Li et al, 1997 ; Fellers et al., 1998 ; Darros et al., 1999). D'autres expériences ont démontré que cette interaction peut stimuler l'activité de la RdRp (Li et al., 1997 ; Fellers et al., 1998). Ce qui suggère que la VPg pourrait être impliquée dans la réplication du virus.

Chez les mammifères, le poliovirus, un virus apparenté au groupe des potyvirus, la VPg interagit avec l'extrémité 3' de l'ARN viral pour initier la synthèse du

brin négatif (Paul et al., 1998). Par analogie avec ce virus, certains chercheurs croient que la VPg de potyvirus pourrait avoir une fonction similaire d'amorce de la réplication du génome viral. Une interaction entre la VPg du Tobbaco etch virus (TEV) et l'ARN viral appuie cette hypothèse (Merits et al., 1998)

1.2.3.3.2 Déterminant pathogénique

Les mécanismes qui mènent à une infection systémique de la plante par un potyvirus sont encore mal connues. Cependant, certaines fonctions ont été attribuées aux protéines du potyvirus pour propager l'ARN viral dans la plante. Des travaux ont montré que la CP, la P1 et la HC-Pro sont impliquées pour le mouvement du virus de cellule en cellule et pour le mouvement du virus sur de longue distance dans la plante (Dolja et al., 1994 ; Dolja et al., 1995; Rojas et al., 1997; Saenz et al., 2002). Il est maintenant connu que la VPg à des fonctions similaires pour propager le virus dans la plante. Des travaux ont montré que la VPg du virus TEV (Schaad et al., 1997), du TVMV (Nicolas et al., 1997), du PSbMV (Keller et al., 1998) et celle du PVA (Rajamaïki et Valkonen, 1999) déterminait la spécificité de l'hôte lors de l'infection virale. Par exemple, lorsqu'on regarde un cultivar de tabac normalement résistant au virus du TVMV, mais susceptible à l'infection par une souche naturelle (différente) du TVMV. Il a été montré qu'une différence de 6 acides aminés dans la VPg a permis à la souche naturelle de surmonter la résistance au virus (Nicolas et al., 1997). Ceci suggère qu'une configuration adéquate de la VPg est nécessaire pour interagir avec les composants de l'hôte et faciliter le mouvement systémique du virus dans la plante. Des résultats similaires ont été montrés pour le TEV, le PVA et le PSbMV.

Lors de mes débuts dans le laboratoire du docteur Laliberté, on cherchait à connaître le mécansime de réplication du TuMV. Le système du double-hybride LexA a été employé afin d'identifier les protéines cellulaires interagissant avec la protéine virale liée de façon covalente à l'ARN, soit la VPg. Le criblage en utilisant une banque d'ADN complémentaire (ADNc) d'*Arabidopsis thaliana* a permis d'isoler quatre clones interagissant avec la VPgPro. L'un d'entre eux fut identifié comme étant un facteur d'initiation de la traduction soir l'eIF(iso)4E d' *A.thaliana.* Afin de bien comprendre la signification de cette interaction, la prochaine section nous décrira le rôle de l'eIF4E lors du mécanisme d'initiation de la traduction cellulaire.

2. Initiation de la traduction cellulaire

2.1 Introduction

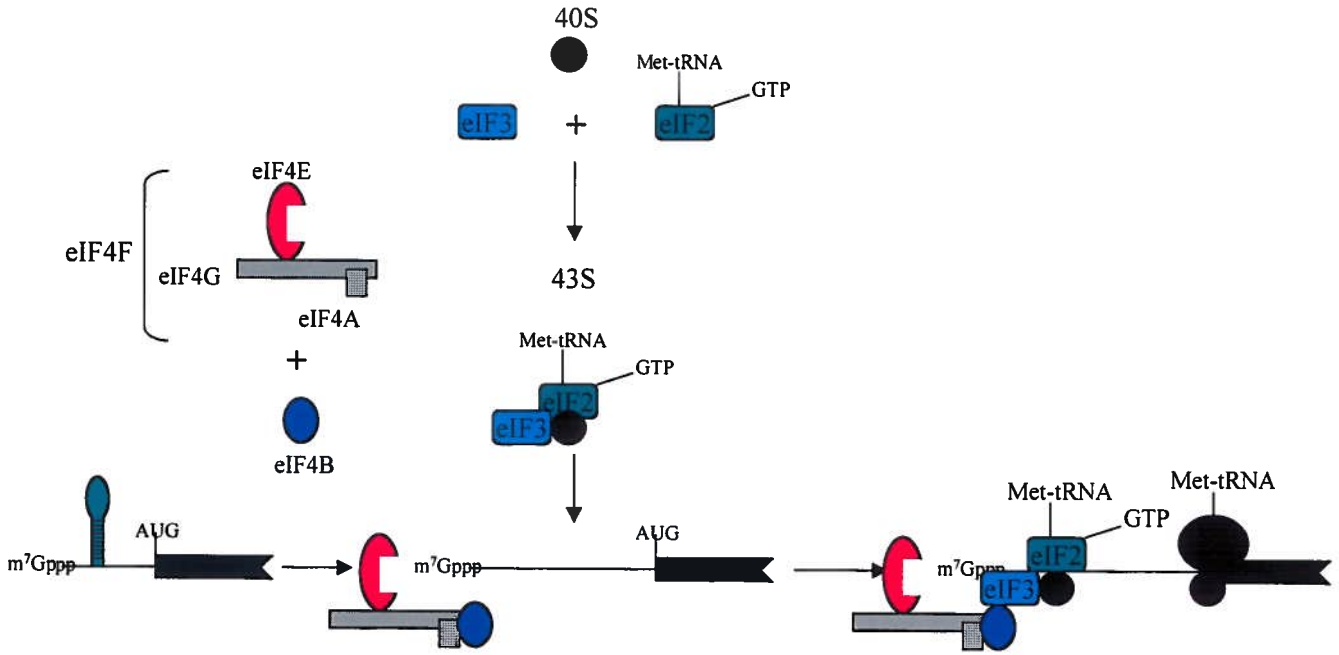
La synthèse de protéines est un processus complexe nécessitant un large arsenal de macromolécules tant chez les cellules eukaryotes que procaryotes : facteurs d'initiation, facteurs d'élongations, facteurs de terminaison, ribosomes, ARNm, aminoacyl synthétases et ARN de transferts. Pour les besoins de cette thèse, nous n'entrerons pas en détail pour chacune des étapes de la synthèse protéique. Cependant, nous nous attarderons à l'étape d'attachement des ribosomes aux ARNm cellulaires : l'initiation de la traduction cellulaire.

2.2 Mécanisme d'initiation de la traduction cellulaire

Tous les ARNm cellulaires eukaryotes (à l'exception des ARNm des organelles) possèdent à leurs extrémités 5' la structure m^7GpppX (X représente n'importe quel nucléotide) nommée la coiffe. L'initiation de la traduction cellulaire consiste en de nombreuses étapes et est catalysée par la liaison de plusieurs protéines nommées eIF (eukaryotic initiation factors). Parmi ces protéines, l'eIF4F lient les ARNm coiffés en leur extrémité 5'. Cette liaison, avec l'aide également d'autres protéines, permet le recrutement des ARNm aux ribosomes. Un modèle simplifié de l'initiation de la traduction cellulaire est présenté à la figure 3. Nous pouvons voir que la coiffe est d'abord reconnu par l'eIF4F à l'extrémité 5' de l'ARN messager. Le facteur eucaryotique 4F est composé d'un complexe de trois protéines : a) l'eIF4A, une ARN-dépendante ATPase et ARN hélicase b) l'eIF4E, une protéine de 24 kDa responsable de la reconnaissance avec la coiffe des ARNm et c) l'eIF4G, une protéine de haut poids moléculaire contenant des sites de liaison pour l'eIF4E, l'eIF4A et l'eIF3. Une fois l'eIF4F fixé sur l'ARNm, l'eIF4B vient se joindre au complexe et permet le déroulement de structures secondaires présentes à l'extrémité 5' (voir figure 3). Cette étape permet au complexe 43S (comprenant la sous-unité ribosomique 40S, le Met-tRNA, l' eIF2 GTP et l'eIF3) de se fixer en 5' de l'ARNm. Une interaction entre l'eIF3, l'eIF4G et la sous-unité ribosomique 40S permet cette liaison. La sous unité 40S peut ensuite effectuer une lecture de l'ARNm jusqu'au codon initiateur AUG. La sous-unité 60S peut alors s'y fixer et enclencher la synthèse protéique avec l'aide du Met-tRNA. Nous verrons dans une section ultérieure comment cette étape peut être limitante lors de la traduction cellulaire.

Figure 3 : Mécanisme d'initiation de la traduction cellulaire.

1- Reconnaissance de la coiffe des ARNm par le complexe eIF4F.
2- Déroulement des structures secondaires de l'ARNm à l'aide de l'eIF4B.
3- Fixation du complexe 43S sur l'ARNm.
4- Fixation de la sous-unité 60S et début de la traduction cellulaire au codon initiateur AUG



2.3 eIF4E

2.3.1 Fonction

Le complexe eIF4F est essentiel pour la traduction coiffe dépendante. Des travaux ont montré que des mutants altérants l'affinité de l'eIF4E pour la coiffe modifient le profile de traduction des ARNm dans la levure (Altmann et al., 1989). De plus, la déplétion de l'eIF4E réduit dramatiquement la traduction des ARNm cellulaires, cependant l'addition d'eIF4E recombinante restaure complètement cette activité (Svitkin et al., 1996). L'utilisation d'ARN anti-sens a d'ailleurs permis de montrer que le taux de synthèse protéique cellulaire est directement proportionnel à la quantité d'eIF4E dans la cellule (De Benedetti et al., 1991). L'eIF4E est la protéine la moins abondante des facteurs initiant la traduction cellulaire. Seulement 0.01-0.2 molécule par ribosome ont été retrouvé dans les cellules HeLa (Duncan et al., 1987). Pour cette raison, l'eIF4E est non seulement un facteur clé mais limitant de la traduction cellulaire.

L'eIF4E joue également un rôle important lors du contrôle de la croissance cellulaire (Sonenberg et al., 1998). Dans la levure (*Saccharomyces cerevisae*) l'abolition du gène codant pour l'eIF4E est létale pour ce micro-organisme (Altmann et al., 1989).

2.3.2 Propriétés biophysiques

Les structures tridimentionnelles de l'eIF4E de souris et de levure liant le m^7GDP ont pu être résolu grâce à la cristallographie à rayon X et à la spectroscopie de résonance magnétique nucléaire (voir figure 4) (Marcotrigiano et al., 1997 ; Matsuo et al., 1997). Ce complexe nous montre que l'eIF4E à la forme d'un creux de main où le m^7GDP est situé dans la portion concave de la protéine. L'eIF4E est constituée de huit brins bêta anti-parallèles avec quatres hélices sur la face convexe de la protéine. Les résidus tryptophanes (Trp56 et Trp102 chez la souris, Trp58 et Trp104 chez la levure) sont impliqués dans l'interaction avec la coiffe des ARNm (non montré). Une des caractéristique structurale de l'eIF4E est la présence de 8 résidus tryptophanes qui sont conservés en nombre et en location tant chez la souris que la levure (Altmann et al., 1988). L'eIF4E possède également un site de phosphorylation situé sur la serine en position 209 de la protéine (Joshi et al., 1995 ; Flynn et al., 1995 ; Rousseau et al., 1996 ; Feigenblum et al., 1993)

L'eIF4E a été isolée et purifiée chez des espèces différentes : la plante, la mouche, l'humain etc. Le tableau III nous indique le pourcentage d'identité en acides aminés qui existe entre diverses eIF4E ou eIF(iso)4E de plantes et de mammifères (Browning, 1996). Environ 50 % d'identité en acides aminés existe si l'on compare les deux isomères d'eIF4E entre eux (49 % pour le blé et 47% pour le riz). De plus, l'eIF4E et l'eIF(iso)4E de blé ressemblent beaucoup à celle de riz (respectivement 78 et 82 %), mais cette similarité est plus faible lorsqu'on compare à l'eIF4E humaine (respectivement 39 et 35 %).Toutefois, les résidus tryptophanes responsables de la liaison avec la coiffe des ARNm sont conservés

chez toutes les formes d'eIF4E. Ce qui suggère que l'eIF4E pourrait avoir une structure similaire selon les espèces.

Figure 4 : Structure tridimensionnelle de l'eIF4E liée au m^7GDP.

Sur cette figure on peut voir l'eIF4E qui contient huit brins bêta (en or). Deux hélices alpha sont colorés en bleues et deux autres hélices alpha sont colorées en mauve. Le m^7GDP est liée à l'eIF4E sur sa face concave. (Matsuo et al., 1997).

Tableau 3 : Comparaison en acides aminés de différentes eIF4E eucaryotes en %.

	EIFiso4E Blé	eIF4E Blé	eIFiso4E Riz	EIF4E Riz	eIF4E Levure	eIF4E Drosophile	eIF4E Xénopus	eIF4E Humaine
eIFiso4E Blé	100	49	78	48	31	32	40	39
eIF4E Blé	----------	100	47	82	31	29	35	35
eIFiso4E Riz	----------	----------	100	47	33	33	39	39
eIF4E Riz	----------	----------	----------	100	32	29	36	36
eIF4E Levure	----------	----------	----------	----------	100	34	31	31
eIF4E Drosophile	----------	----------	----------	----------	----------	100	41	44
eIF4E Xénopus	----------	----------	----------	----------	----------	----------	100	89
eIF4E Humaine	----------	----------	----------	----------	----------	----------	----------	100

2. EIF4E et eIF(iso)4E chez les végétaux

La traduction dans la plante est comparable à celle des mammifères. Ainsi, les règles établies pour définir la traduction des ARNm de mammifères sont donc aussi valables pour les ARNm des plantes. Toutefois, le complexe eIF4F des plantes contient seulement deux sous-unités : l'eIF4G et l'eIF4E. Les plantes possèdent également un second isomère d'eIF4F (Browning et al., 1992 ; Browning, 1996); ce second isomère, l'eIF(iso)4F est aussi composé de 2 protéines : l'une de 28 kDa l'eIF(iso)4E et une autre de 86 kDa l'eIF(iso)4G. L'eIF(iso)4F est un homologue fonctionnel de l'eIF4F mais il a une antigénicité différente (Browning et al., 1992). Les deux isomères ont également un profil d'expression différent : l'ARN codant pour l'eIF4E est exprimé dans tous les tissus à l'exception des racines tandis que l'ARN codant pour l'eIF(iso)4E est particulièrement abondant dans les fleurs et dans les organes en développement (Rodriguez et al., 1998).

2.3.4 Régulation de l'eIF4E

La cellule peut exercer un contrôle sur la traduction cellulaire en modulant l'activité de l'eIF4E par diverses façons. L'activité de l'eIF4E est régulée par phosphorylation, ce qui modifie son affinité pour la structure de la coiffe, pour l'eIF4G, ainsi que pour les sous unités inhibitrices que sont les *4E-binding proteins (4E-BP).* Ces dernières sont une famille de protéines qui, lorsqu'elles sont déphosphorylées, lient l'eIF4E pour la séquestrer et l'empêcher de lier la coiffe. Le site de phosphorylation de l'eIF4E est le résidu Ser-209 (Joshi et al., 1995 ; Flynn et al., 1995 ; Rousseau et al., 1996 ; Feigenblum et al., 1993).

Lorsque cet acide aminé est phosphorylé, l'affinité de l'eIF4E pour la coiffe est accrue (Joshi et al., 1995). Il y a donc une corrélation entre l'augmentation de l'affinité de la 4E pour la coiffe après phosphorylation et l'augmentation de la traduction cellulaire. Cependant ce phénomène demeure controversé (Scheper et al., 2002). La serine en position 209 est un site susceptible d'être phosphorylé par l'intermédiaire de la protéine kinase C (PKC) ainsi que par la MNK1 (MAP kinase interacting kinase 1), impliquée dans la cascade de signalisation des MAPK (mitogen activated protein kinase) (Waskiewicz et al., 1997 ; Fukanaga et al., 1997 ; Kleijn et al., 1998). Ainsi, la synthèse protéique, qui est un processus coûteux en énergie, est strictement contrôlée dans la cellule (Mathews et Shenk, 1991). De plus, des stimulus externes comme des hormones, des facteurs de croissance et agents mitogènes peuvent, au besoin, promouvoir la croissance cellulaire et augmenter le taux de traduction cellulaire en allant directement phosphoryler l'eIF4E. D'autre part, le mécanisme de phosphorylation de l'eIF4E chez la plante reste encore à démontrer. Peu de travaux ont été effectués sur le sujet. Des expériences ont cependant démontré que les isomères d'eIF4E sont phosphorylés dans la plante (Gallie et al., 1997).

2.3.5 Propriétés oncogéniques

L'eIF4E possède des propriétés oncogéniques. C'est-à-dire, que lorsqu'anormalement exprimé, elle peut contribuer au développement d'un cancer. Chez les mammifères, des travaux ont indiqué qu'une surexpression de l'eIF4E résulte en la transformation maligne de cellules (De Benetti et al., 1991 ; Lazaris-Karatzas et al., 1990) De plus, des expériences immunohistochimiques

ont révélé que l'eIF4E est surexprimée dans plusieurs carcinomes humains (cancer du colon, du cou et de la poitrine) (revue par Zimmer et al., 2000). D'autres expériences ont montré que cette protéine affecte non seulement l'invasion tumorale mais également la capacité métastasique de la tumeur. Cette protéine est donc une cible potentielle pour le traitement de ces cancers et fait actuellement l'objet de nombreux travaux.

2.4 Mécanisme circulaire de la traduction cellulaire : Liaison entre l'extrémité 5' et 3' de l'ARNm.

2.4.1 Effet stimulateur de la queue poly (A).

Il y a 45 ans, il a été suggéré que l'extrémité 3' des ARNm pouvait être physiquement à proximité de l'extrémité 5' lors de la traduction cellulaire. Des chercheurs avaient remarqué que des polysomes apparaissaient de forme circulaire ou montraient une forme en spirale (Philipps, 1965 ; Baglioni et al., 1969). C'est seulement récemment que d'autres études ont été réalisées pour démontrer une relation entre la coiffe et la queue poly(A) lors de la traduction. Maintenant, des études biochimiques ainsi que génétiques ont établi qu'une interaction entre les deux extrémités d'un ARNm est requise pour obtenir une traduction efficace (Revue par Gallie et al., 1998). Des travaux ont montré que l'ARN doté à la fois d'une coiffe et de la queue poly(A) était bien traduit dans les plantes, dans les cellules de mammifères ainsi que dans les levures (Gallie et al., 1991). La présence de ces deux structures stimulait la traduction cellulaire

par un facteur allant de 5 à 10 fois. Cependant comme nous le verrons dans le prochain paragraphe d'autres protéines sont également requises pour une traduction efficace d'un ARNm dans la cellule.

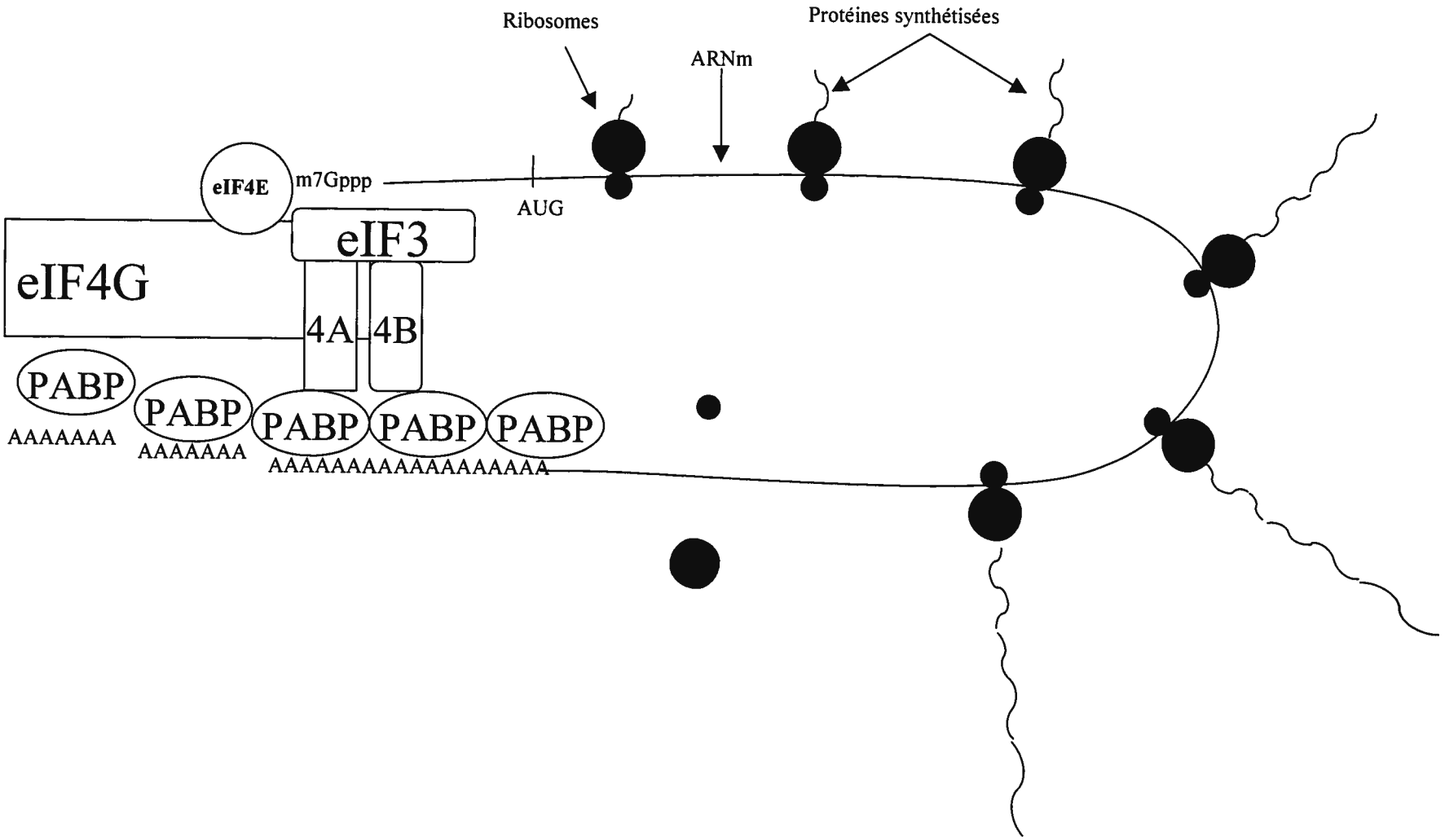
2.4.2 Interaction entre PABP et eIF4G-eIF4B

L'augmentation du taux de traduction par la coiffe et la queue poly(A) peut être expliquée physiquement. Historiquement, de nombreuses études ont démontré que l'addition exogène de poly(A) inhibait la traduction cellulaire dans un lysat *in vitro* (Lodish et Nathan, 1972; Jacobson et Favreau, 1983; Grossi de Sa et al., 1988; Munroe et Jacobson, 1990; Gallie et Tanguay, 1994). Cette étude suggérait donc que la queue de poly(A) séquestrait les composants nécessaires à la traduction. Dans la cellule la queue de poly(A) est associée à de nombreuses copies de la PABP ((Poly(A) binding protein)) un polypeptide de 70 kDa. Il fut par la suite montré que la PABP est essentiel pour la croissance dans la levure et joue un rôle important pour la stabilité et une traduction efficace des ARNm (Baer et Kornberg 1983, Sachs et Kornberg 1985, Adam et al. 1986, Sachs et Davis 1989, 1990). Conséquemment, des études d'interaction ont été menées entre la PABP et les facteurs initiant la traduction cellulaire associés à la structure de la coiffe. Des associations entre la PABP et l'eIF4G dans la levure, dans la plante ainsi que chez les mammifères ont été démontrée (Tarun et Sachs, 1996; Le et al., 1997;Craig et al., 1998). Chez la plante, le facteur eIF4B peut aussi interagir avec la PABP (Le et al., 1997).

2.4.3 Circularisation de l'ARNm

D'autres recherches ont montré que la PABP était impliquée lors de l'initiation de la traduction cellulaire évoquant que la PABP effectue la jonction entre l'extrémité 5' et 3' des ARNm. L'eIF4G et l'eIF4B chez la plante, exercent un effet synergétique sur l'affinité de la PABP pour la queue poly(A). La jonction de ces trois protéines augmentent également l'affinité du complexe eIF4F pour la coiffe dans la plante (Wei et al., 1998). Plus récemment, dans un lysat de germe de blé, il a été établi que la PABP augmentait l'activité ATPase et hélicase des facteurs d'initiation eIF4A, eIF4B et eIF(iso)4F (Bi et al., 2000). L'implication de la PABP durant la traduction cellulaire est illustrée dans la figure 5. On y voit que l'interaction entre la PABP, eIF4G et l'eIF4B ne lie l'extrémité 3' à la coiffe. Ces interactions résultent en la circularisation de l'ARNm et présentent de nombreux avantages pour une traduction efficace.

Figure 5 : Modèle circulaire de la traduction des ARNm cellulaires.



2.4.4 Avantage de la circularisation de l'ARNm.

Le modèle de traduction circulaire présente, selon les chercheurs, de nombreux avantages pour une traduction optimale et efficace. Premièrement, l'association de la PABP aux facteurs initiant la traduction cellulaire à un effet stabilisateur sur l'ARNm. La liaison de la PABP à l'extrémité 5' de l'ARNm le protège contre l'attaque d'enzymes de dégradation ("decapping enzyme", dégradation exonucléolytique 5'→3', processus de déadénylation, ...) (Sachs et Kornberg 1985) Deuxièmement, l'interaction 5'-3' peut être un moyen de vérifier l'intégrité des ARNm avant leur traduction. Des études ont montré que la coiffe sert non seulement au recrutement de facteurs de traduction mais également est requise pour la liaison de la sous-unité ribosomique 40S en 5' de l'ARNm (Preiss et Hentze, 1998). Conséquemment, l'association de la queue poly(A) à l'extrémité 5' via la PABP pourrait prévenir la traduction d'ARNm partiellement dégradés ou incomplets. Finalement, le modèle circulaire de traduction pourrait faciliter la réinitiation de la traduction. En effet, une fois que le ribosome aurait terminé la traduction de l'ARNm, il serait à proximité de l'extrémité 5' et pourrait rapidement démarrer un autre cycle de traduction.

Maintenant que nous avons vu par quel processus la cellule effectue la traduction de ses ARNm, nous regarderons comment les virus, apparentés au potyvirus, s'y prennent pour traduire leur propre ARN au dépend de la traduction cellulaire.

3. Contrôle traductionnel par les virus

3.1 Généralités

Les virus sont des parasites obligatoires, ils ne possèdent donc pas tout l'appareillage nécessaire (enzymes, protéines, voies métaboliques, etc.) à leurs biosynthèses. Les cellules qu'ils infectent leur fournissent l'énergie, leurs constituants biochimiques et tout ce dont ils ont besoin pour se multiplier. Les virus ont élaboré des stratégies permettant la traduction préférentielle de leur ARNm, bloquant ainsi bien souvent la traduction des ARNm cellulaires. Ce phénomène est généralement connu sous le nom de "Host translational shutdown".

3.2 Picornavirus

Dans bien des cas, les facteurs initiant la traduction cellulaire sont des cibles de choix pour les virus d'animaux. Les exemples les plus connus de virus qui induisent un blocage de la traduction cellulaire sont observés chez les picornavirus. Pour certains membres de cette famille (poliovirus, coxsackievirus et le rhinovirus), l'inhibition s'effectue par le clivage de l'eIF4G par des protéases virales. L'hydrolyse du facteur eIF4G empêche l'eIF4E de lier le complexe ribosomique 43S et interfère dans le recruitement des ARNm coiffés (Kirchweger et al., 1994, Lamphear et al., 1995, Liebig et al., 1993). Cependant, l'inhibition de la traduction s'effectue selon un autre mécanisme chez l'EMCV, un autre membre des picornavirus (virus de l'encephalomyocardite). Lors de l'infection l'EMCV modifie l'état de phosphorylation de la 4E-BP1 et la maintient déphosphorylée. Cette déphosphorylation augmente l'affinité de la 4E-

BP1 pour l'eIF4E ce qui résulte en l'inhibition de la traduction des ARNm coiffés (Gingras et al., 1996).

3.3 Adénovirus et virus de l'influenza.

Le blocage traductionnel visant les facteurs initiant la traduction cellulaire s'effectue également chez d'autres types de virus. L'adénovirus prévient la synthèse protéique cellulaire lors de sa phase tardive de réplication en déphosphorylant l'eIF4E (Huang et al., 1991) qui devient moins efficace pour lier les ARNm coiffés. L'ARN viral peut être quand même efficacement traduit puisqu'il dépend très peu du facteur eIF4E. L'infection par le virus de l'influenza est aussi accompagnée d'un phénomène de "Host shutdown" de la synthèse protéique et est due aussi à une déphosphorylation et une inactivation de l'eIF4E (Feigenblum et al., 1993). Cependant, le facteur viral impliqué est encore inconnu.

3.4 Circularistation des ARNm viraux

Le rotavirus, un virus de mammifère, possède un ARN viral coiffé mais non polyadénylé. Il possède une protéine NSP3 (non structural protein 3) qui présente plusieurs similarités à la PABP. La NSP3 lie l'extrémité 3' des ARN viraux en reconnaissant la séquence consensus (UGACC) retrouvé chez les 11 ARN génomiques du rotavirus. Le groupe de Poncet a montré que la NSP3 peut être fixée à l'extrémité 3' d'un ARN de rotavirus (Poncet et al., 1993; Poncet et al., 1994). La NSP3 peut être également coimmunoprécipitée avec le facteur eIF4G (Piron et al., 1998). Ces travaux suggèrent que la protéine NSP3 liée à l'extrémité 3' peut interagir avec des facteurs initiant la traduction cellulaire et

circulariser l'ARN viral. Récemment, des études ont mis en évidence qu'une traduction efficace d'un ARN viral requiert simultanément l'interaction entre la protéine NSP3, l'eIF4G et l'ARNm (Vende et al., 2000). Lors d'une infection par le rotavirus, la PABP qui entre en compétition avec la NSP3 pour la liaison avec l'eIF4G, est probablement évincée du processus de traduction, empêchant ainsi la traduction des ARNm polyadénylés. Ce qui expliquerait le phénomène d'inhibition de la traduction observé lors d'une infection par le rotavirus (Vende et al., 2000).

De plus, la coopération entre l'extrémité 5' et 3' des ARN viraux semblent se produire également chez les picornavirus. La rupture de l'interaction entre l'eIF4G et la PABP ou le clivage du facteur eIF4G abolissent ou inhibent grandement l'effet de stimulation qu'amène la queue de poly(A) pour la traduction des ARNm du poliovirus, de l'EMCV et de virus de l'hépatite A (Michel et al., 2001). De plus, la PABP est spécifiquement dégradée lors d'un infection par le poliovirus et également par le coxsackievirus. La PABP est clivée *in vitro* par les protéases 2A et 3C du poliovirus ainsi que les protéases B3 et 2A du coxsackievirus (Joachims et al., 1999). Le clivage de la PABP concorde avec l'inhibition de la traduction observée suite à l'infection du poliovirus *in vitro*. La circularisation des ARN viraux du poliovirus serait également requise pour la réplication du virus (Herold et al., 2001).

3.5 LCMV

Une autre protéine (la "RING Z") d'un virus humain le LCMV (Lymphocytic choriomeningitis virus) interagit avec le facteur eIF4E et inhibe sélectivement la synthèse protéique (Campbell Dwyer et al., 2000). De plus, la cycline D1, une protéine sensible au taux d'eIF4E, était sous exprimée durant l'infection tandis qu'une protéine indépendante d'eIF4E la GAPDH (glycéraldehyde-3-phosphate déhydrogénase) ne montrait aucune variation de son taux d'expression. Ce qui indique que la "RING Z" protéine du LCMV peut affecter directement le rôle de l'eIF4E dans la cellule. La "RING Z" protéine supprime également la transformation oncogénique en réduisant l'affinité de l'eIF4E pour l'ARNm (Cohen et al., 2001).

3.6 Interaction protéine virale - protéine de l'hôte chez les plantes.

Contrairement aux virus d'animaux, la nature des interactions entre le virus et l'hôte menant à la production virale est mal connue chez les plantes. Cependant, dans la littérature, plusieurs protéines sont évincées du processus de réplication/traduction de la plante par la polymérase virale et aident grandement la réplication du virus (revue par Lai, 1998, Strauss et Strauss, 1999). Pour les virus de plantes, les exemples les mieux connus sont probablement ceux du BMV (brome mosaic virus) et du TMV (tobacco mosaic virus) qui lient l'eIF3 nécessaire à l'activité de leur polymérase (Osman et Buck, 1997; Quadt et al., 1993).

Les travaux de Wang et Maule suggèrent que l'inhibition de l'expression génique induite suite à une infection virale s'effectue dans la plante. Ces derniers ont montré, lorsqu'ils ont examiné le front d'infection du pea seed borne mosaic virus (PSbMV) dans des embryons de pois, qu'au moins dix gènes cellulaires différents montraient une inhibition de leur expression (Wang et Maule, 1995). Cependant, aucune expérience n'a été clairement démontré pour expliquer ce taux réduit d'ARNm. Ultérieurement, une autre interaction protéine virale - protéine de l'hôte fut démontré dans la plante. La polymérase RdRp (RNA dependant RNA polymerase) du Zucchini Yellow Mosaic potyvirus interagit avec la PABP du concombre (Wang et al., 2000). Ce lien évoque la possibilité d'une inhibition de la traduction et/ou du recrutement de la polymérase sur la queue de poly(A) pour faciliter la réplication virale.

Par ailleurs, il a été démontré dans notre laboratoire que la VPg du virus de la mosaïque du navet interagit avec le facteur initiant la traduction eIF4E (Wittmann et al., 1997). Les conséquences de cette interaction ainsi que la caractérisation de cette liaison ont donc été investiguées.

Chapitre 2 :

Étude de l'interaction entre la VPg (viral protein genome linked) du virus de la mosaïque du navet (TuMV) et le facteur eucaryotique inititant la traduction cellulaire 4E.

Introduction à l'article I

Lorsque j'ai débuté mon projet de recherche, une nouvelle interaction venait d'être mise en évidence dans notre laboratoire : la VPg (viral protein genome linked) du virus de la mosaïque du navet (TuMV) interagissait avec le facteur eucaryotique d'initiation de la traduction 4E (eIF4E) (cap binding protein) d'*Arabidopsis thaliana* (Wittmann et al., 1997). Cette interaction avait été montrée non seulement dans la levure à l'aide du système double-hybride, mais également *in vitro* à l'aide d'un test de type ELISA. Bien que cette interaction ait été démontrée *in vitro*, nous ne connaissions pas les conséquences de l'association VPg-eIF4E *in vivo*. Cependant, plusieurs travaux nous laissaient croire que cette interaction est importante pour l'établissement de l'infection par le TuMV. En effet, d'autres parasites, comme le virus de l'influenza, le virus de l'encéphalomyocardite et l'adénovirus, agissent sur l'eIF4E de façon à modifier l'activité de ce facteur d'initiation de la traduction. Suggérant ainsi que l'interaction VPg-eIF4E pourrait être une étape importante pour l'établissement de l'infection par un potyvirus.

À l'exception de la figure 1, mon implication dans cet article fut entière.

Article I

Complex Formation between Potyvirus VPg and Translation Eukaryotic Initiation Factor 4E Correlates with Virus Infectivity

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Sommaire traduit

Il a récemment été démontré que la VPg du virus de la mosaïque du navet (TuMV) interagit avec le facteur eucaryotique d'initiation de la traduction iso 4E ((eIF(iso)4E)) d'*Arabidopsis thaliana*. L'eIF(iso)4E lie la structure de la coiffe (m^7GpppN, ou N représente un nucléotide) des ARNm et joue un rôle important dans la régulation de l'initiation de la traduction cellulaire. Dans la présente étude, nous avons montré que non seulement la VPg interagit avec l'eIF(iso)4E mais également avec l'isomère eIF4E d'*Arabidopsis thaliana* et aussi avec l'eIF(iso)4E de *Tritricum aestivum* (blé). Le domaine d'interaction de la VPg pour l'eIF(iso)4E a été délimité à une région de 35 acides aminés et la substitution d'un acide aspartique dans cette région abolie complètement l'interaction. L'analogue de la coiffe, le m^7GTP, mais non le GTP, inhibe la formation du complexe VPg-eIF(iso)4E, suggérant que la VPg et les ARNm cellulaires compétitionnent pour la liaison à l'eIF(iso)4E. La signification biologique de cette interaction a été investiguée. Des plants de *Brassica perviridis* ont été infectés avec un plasmide infectieux du TuMV (p35Tunos) et avec le p35TuD77N, un mutant qui contient une substitution de l'acide aspartique responsable de l'interaction pour l'eIF(iso)4E. Après 20 jours, les plantes bombardées par le p35Tunos montraient des symptômes d'une infection virale, tandis que les plants bombardés avec le p35TuD77N demeuraient asymptômatiques. Ces résultats suggère que l'interaction VPg-eIF(iso)4E est un événement critique pour la production virale.

ABSTRACT

The interaction between the viral protein linked to the genome (VPg) of turnip mosaic potyvirus (TuMV) and the translation eukaryotic initiation factor eIF(iso)4E of Arabidopsis thaliana has previously been reported. eIF(iso)4E binds the cap structure (m^7GpppN, where N is any nucleotide) of mRNAs and has an important role in the regulation in the initiation of translation. In the present study, it was shown that not only did VPg bind eIF(iso)4E but it also interacted with the eIF4E isomer of *A. thaliana* as well as with eIF(iso)4E of *Triticum aestivum* (wheat). The interaction domain on VPg was mapped to a stretch of 35 amino acids, and substitution of an aspartic acid residue found within this region completely abolished the interaction. The cap analogue m^7GTP, but not GTP, inhibited VPg-eIF(iso)4E complex formation, suggesting that VPg and cellular mRNAs compete for eIF(iso)4E binding. The biological significance of this interaction was investigated. *Brassica perviridis* plants were infected with a TuMV infectious cDNA (p35Tunos) and p35TuD77N, a mutant which contained the aspartic acid substitution in the VPg domain that abolished the interaction with eIF(iso)4E. After 20 days, plants bombarded with p35Tunos showed viral symptoms, while plants bombarded with p35TuD77N remained symptomless. These results suggest that VPg-eIF(iso)4E interaction is a critical element for virus production.

INTRODUCTION

Potyviruses belong to the supergroup of "picorna-like" viruses. The viral genome is a single RNA molecule of positive polarity of close to 10,000 nucleotides with a poly(A) tract at its 3' end. It codes for one large polyprotein which is processed into at least 10 mature proteins by three viral proteinases (Pro) (47). The 5' end of the viral RNA does not have a cap structure (m^7GpppN, where N is any nucleotide) but is covalently linked to a virus-encoded protein termed VPg via a tyrosine residue (39, 40). VPg has several suggested roles in the virus life cycle. Interactions of VPg with the viral RNA polymerase in yeast (25, 33) and in vitro (15) support a role in viral RNA synthesis. Additionally, VPg has been implicated in overcoming resistance in plants (27, 35, 41, 42, 55). VPg also performs a yet-to-be-defined function in the nucleus. Indeed, Nia protein of tobacco etch potyvirus, a precursor form of VPg, has been found in the nucleus (10, 23, 46), and mutations in the VPg domain resulting in the inhibition of nuclear transport debilitated viral genome amplification (54). Recently, an interaction was shown to take place between the VPg of turnip mosaic potyvirus (TuMV) and the translation eukaryotic initiation factor (eIF) iso 4E of *Arabidopsis thaliana* (65). eIF4E is a component of the eIF4F complex and binds the cap structure of cellular mRNAs (6, 36, 38, 43). The cap mediates attachment of mRNAs to small ribosomal subunits, and the association is mediated by eIF4F (through binding to eIF4E) and eIF3 (38, 43). The interaction between VPg and eIF(iso)4E suggests the participation of the viral protein in the initiation of translation of the viral RNA.

Initiation is the rate-limiting step of translation in eukaryotes, and eIF4E has a regulatory role in this cellular event (38, 43, 60). In mammals, eIF4E is the least abundant of the initiation factors (13), although this assertion has been challenged (45). Its cap-binding activity is modulated by phosphorylation (62, 64). It is also regulated by eIF4E-binding proteins (4E-BPs) (31) which, by binding eIF4E, prevent the formation of the eIF4F complex (21, 34, 44). As a consequence, eIF4E plays an important role in the control of cell growth (58). In *Saccharomyces cerevisiae*, disruption of the gene coding for eIF4E is lethal, and mutants with altered mRNA cap-binding affinity reprogram mRNA selection by ribosomes (2). In mammals, overexpression of eIF4E has been shown to transform cells in tissue culture (11, 32). Elevated eIF4E expression results in the selective increase of a few proteins whose mRNAs are normally translationally repressed, such as ornithine decarboxylase and cyclin (49, 50). Just as elevated levels of eIF4E contribute to the development of a transformed cellular state, the reduction of eIF4E levels, using antisense RNA, has been shown to lengthen cell division times (12). The results of these *in vitro* studies, which emphasize the importance of eIF4E in the regulation of the cell division cycle, have been extended to clinical observations: eIF4E amounts have been found to be elevated in some human carcinomas (16, 27).

eIF4F is targeted by several animal viruses in their attempt to control host translation for preferential viral mRNA translation. For instance, adenoviruses and influenza viruses affect the phosphorylation state of eIF4E (14, 66). Encephalomyocarditis virus inactivates the initiation factor by enhancing 4E-BP1 binding (18). Finally, picornaviruses induce the cleavage eIF4G, with the consequence that cellular mRNAs linked to eIF4E cannot interact with 40S ribosome complexes (22, 59).

Although most of these observations relating to the role of eIF4E have been made in mammalian cells, the similarities in translation initiation in mammals, plants, and yeasts and the sequence homologies of different translation initiation factors (6, 17) suggest that the plant eIF4E plays as important a role as its mammalian homologue in the regulation of cellular processes. In this study, we investigated the interaction between the VPg of TuMV and eIF(iso)4E and its consequences for viral infection. We found that the cap analogue m^7GTP competed with VPg for eIF(iso)4E binding. Furthermore, TuMV whose VPg was mutated at a single residue which abolished *in vitro* interaction with eIF(iso)4E was debilitated for viral infection in whole plants.

MATERIALS AND METHODS

Microorganisms and media. Manipulations of bacterial as well as yeast strains and of nucleic acids and proteins were done by standard methods (19, 52). *Escherichia coli* XL1-Blue was used for subcloning, and *E. coli* BL21(DE3) (Novagen) was used for protein expression. *S. cerevisiae* EGY48 (MATa trp1 his3 ura3 8op-Leu2) (19) was used for the interaction study.

Yeast two-hybrid system. Plasmids employed for the interaction study were as described by Golemis et al. (19). pEG202 was used for the fusion of VPg and its derivatives to the DNA-binding domain of LexA. pJG4-5 was used to express eIF(iso)4E of *A. thaliana* (pSW56) (65) as a translation fusion to a cassette consisting of the simian virus 40 nuclear localization sequence, the acid blob B42, and the hemagglutinin epitope tag; expression was under the control of the GAL1 inducible promoter. The lacZ reporter plasmid was pSH18-34 containing eight lexA operators. Strength of the interaction was quantified using the β-galactosidase liquid assay (19). β-Galactosidase units were calculated using the following equation: units = 1,000 × (optical density at 420 nm [OD420] 1.75 × OD550)/(T × V × OD660), where T is time in minutes and V is the volume of culture used in milliliters.

The pLex-VPg plasmids were constructed as follows. The region coding for VPg in plasmid pETPro/24 (30) was amplified by PCR using the 5' and 3' primer pairs listed in Table 1. The amplified fragment was digested with BamHI and XhoI, ligated with

similarly restricted pEG202, and introduced into *E. coli* XL1 and ultimately into *S. cerevisiae* EGY48. pEGVPg59-93 was produced by amplification of pETPro/24 with a first set of primers (Table 1); the amplified fragment was digested with EcoRI and XhoI and ligated into similarly digested pKS pBluescript I (Stratagene) to produce pKS-VPg3'. Plasmid pETPro/24 was also amplified with a second set of primers, and the amplified fragment was digested with BamHI and EcoRI and ligated in similarly digested pKS-VPg3'. This plasmid was digested with BamHI and XhoI, and the VPg-containing fragment was ligated into BamHI- and XhoI-digested pEG202.

TABLE 1. List of oligonucleotides used in this study for plasmid construction and site-directed mutagenesis

Plasmid construct	5' Oligonucleotide sequence (5'3')	3' Oligonucleotide sequence (5'3')
pEGVPg7-191	AAAGGCAGGATCCAAAGACAG	AGTTACTCTCGAGGTCCACT
pEGVPg94-191	CCATTCACGGAATTCACCCTTGTA	AGTTACTCTCGAGGTCCACT
pEGVPg62-191	GAACAGGAGGATCCTTAACA	AGTTACTCTCGAGGTCCACT
pEGVPg7-63	AAAGGCAGGATCCAAAGACAG	GATCAAACTCGAGCATGTTA
pEGVPg59-93	AAAGGCAGGATCCAAAGACAGa	CATGTTAATGAATTCCCTGTTCTT
	CCATTCACGGAATTCACCCTTGTA	AGTTACTCTCGAGGTCCACT
pETeIF4Eat	TAATTTAGGGAATTCGGAGAAACA	GCAAAGATTCTCGAGGTTTCAAGC
pEGVPgF59A	CATGAAGCGGAATTCAAGAGGCAA	CTGACTGTTCTCGAGTGGCATTAT
	AACAGGGCAATCAACATGTAT	ATACATGTTGATTGCCTTCCTGTT
pEGVPgY63A	CATGAAGCGGAATTCAAGAGGCAA	CTGACTGTTCTCGAGTGGCATTAT
	TTCATCAACATGGCCGGCTTTGAT	ATCAAAGCGGGCCATGTTGATGAA
pEGVPgD77A	CATGAAGCGGAATTCAAGAGGCAA	CTGACTGTTCTCGAGTGGCATTAT

a Oligonucleotides on the same line were used in pairs, and amplified fragments were assembled as described in Materials and Methods.

Recombinant protein expression in *E. coli* and purification. Plasmid pETtag(iso)4EAt codes for eIF(iso)4E of *A. thaliana* and was produced by digestion of plasmid pSW56 with EcoRI and XhoI and ligation of the 0.7-kb insert with similarly restricted pET21a (Novagen). The resulting eIF(iso)4E is fused at its N-terminal end to the 11-amino-acid N-terminal peptide of the T7 gene 10 protein (T7 tag), which is recognized by the anti-T7 tag monoclonal antibody (Novagen). Plasmid pETtag(iso)4ETa codes for eIF(iso)4E of *Triticum aestivum* (wheat) and was produced by digestion of plasmid pGAG424/eIF(iso)4E (a generous gift from K. S. Browning, University of Texas) with EcoRI and SalI and ligation with EcoRI- and XhoI-restricted pET21a. The resulting protein is fused at its N-terminal end with the T7 tag. Plasmid pETtag4EAt codes for eIF4E of A. thaliana and was produced by amplification of plasmid pET14b/eIF4E (kindly provided by C. Robaglia, Centre d'Énergie Atomique) with the primers listed in Table 1; the amplified fragment was digested with EcoRI and XhoI and ligated with EcoRI- and XhoI-restricted pET21a. The resulting recombinant protein is fused at its N-terminal end with the T7 tag. Plasmids were introduced into E. coli BL21(DE3). Recombinant proteins were purified as described earlier (65).

VPgPro was purified as previously described (37). VPgPro was produced as follows. pETPro/24 and pEGVPg59-93 were digested with NcoI and StuI. The 5.5- and 0.4-kb fragments from pETPro/24 and pEGVPg59-93, respectively, were purified and ligated. The ligation product was introduced into *E. coli* XL1-Blue and ultimately into

BL21(DE3). The recombinant protein was expressed and purified as described above for VPgPro.

ELISA-based binding assay. Purified VPgPro was adsorbed to the wells of an enzyme-linked immunosorbent assay (ELISA) plate (1.0 μg/well) by overnight incubation at 4°C, and the wells were blocked with 5% Blotto in phosphate-buffered saline (PBS). Purified initiation factor was diluted in 1% Blotto in PBS with 0.2% Tween and was incubated for 1 h at 4°C with the previously coated wells. Detection of bound initiation factor was achieved as in the ELISA assays with the anti-T7 tag antibody and peroxidase-labeled goat anti-mouse immunoglobulin G (KPL). Wells were washed three times with 0.05% Tween between incubations.

Site-directed mutagenesis. PCR site-directed mutagenesis by the overlap extension method was done as described previously (24). Primers used for mutagenesis are listed in Table 1, and plasmid p35Tunos (53) was used as a template. Amplification was performed with the Pwo DNA polymerase (Roche).

Particle bombardment. Plasmid p35TuD77N was constructed by digesting p35Tunos (53) with ClaI and ligating the 3.8-kb fragment with similarly digested pKS pBluescript I (Stratagene), resulting in the recombinant plasmid pKS-Tunos/Cla. Plasmid pEGVPgD77N was digested with PmlI and SpeI, and the corresponding fragment was inserted into pKS-Tunos/Cla linearized with SpeI and partially digested with PmlI. This last construction was digested with ClaI, and the fragment was ligated back into p35Tunos. Proper assembly was verified by nucleic acid sequencing. Particle

bombardment was done in the Biolistic PDS-1000/He instrument (Bio-Rad). Then, 7 μg of DNA was mixed with 3 mg of gold particles in 2.5 M CaCl2 and 0.1 M spermidine. This mixture was diluted 1:5 in ethanol, and 5 μl was placed in the center of a 900-lb/in2 rupture disk. *Brassica perviridis* plants at the two-leaf stage were used.

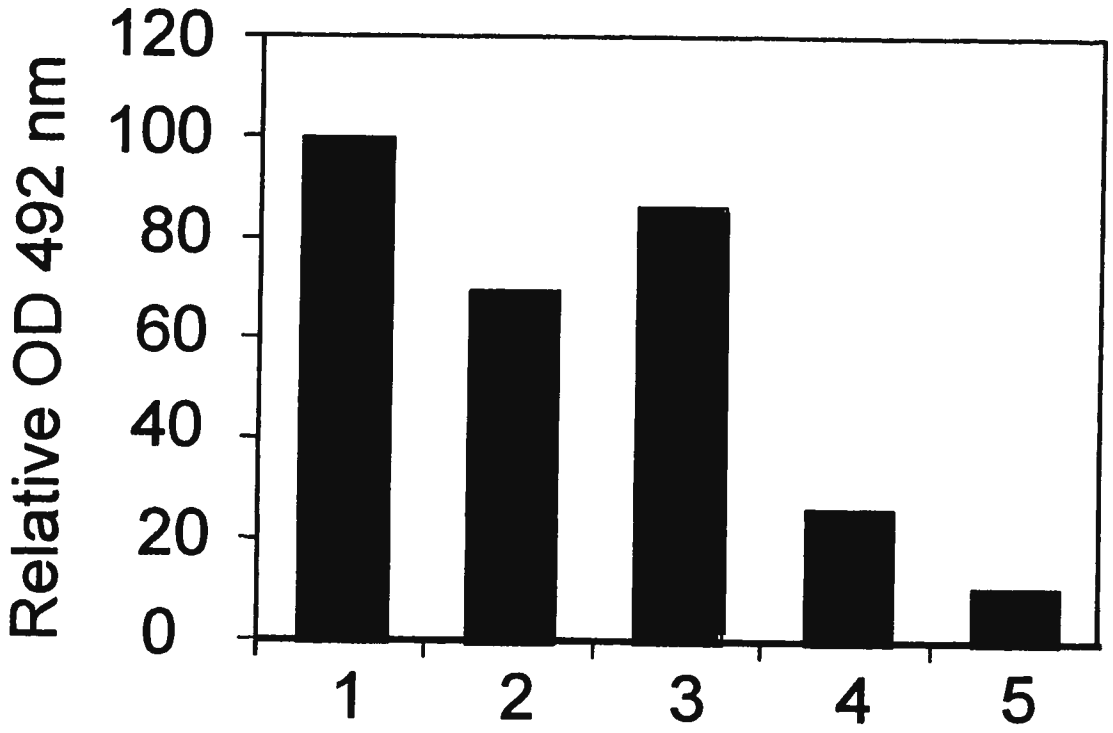
RESULTS

Interaction of VPg with eIF4E of *A. thaliana* and eIF(iso)4E of *T. aestivum*. Plants have two isomers of the cap-binding initiation factor, namely, eIF(iso)4E and eIF4E (7, 8). A third factor was recently identified (51), but its involvement in translation initiation relative to the eIF4E isomers is unclear. Since both isomers of eIF4E participate in translation initiation, it was speculated that VPg would bind to both forms and to eIF(iso)4E from a monocotyledenous species such as *T. aestivum* (wheat). *A. thaliana* is a dicotyledenous plant and is infected by TuMV, whereas wheat is not a host of the virus. Interactions between the viral protein and these initiation factors were investigated using an ELISA-based binding assay. The initiation factors were produced in *E. coli* as recombinant proteins fused at their N-terminal end to the 11-amino-acid N-terminal peptide of the T7 gene 10 protein (T7 tag), which is recognized by an anti-T7 tag monoclonal antibody. The proteins were purified by m^7GTP-Sepharose chromatography. ELISA plate wells were coated with 1.0 μg of recombinant VPgPro (see protein purity in Fig. 2A, lane 1) and incubated with 2.0 μg of the different initiation factors. VPgPro, a precursor form of VPg, was used because it is purified more easily than VPg in *E. coli* and because it had been shown that the Pro domain does not participate in eIF(iso)4E binding (65). Complex formation was detected using anti-T7 tag antibodies. Figure 1 shows that VPgPro interacted most effectively with eIF(iso)4E of *A. thaliana*, and the level of that interaction was given as a relative value of 100 (lane 1). The interaction was specific for the viral protein since the initiation factor was not retained when wells were not coated with VPgPro (lane 5), nor was it retained with an *E. coli* lysate not containing

VPgPro (65). Figure 1 also shows that eIF4E from *A. thaliana* (lane 2) and eIF(iso)4E from wheat (lane 3) interacted with VPgPro. Once the OD values were corrected for background noise (i.e., the OD value obtained in the absence of initiation factors [lane 4]), the binding values of VPgPro to eIF4E from *A. thaliana* and eIF(iso)4E from wheat were 60 and 80%, respectively, of the binding to eIF(iso)4E from A. thaliana. This experiment indicated that the VPg of TuMV interacted with several initiation factor species, with similar binding affinities.

Figure. 1.

VPg interaction with eIF4E isomers as demonstrated by ELISA-based binding assay. Wells precoated with 1.0 µg of VPgPro were incubated with 2.0 µg of eIF(iso)4E (lane 1) and eIF4E (lane 2) from *A. thaliana*, eIF(iso)4E from *T. aestivum* (lane 3), or no added initiation factor (lane 4). In lane 5, wells were coated with Blotto only and incubated with 2.0 µg of eIF(iso)4E from *A. thaliana*. Complexes were detected using anti-T7 tag antibodies. Values are averages of two replicates from a typical experiment.



Mapping of the VPg interaction domain. Since VPg interacted with the different isomers of the initiation factor and since the interaction is likely to be important for all potyviruses, it was hypothesized that the VPg domain responsible for the eIF(iso)4E interaction would be conserved among different potyviral VPgs. The VPg domain involved in the interaction with eIF(iso)4E was mapped using the yeast two-hybrid system. Deletions in the VPg gene were made by PCR and were fused to the gene coding for the DNA-binding domain of LexA in pEG202. These recombinant plasmids were introduced into the yeast EGY48 strain, which contained either pJG4-5 (carrying the activation domain without insert) or pSW56 which codes for eIF(iso)4E of *A. thaliana* fused to the activation domain of pJG4-5. The lacZ reporter plasmid pSH18-34 was also present in the yeast cells. Interaction between the different deleted VPg domains and eIF(iso)4E was measured by β-galactosidase assay. The near-full-length VPg comprising amino acids 7 to 191 (VPg7-191) strongly interacted with eIF(iso)4E, providing on average 659 U of β-galactosidase activity (Table 2). No activity was measured when the initiation factor was omitted. VPg fragments comprising amino acids 7 to 63 (VPg7-63) or amino acids 94 to 191 (VPg94-191) failed to interact with the initiation factor. However, the VPg fragment comprising amino acids 62 to 191 (VPg62-191) strongly interacted with eIF(iso)4E. This suggests that the region comprising amino acids 62 to 93 was involved in the interaction. This was confirmed by the deletion of amino acids 59 to 93 from VPg; this deletion mutant (VPgΔ59-93) exhibited extremely low levels of interaction (17 U of β-galactosidase).

TABLE 2. β-Galactosidase activity displayed by various VPg deletions in yeast expressing eIF(iso)4E from *A. thaliana* fused to the B42 activation domain

Schematic representation of VPg deletion fused to DNA binding domain of LexA	VPg residues (range)[a]	Interactor	β-Galactosidase units
	7–191	None[b]	0[c]
		eIF(iso)4E	659
	7–63	None	6
		eIF(iso)4E	6
	94–191	None	5
		eIF(iso)4E	6
	62–191	None	9
		eIF(iso)4E	1,081
	Δ59–93[d]	None	5
		eIF(iso)4E	17

a Numbers represent first and last residues of VPg fused to DNA binding domain of LexA.

b Yeast containing pJG4-5.

c Average value of two replicates from a typical experiment.

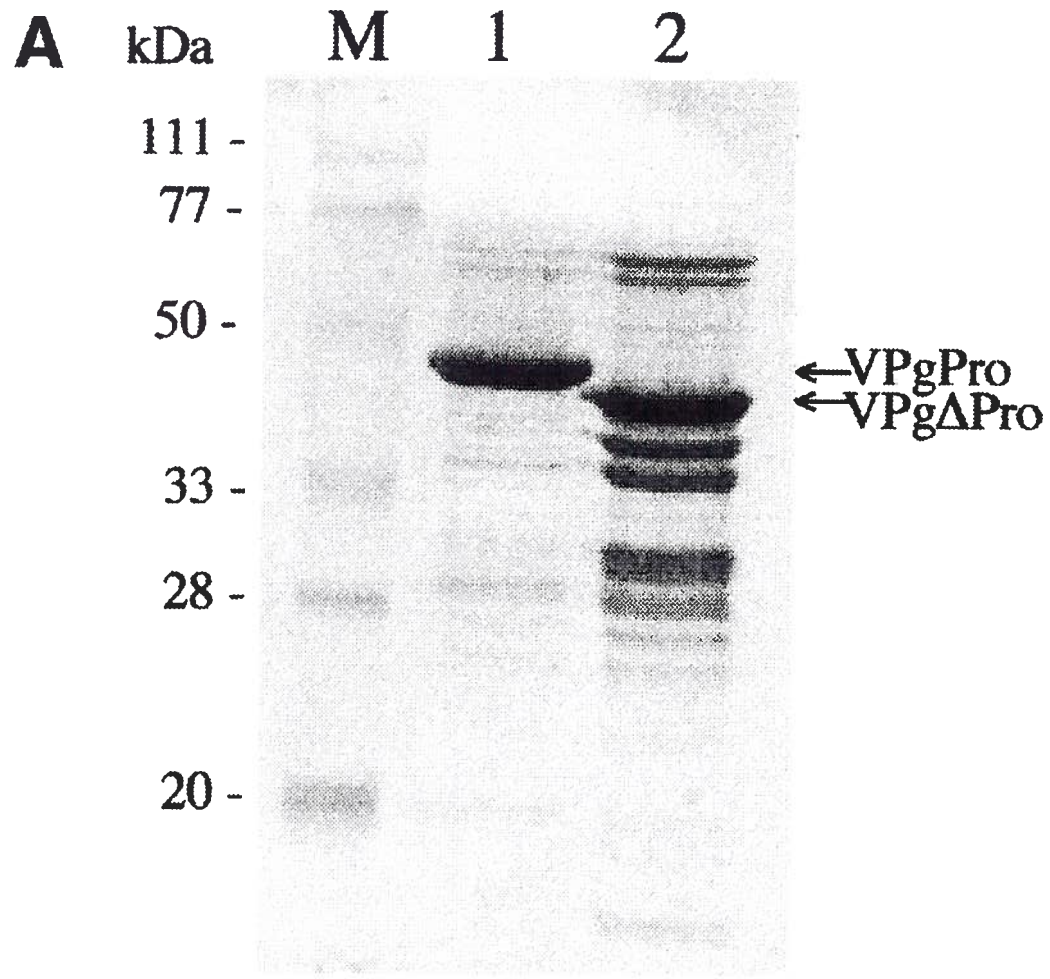
d Symbol and numbers represent deleted residues on VPg7-191.

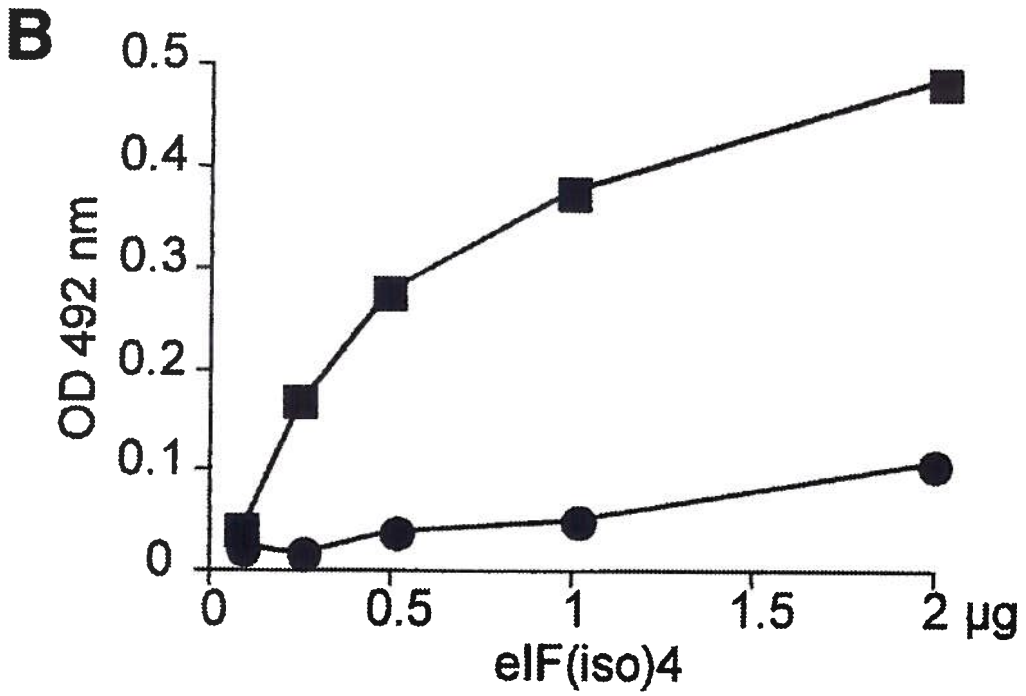
The lack of interaction with eIF(iso)4E by VPgΔ59-93 could, however, have been the result of degradation of the fusion protein or lack of nuclear transport in the yeast. To test that this was not the case, *in vitro* binding assays with purified proteins were performed. The deletion mutant gene was subcloned in the plasmid pET21a and expressed as a Pro fusion (VPgΔPro) in *E. coli*. The protein was purified using the same procedure as for VPgPro. While VPgPro was purified as a 49-kDa species (Fig. 2A, lane 1), multiple forms of VPgΔPro, with a main band at 46 kDa, were observed (lane 2). This degradation of VPgΔPro suggests that deletion of the amino acids caused the protein to be more susceptible to degradation than the complete VPgPro in *E. coli*. Once purified, VPgΔPro was not susceptible to further degradation. Conditions for the binding assay were adjusted so that similar concentrations of VPgPro and nondegraded VPgΔPro were used. ELISA plate wells were coated with either 1.0 μg of VPgPro or 4.0 μg of VPgΔPro and then incubated with increasing concentrations of eIF(iso)4E. Compared with wild-type VPgPro, VPgΔPro bound approximately fivefold less initiation factor (Fig. 2B). This experiment suggests that amino acids 59 to 93 of VPg are largely responsible for the binding of eIF(iso)4E.

Figure. 2. VPgPro and VPgΔPro interaction with eIF(iso)4E of *A. thaliana* as demonstrated by ELISA-based binding assay.

(A) Purification of VPgPro and VPgΔPro. Expression and purification were as described in Materials and Methods. Samples were loaded on a sodium dodecyl sulfate-polyacrylamide gel and stained with Coomassie blue. Lane 1, VPgPro (5 μg); lane 2, VPgΔPro (20 μg); lane M, molecular mass standards.

(B) ELISA-based binding assay. Wells were coated with 1.0 μg of VPgPro (■) or 4.0 μg of VPgΔPro (●) and then incubated with increasing concentrations of eIF(iso)4E from A. thaliana. Complexes were detected using anti-T7 tag antibodies. Values are averages of two replicates from a typical experiment.


A
kDa
M
1
2
111 -
77 -
50 -
33 -
28 -
20 -
←VPgPro
←VPgΔPro


B
OD 492 nm
0.5
0.4
0.3
0.2
0.1
0
0
0.5
1
1.5
2 µg
eIF(iso)4

The 35 amino acids identified above are listed in Fig. 3 and were compared with the corresponding region of eight potyviruses. The comparison indicates that the region is highly conserved among the different potyviruses: of the 35 amino acids, 8 residues are identical for all listed viruses, 13 are identical for most of the listed viruses, and 7 residues belong to the same class.

Figure. 3. Amino acid sequence of the eIF(iso)4E-binding domain of VPg and comparison with corresponding region from other potyviruses. Amino acid sequences were aligned using BLAST software with the BLOSUM62 matrix provided on the NCBI World Wide Web server. The numbers for TuMV represent the first and last residue positions of VPg; for the other viruses, the numbers represent the first and last residue positions of the polyprotein. Dashes indicate amino acids identical to that of the TuMV VPg. PPV, plum pox potyvirus (accession number S47508); LMV, lettuce mosaic potyvirus (P89876); TVMV, tobacco vein mottling potyvirus (P09814); PVY, potato mosaic potyvirus (1906388); TEV, tobacco etch potyvirus (P04517); BCMV, bean common mosaic potyvirus (Q65399); PRSV, papaya ringspot potyvirus (Q01901); ZYMV, zucchini yellow mosaic potyvirus (Q89330).

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TuMV    59  FINMYGFDPEDFSAVRFVDPLTGATLDDNPFTDIT   93
PPV   1915  -V----Y--T-YNF---------H---E--LM--N  1949
LMV   2080  -V----YN---Y-FI--L-----K-M-EQV----S  2114
TVMV  1856  -V----VS-DEY-Y--YL--V------ES-M--LN  1890
PVY   1923  ----------TEY-FIQ--------QIEE-VYA--R  1957
TEV   1907  ----------T---YI--------H-I-ESSTNA-D  1941
BCMV  1920  ------VE--NY-TL--------H-M-ES-RV--R  1954
PRSV  2152  -VAT---K---Y-Y--YL-----E---ES-Q---S  2186
ZYMV  1911  -VHL--VE--NY-FI--------H---ESTH---S  1945
```

Site-directed mutagenesis of the phenylalanine at position 59, the tyrosine at position 63, and the aspartic acid at position 77 of the VPg was undertaken to determine their importance for eIF(iso)4E binding. Phe59 and Asp77 are conserved in all listed potyviruses and are adjacent to other highly conserved residues; Tyr63 is the residue which is covalently linked to the viral RNA (39, 40, 47). The VPg from an infectious TuMV cDNA clone (p35Tunos) derived from the UK1 strain (53) was used for these mutagenesis experiments since introduced mutations could be transferred back into infectious cDNA plasmids without introducing changes elsewhere in the viral genome. The VPg sequence of the Quebec and UK1 strains differ at several nucleic acid positions (mainly at position 3 of the codons) but differ by only four amino acid residues clustered in the middle of the protein. However, these residues are outside of the eIF(iso)4E binding region mapped above. The affinity of VPg from both strains for eIF(iso)4E of A. thaliana was similar, as determined with the yeast two-hybrid system (data not shown).

PCR site-directed mutagenesis by overlap extension was used to introduce substitutions, and the interaction of the VPg mutants with eIF(iso)4E was measured using the yeast two-hybrid system. Here, a portion of Pro was introduced along with VPg in pEG202 for subsequent subcloning into p35Tunos. Mutants VPgF59A and VPgY63A, which introduced alanine residues at positions 59 and 63, respectively, produced -galactosidase activity levels similar to that of the wild-type VPg, indicating that their modification did not affect VPg interaction with the initiation factor (Table 3). Mutants VPgD77A, VPgD77E, and VPgD77N, which introduced either an alanine, a glutamic acid, or an asparagine, respectively, at position 77, failed, however, to interact with the translation factor. The importance of the aspartic acid in the interaction is stressed by the fact that

replacement with related amino acids such as glutamic acid and asparagine abolished binding.

TABLE 3. β -Galactosidase activity displayed by mutants of VPg in yeast expressing eIF(iso)4E from *A. thaliana* fused to the activation domain B42

VPg	Interactor	βGalactosidase units
Wild type	None[a]	0[b]
	eIF(iso)4E	178
F59A[c]	None	0
	eIF(iso)4E	125
Y63A	None	0
	eIF(iso)4E	198
D77A	None	0
	eIF(iso)4E	0
D77E	None	0
	eIF(iso)4E	0
D77N	None	0
	eIF(iso)4E	0

a Yeast containing pJG4-5.

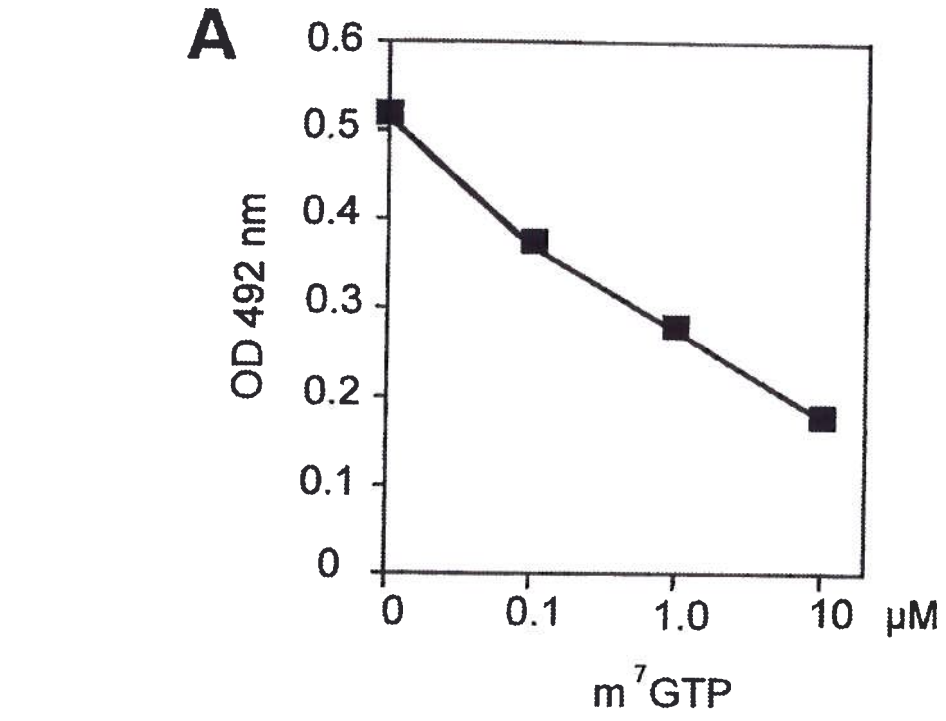
b Average value of three replicates from a typical experiment.

c First and second letters represent original and modified residues, respectively; the number is the residue position on VPg.

Effect of m^7GTP on the formation of VPg-eIF(iso)4E complex. eIF(iso)4E's role in the cell is to initiate assembly of the translation apparatus by binding to the 5' m^7G residue of the mRNAs. In order to test whether the VPg and mRNAs would compete for eIF(iso)4E interaction, the influence of the cap analogue m^7GTP on the formation of the VPg-eIF(iso)4E complex was tested. ELISA plate wells were coated with 1.0 μg of recombinant VPgPro and incubated with 2.0 μg of eIF(iso)4E and various concentrations of m^7GTP. Complex formation was detected with anti-T7 tag antibodies. Figure 4A shows that increasing concentrations of the analogue progressively prevented the formation of the VPg-eIF(iso)4E complex. These concentrations appear to be physiologically relevant since m^7GTP at 4 μM greatly inhibited in vitro translation of RNAs in rabbit reticulocyte lysates (9). The cap analogue m^7GTP used at a concentration of 10 μM inhibited complex formation by 60%, while GTP used at the same concentration had no effect on the formation of the complex. To determine what type of ligand relationship (i.e., competitive or noncompetitive) existed between VPg and m^7GTP, ELISA plate wells were coated with 1.0 μg of recombinant VPgPro and incubated with increasing concentrations of eIF(iso)4E in the absence or in the presence of 0.5 and 1.0 μM m^7GTP. Binding data were treated as enzyme kinetic data and were represented as a Lineweaver-Burk plot [i.e., 1/OD492 versus 1/eIF(iso)4E] (Fig. 4B). The experimental points were not expected to fall on a straight line since VPg and eIF(iso)4E are in the same concentration range, while in enzyme kinetics the substrate concentrations are much higher than the enzyme concentrations. Curves were fitted across the experimental points using least-square analysis, assuming a binomial equation of the following type: $y = ax + bx^2 + c$. The three lines crossed at a single point left of the

y axis. Such a pattern is indicative of mixed-type noncompetitive ligand binding, meaning that VPg and m^7GTP can simultaneously bind eIF(iso)4E, but the binding of one ligand decreases the binding affinity for the second ligand (56). This binding relationship is depicted in Fig. 5, where K1 and K2 are the dissociation constants for the respective complexes, and "a" is the factor by which the constants increase when the other ligand is already bound. Data of the type shown in Fig. 4B may be used to extract the dissociation constants (Kd) for the VPg-eIF(iso)4E and the m^7GTP -eIF(iso)4E complexes (56). When 1/[eIF(iso)4E] approaches zero (i.e., [eIF(iso)4E] > [VPgPro]), the bx2 term becomes negligible and the equation is now y = ax + c and has the same form as the Lineweaver-Burk equation, 1/v = Kapp/(Vmax[S]) + 1/Vmax. Using the values estimated for the constants a and c for each curve, the calculated Kd for the VPg-eIF(iso)4E complex is 0.9 μM, and the Kd for m^7GTP is 0.4 μM. The dissociation constant for m^7GTP measured here is slightly lower when compared with the Kd of 2 to 9 μM previously obtained for the dissociation of m^7GTP with wheat eIF(iso)4E (57, 63) and can be explained by the different experimental procedures used to determine the constant value. Furthermore, the factor by which the Kd of one ligand increases when the other ligand occupies its binding site is estimated to be 4.3.

Figure. 4. Inhibition m^7GTP of VPg-eIF(iso)4E complex formation as determined by ELISA-based binding assay. (A) Wells were coated with 1.0 μg of VPgPro and incubated with 2.0 μg of eIF(iso)4E from A. thaliana with increasing concentration of m^7GTP. Values are averages of two replicates from a typical experiment. (B) Lineweaver-Burk reciprocal representation of binding data. Wells were coated with 1 μg of VPgPro and incubated with increasing concentrations of eIF(iso)4E from A. thaliana in the absence (■) or presence, at 0.5 μM (●) or 1.0 μM (▲), of m^7GTP. Values are averages of two replicates from a typical experiment. Solid lines present the best fit of the data to equation $y = ax + bx^2 + c$.


A
0.6
0.5
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0.3
0.2
0.1
0
OD 492 nm
0
0.1
1.0
10
μM
m 7 GTP

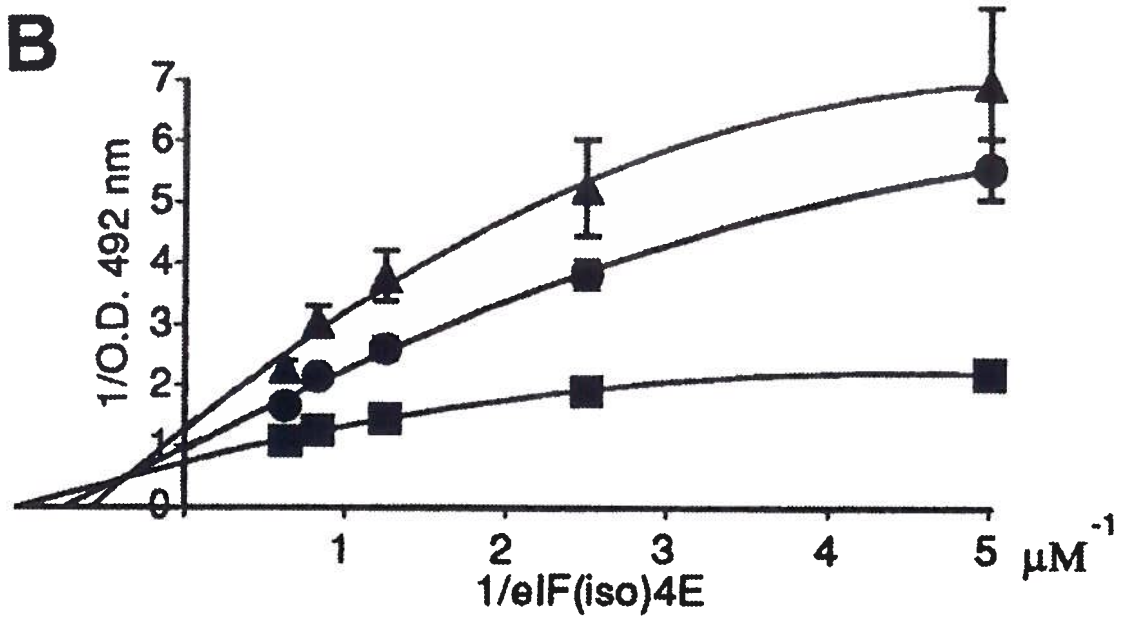
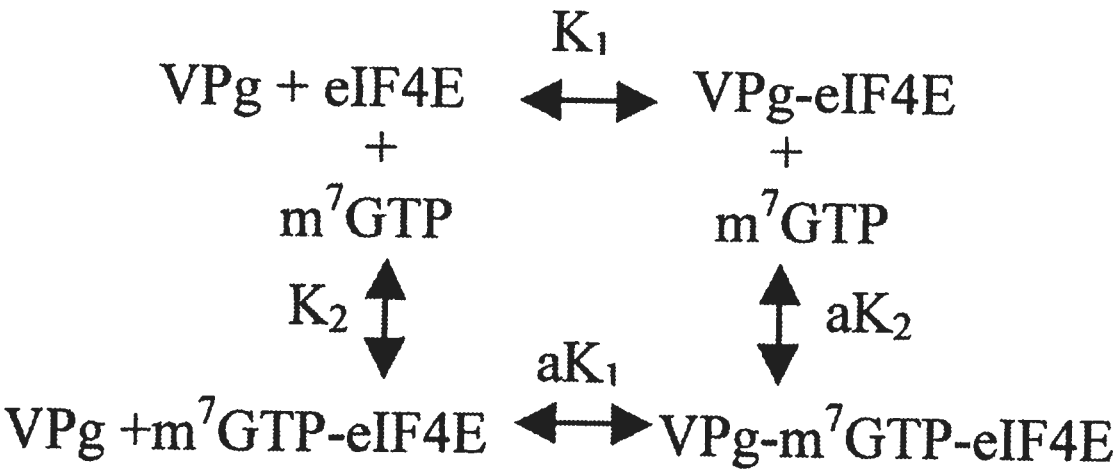
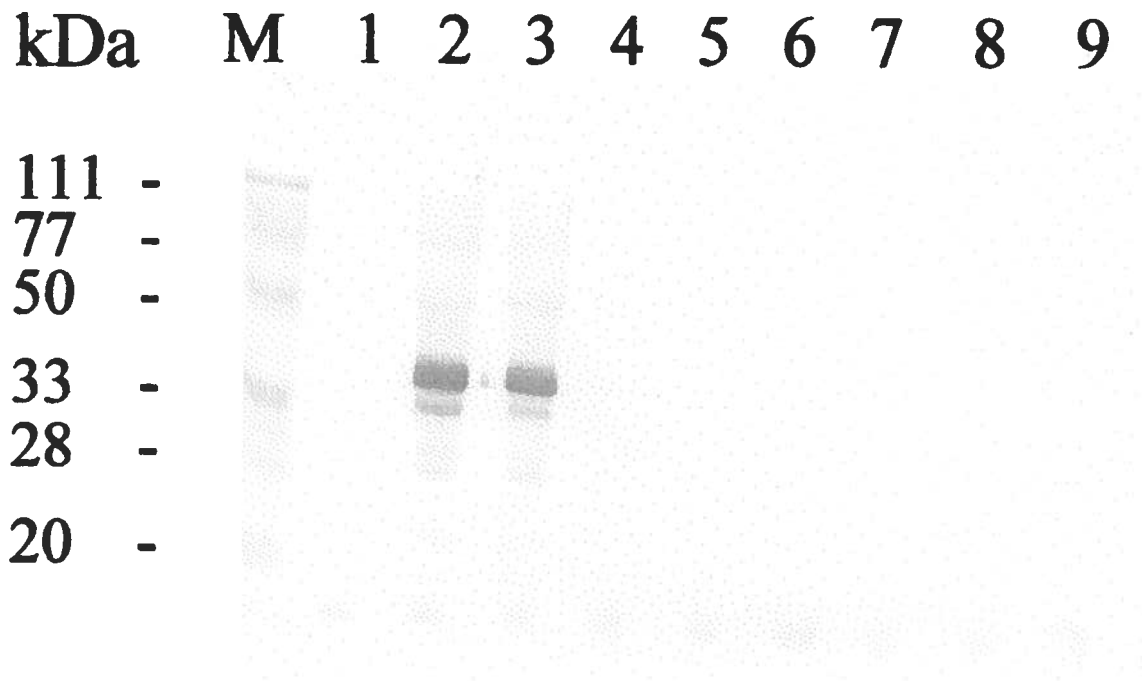

B
7
6
5
4
3
2
1
0
1/O.D. 492 nm
1
2
3
4
5
μM -1
1/eIF(iso)4E

Figure 5. Binding of VPg and m^7GTP to eIF(iso)4E.



Infection of whole plants. To determine if there is a correlation between the lack of in vitro interaction between VPg and eIF(iso)4E and debilitation of viral production, *B. perviridis* plants were infected with p35Tunos and p35TuD77N by particle bombardment. p35Tunos is an infectious cDNA clone of TuMV (53), and p35TuD77N is a p35Tunos derivative which contained the D77N mutation in the VPg domain that abolished the interaction with eIF(iso)4E. After bombardment, the plants were kept under an 18-h light regime at 22°C. After 8 days, plants bombarded with the wild-type infectious plasmid showed initial vein clearing followed by systemic mosaic symptoms. After 20 days, 14 of the 15 plants thus bombarded showed full symptoms of TuMV infection. On the other hand, plants bombarded with p35TuD77N remained symptomless. The presence or absence of viral proteins was confirmed by immunoblot analysis using a rabbit anti-TuMV capsid serum. No immunoreactive signal was found in mock-bombarded plants (Fig. 6, lane 1), while a strong signal of the expected molecular weight for the capsid protein was observed in plants bombarded with p35Tunos (lanes 2 and 3). No immunoreactive species were found in those plants bombarded with p35TuD77N (lanes 4 to 9).

Figure. 6. Immunoblot analysis of *B. perviridis* plants bombarded with TuMV plasmid cDNA. After bombardment, plants were placed in a growth chamber for 10 days. Proteins were extracted from the new leaf emerging above the one bombarded, separated on a sodium dodecyl sulfate-polyacrylamide gel, transferred on a nitrocellulose membrane, and incubated with a rabbit anti-TuMV capsid serum. Lane 1, plant bombarded with gold particles not coated with DNA; lanes 2 and 3, plants bombarded with p35Tunos; lanes 4 to 9, plants bombarded with p35TuD77N; lane M, molecular mass standards.



DISCUSSION

Viruses use the cellular machinery for their replication, and this implies that viral proteins interact with proteins from the host. In this study, experiments were undertaken to investigate the biological importance of the interaction between the VPg of TuMV and eIF(iso)4E of *A. thaliana*. In *A. thaliana*, eIF4E and eIF(iso)4E share 70% identity in their amino acid sequence, and the identity between eIF(iso)4E from *A. thaliana* and from wheat is equally high at 70% (48). This high sequence homology is found in other plant species as well (6). The two factors are mechanistically equivalent for the translation process but exhibit differences in their ability to bind m^7GTP and other cap analogues (8), as well as in their expression in different organs (48). Because of this homology in sequence and function, VPg binding to eIF4E and eIF(iso)4E *from A. thaliana* as well as to eIF(iso)4E from wheat was expected and indicated that it can take place in many cell types and plant species, both monocotyledenous and dicotyledenous. In addition, the identification of the VPg domain interacting with eIF(iso)4E in a conserved region among potyviruses suggests that this interaction exists with other potyviruses as well. Preliminary experiments with the VPg of tobacco vein mottling potyvirus and plum pox potyvirus showed that indeed they can interact with eIF(iso)4E of *A. thaliana* (M. G. Fortin et al., unpublished results). Interaction with various initiation factor isomers and the identification of the binding domain in a highly conserved region of the VPg are indications that the interaction plays an important role in the viral life cycle. This presumed important role is supported by the fact that a mutation in VPg which abolished the interaction with the translation factor in vitro debilitated viral infection in whole plants.

The ELISA-based binding experiments indicated that the initiation factor can simultaneously make a complex with VPg and m^7GTP. Ligand binding showed negative cooperativity (i.e., one ligand decreases the affinity of the initiation factor for the other ligand). Lower ligand affinity can result from the binding of the first ligand physically hindering the binding of the second ligand. It can also be the consequence of eIF(iso)4E undergoing a conformational change, which is known to take place when eIF(iso)4E binds m^7GTP (57). This binding cooperativity seems to be a feature of eIF4E to regulate its activity. For instance, binding of mammalian eIF4G to eIF4E increased the affinity of the latter for the cap analogue (22), and the wheat germ poly(A) binding protein enhanced the binding affinity of eIF4E isomers for the cap analogue (63). A consequence of negative binding cooperativity would be that the interaction of VPg with eIF(iso)4E can lower the affinity of the initiation factor for the cap structure of mRNAs *in planta*, which may lead to a decrease in host protein synthesis.

Interaction of plant viruses with the host translation machinery and its consequence on protein synthesis has not been intensively investigated (4). Recently, inhibition of host gene expression has been associated with potyvirus replication. By examining the front of virus invasion in immature pea embryos infected with pea seed-borne mosaic potyvirus, decreased levels of host transcripts were observed (61), but not for all transcripts (5). Although no experimental explanation was provided, reduced transcript levels can result from an inhibition of transcription and/or from hydrolysis of mRNAs. However, transcript hydrolysis may be the consequence of inhibition of translation since there is a relationship between translatability and mRNA stability (1); it has been proposed that

factors that stimulate translation initiation minimize the rate of entry of mRNA into decay pathways (26). For instance, a cis-acting mRNA stability determinant is the m^7Gppp cap. If less eIF(iso)4E is available for cap binding, the cap structure of mRNAs may become more susceptible to hydrolysis by decapping enzyme(s) (29), which then leads to degradation by 5'3' exonuclease(s) (1, 26). It remains to be seen if the interaction between VPg and eIF4E has any part to play in the observed inhibition of host gene expression during potyvirus infection.

This study showed that the ability of VPg to make a complex with eIF(iso)4E *in vitro* correlated with viral infection in planta. We are now attempting to elucidate the precise role of the VPg-eIF(iso)4E interaction in virus replication, i.e., whether VPg, when linked to viral RNA, can still bind the initiation factor and provide for the viral RNA a competitive edge over cellular mRNAs in translation initiation.

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FOOTNOTES

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Chapitre 3 :

Étude du mécanisme d'infection du potyvirus : effet sur l'expression des isomères d'eIF4E et interaction entre la VPgPro et la PABP "poly(A) binding protein".

Introduction à l'article II

Nous avons démontré dans notre laboratoire qu'il y a une corrélation entre la formation du complexe VPg-eIF4E et l'infectivité du virus de la mosaïque du navet (Léonard et al., 2000). Ces résultats suggèrent que l'interaction VPg-eIF4E est importante pour l'établissement de l'infection virale. Nous avons également montré que la VPg compétitionnait avec un analogue de la coiffe pour la liaison avec l'eIF4E *in vitro*. Par analogie avec d'autres virus de mammifères et à la lumière de nos résultats, nous croyons que la VPg agit sur l'eIF4E de façon à modifier l'activité de ce facteur d'initiation de la traduction.

Nous avons donc vérifié premièrement si l'infection du TuMV modifiait l'expression du facteur d'initiation de la traduction 4E *in vivo*. Nous avons également observé si la VPgPro était susceptible de participer à la réplication ainsi qu'à la traduction du génome viral.

Nous croyons que la VPgPro est une protéine multi-fonctionnelle importante notamment pour la réplication ainsi que pour la traduction du virus. Dans un premier temps, j'ai montré une nouvelle interaction entre la VPgPro et la PABP avec l'aide d'un test ELISA. J'ai effectué par la suite la panoplie d'interactions *in vitro* (présentée dans l'article) entre la VPg, la VPgPro, l'eIF4E, la RdRp et la PABP (figure 5 et figure 6 de l'article) J'ai également contribué à la réalisation des expériences d'expression de l'eIF4E suite à l'infection du TuMV (figure 1 et figure 2). Ces dernières manipulations ont été effectuées principalement par la technicienne du laboratoire (Nicole Daigneault).

Multi-protein interaction with VPgPro of turnip mosaic virus in infected cells.

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Sommaire traduit

La protéine VPg liant l'extrémité du génome du virus de la mosaïque du navet (TuMV) interagit *in vitro* avec le facteur eucaryotique initiant la traduction cellulaire (eIF) 4E. Dans la présente étude, nous avons observé les conséquences de l'infection du TuMV sur l'expression de l'eIF4E ainsi que l'interaction de la VPgPro avec d'autres protéines virales ou ayant la plante comme origine. Deux isomères sont présents dans les plantes l'eIF4E et l'eIF(iso)4E. Cette dernière est détectée dans les plants infectés et non-infectés de *Brassica perviridis* cependant l'isomère eIF4E a été seulement retrouvé dans les plants infectés. Un immunobuvardage de type Northern a montré que l'induction de la synthèse d'eIF4E lors de l'infection virale était sous le contrôle post-traductionnel. Les interactions entre la VPg avec les autres protéines ont été réalisée par des expériences de co-localisation sur membrane. Un gradient de sucrose a été utilisé pour séparer les membranes récoltées de plantes infectées ou non par le TuMV. Des immunobuvardages ont montré que la 6KVPgPro/VPgPro, l'ARN dépendante ARN polymérase (RdRp), l'eIF(iso)4E/eIF4E et la poly(A) binding protein (PABP) sont co-localisées dans les mêmes fractions, suggérant que ces protéines ont l'opportunité d'interagir. *In planta* l'interaction a été confirmée par purification de la 6KVPgPro/VPgPro par chromatographie d'affinité par chelation de métaux: la RdRp, l'eIF(iso)4E et la PABP2 ont été co-purifié avec la VPgPro. Des interactions directes entre la VPgPro et la RdRp ou la PABP ont également été montré en utilisant un test de type ELISA. Ces expériences suggèrent qu'un complexe multi-protéine peut se former autour de la VPgPro du TuMV.

Abstract

The viral protein linked to the genome (VPg) of turnip mosaic virus (TuMV) interacts *in vitro* with the translation eukaryotic initiation factor (eIF) 4E. In the present study, we investigated the consequence of TuMV infection on eIF4E expression and the interaction of VPgPro with other proteins of viral or plant origins. Two isomers are present in plants, namely eIF4E and eIF(iso)4E. The latter was detected in both TuMV-infected and mock-inoculated *Brassica perviridis* plants, but eIF4E was found only in infected plants. Northern blot experiments indicated that induction of eIF4E production during viral infection was under post-transcriptional control. Interaction of VPg with other proteins was investigated by membrane co-localization experiments. Membranes from TuMV-infected or mock-inoculated tissues were separated by sucrose gradient centrifugation and fractions were collected. Immunoblot analyses showed that 6KVPgPro/VPgPro, the viral RNA dependent RNA polymerase (RdRP), eIF(iso)4E/eIF4E and the poly(A) binding protein (PABP) co-localized in the same fractions, suggesting that these proteins have the opportunity to interact. *In planta* interaction was confirmed by purification of 6KVPgPro/VPgPro by metal chelation chromatography: RdRp, eIF(iso)4E/eIF4E and PABP2 were found to co-purify with VPgPro. Direct interaction between VPgPro and RdRp or PABP was shown by an ELISA-based binding assay. These experiments suggested that a multi-protein complex may form around VPgPro of TuMV.

Key words: Plant RNA virus; potyvirus; turnip mosaic virus; viral genome linked protein; VPg; eukaryotic initiation factor; eIF4E; poly(A) binding protein; PABP; protein interaction

Introduction

Turnip mosaic virus (TuMV) is a member of the largest family of plant viruses, the Potyviridae. Potyviruses have a plus sense, single-stranded RNA genome of about 10 kb in length, a poly(A) tail at the 3' end and a VPg (viral protein genome linked) covalently linked to the 5' end. The genome codes for one large polyprotein, which is processed into at least ten mature proteins by three viral proteinases (Pro) (50). Several functions have been attributed to the potyvirus VPg. First, the viral protein and its precursor form VPgPro interact with and have a stimulating effect on the activity of the viral RNA-dependent RNA polymerase (RdRp), suggesting a participation in viral replication (10, 13, 21, 34). VPg has also been shown to have a role in overcoming viral resistance in plants (5, 24, 26, 38, 42, 43, 49, 57). Finally, interaction between the VPg and the translation eukaryotic initiation factor (eIF) 4E has been reported for *Tobacco etch virus* (TEV) (55) and eIF(iso)4E for TuMV (65). This factor binds the cap structure of cellular mRNAs and has an important role in the regulation of translation initiation (58). Further characterization of the interaction involving the VPg of TuMV showed that the cap analogue m^7GTP, but not GTP, inhibited VPg-eIF(iso)4E complex formation, suggesting that the viral protein and cellular mRNAs compete for eIF(iso)4E binding (33). The biological significance of this interaction was investigated by infecting *Brassica perviridis* plants with a TuMV infectious cDNA, p35Tunos, and with p35TuD77N, a mutant which contained an aspartic acid substitution in the VPg domain that abolished the interaction with eIF4E *in vitro*. Plants infected with p35TuD77N remained symptom-less, indicating that there was a correlation between VPg-eIF4E binding *in vitro* and

virus viability *in planta* (33). In the case of TEV, the interaction was shown to have a positive effect on genome amplification (55).

Translation of viral RNAs by the host machinery is a crucial event in the virus cell cycle and proceeds essentially as for cellular mRNAs (14, 61). For cellular mRNAs, one of the first steps in translation is their recruitment by the eIF4F complex. eIF4E is a member of this complex and recognizes the cap structure (m^7GpppN, where N is any nucleotide) at the 5' end of cellular mRNAs. The other members of the eIF4F complex are eIF4G and eIF4A. In conjunction with additional proteins, the eIF4F complex links mRNAs to ribosomes, and promotes the search of the translation start site. Among the proteins that interact with components of eIF4F is the poly(A) binding protein (PABP). A functional consequence of this interaction is an increase in affinity of PABP for the poly(A) tail and of eIF4F for the 5' cap structure of mRNAs (15, 23, 59). In addition, association of PABP with eIF4F results in the circularization of mRNAs and promotes the re-initiation of translation (53).

Animal viruses have elaborated strategies that allow the preferential translation of viral mRNAs (6), with the consequence that host protein synthesis is usually blocked. This phenomenon is known as host translation shutdown. However, plant virus infection and its impact on the cell protein synthesis has not been as extensively studied as for animal virus infections (2). The most studied example of a block in translation involves the picornaviruses. The inhibition is mediated by the cleavage of eIF4G by viral or cellular proteinases. Hydrolysis of eIF4G prevents eIF4E from binding to the 43S ribosome subunit complex and thus precludes the recruitment of capped cellular mRNAs (29, 35). However, this presumption has recently been challenged (1). Equally, adeno- and influenza

viruses inhibit host cell protein synthesis by inactivating eIF4E through de-phosphorylation (12, 22). Finally, the ring Z protein of the lymphocytic choriomeningitis virus was shown to interact with eIF4E and to repress host mRNA translation (8).

RNA viruses may also recruit translation factors in order to further increase their translation efficiency. A case in point is the rotavirus NSP3 protein. It interacts with the 3' end of rotavirus mRNAs, which are capped but not polyadenylated (46, 47). NSP3 also interacts with eIF4G (45). Further studies showed that enhancement of rotavirus mRNA translation requires the simultaneous interaction of NSP3 with eIF4G and the mRNA 3' end (62). During rotavirus infection, PABP is probably evicted from eIF4G, impairing the translation of polyadenylated host mRNAs (45). Recently, it was shown that eIF4G may play a role in the translation competitive advantage conferred by the TEV internal ribosome entry site (16). Recruitment of translation initiation factor has additionally been noticed in the replication of RNA viruses (reviewed in 27). For example, bacteriophage Qβ needs host components of the translation machinery for negative-strand synthesis (4, 25). The exact role of translation initiation factors in viral replication is however not fully understood. Recently, Lellis *et al.* (32) demonstrated that eIF(iso) 4E plays an essential role in viral infection. Following chemical mutagenesis, a population of *Arabidopsis thaliana* was screened for resistance to TuMV infection. Three lines have been isolated and the locus responsible for the resistance has been linked to the gene coding for eIF(iso)4E. The mutations had introduced a premature stop codon and consequently a functional protein was not produced.

In the present study, we investigated whether eIF4E expression was affected following TuMV infection and whether VPg and its precursor form VPgPro could act as a recruitment focal point for other proteins of cellular and viral origins. Our data show that indeed the eIF4E isomers expression profile was modified, and that, in addition to eIF(iso)4E and eIF4E, the viral RdRp and PABP can interact with VPgPro *in planta*.

Materials and methods

Microorganisms and media

Manipulations of bacteria as well as nucleic acids and proteins were done by standard methods (54). *E. coli* XL1-Blue was used for subcloning, and *E. coli* BL21 (DE3) (Novagen) was used for protein expression.

Recombinant protein expression in *E.coli* and purification

Plasmid pETtagPABP codes for the poly(A) binding protein of wheat and was produced by digestion of plasmid pSKPABP (a generous gift from R. Gallie, University of California) with *Eco*RI and *Xho*I and ligation of the 2.2 kb insert with similarly restricted pET21a (Novagen). The resulting PABP was fused at its N-terminal end to the 11-amino-acid N-terminal peptide of the T7 gene 10 protein (T7 tag), which is recognized by the anti-T7 tag monoclonal antibody (Novagen). An overnight culture of *E. coli* BL21 (DE3) was diluted 1:100 in fresh medium and after 3 hr of growth at 37°C protein production was induced at room temperature with 0.4 mM isopropylthio-β-galactoside (IPTG). The cell pellet from 1 liter was resuspended in 5 ml of buffer TEDN (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM DTT) containing 0.5 mM PMSF. The cells were passed twice through a French press and immediately spun at 10 000 *g*. The supernatant containing the recombinant protein was kept at –70°C.

Plasmid pGSTRdRp codes for the TuMV RdRp and was produced by digestion of plasmid pSKRdRp with *BamH*I and *Sal*I and ligation of the insert with similarly restricted pGEX-3X (Amersham Bioscience). The resulting RdRp was fused at its N-terminal end to the GST protein, which is recognized by a rabbit anti-GST serum. Plasmid was introduced into *E. coli* BL21 (DE3). An overnight culture of *E. coli* BL21 (DE3) was diluted 1:100 in fresh medium and after 3 hr

of growth at 37°C, protein production was induced with 1.0 mM IPTG. The cell pellet from 1 liter was resuspended in 5 ml of lysis buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl) containing 1 mM DTT. The cells were passed twice through a French press and immediately spun at 10 000 *g*. The pellet was washed in 2.0 M urea and spun at 8 000 *g*. Inclusion body was solubilized in 4.0 M urea overnight and the resulting suspension was spun at 10 000 *g* for 15 min. Supernatant (2.5 ml) was applied on top of a PD-10 column containing Sephadex G-25 M (Amersham Bioscience) for rapid desalting and lysis buffer exchange. Fractions (0.5 ml) were collected, pooled and extensively dialysed against lysis buffer containing 1 mM DTT. VPgPro and eIF(iso)4E of *A. thaliana* were purified as previously described (65).

Two-dimensional gel electrophoresis

The first-dimension separation gel was done with the IPGphor Isoelectric Focusing System (Amersham Bioscience) using immobilized pH gradients steps pH 4-7, according to the manufacturer's instructions. The strips were equilibrated in buffer pH 4-7 and were resolved in the second dimension gel using standard SDS-PAGE. The proteins were transferred to a nitrocellulose membrane by electroblotting. Following transfer, the nitrocellulose membrane was incubated with a rabbit anti-eIFiso4E serum. The signal was detected by chemiluminescence using the ECL kit (Amersham Bioscience).

Northern analysis

Total RNA was extracted from TuMV-infected or mock-inoculated plants with the RNeasy plant mini kit (Quiagen). RNA was run on a 1.0% agarose gel (MOPS, 7.5% formaldehyde), and transferred to a nylon membrane (Roche). The RNA was immobilized on the membrane by UV light. Subsequently, membranes

were prehybridized for 1 h at 65°C in 5X SSC, 5X Denhardt's, 0.5% SDS, 0.1 mg/ml salmon sperm DNA and then hybridized overnight at 55-65°C with ^{32}P-labelled eIF4E or eIF(iso)4E probes. Blots were analysed with a Phosphor-Imager.

ELISA-based binding assay

The specified proteins were absorbed to wells of an ELISA plate (1.0 µg/well) by overnight incubation at 4 °C, and wells were blocked with 5% Blotto in phosphate-buffered saline (PBS). Appropriate proteins were diluted in 1% Blotto in PBS with 0.2% Tween and incubated for 1 h at 4°C with the previously coated wells. Detection of retained protein was achieved as in ELISA with the anti-T7 tag antibody and peroxydase-labeled goat anti-mouse immunoglobulin G (KPL) or the rabbit anti-GST serum peroxydase-labeled goat anti-rabbit immunoglobulin G (KPL), as appropriate. Wells were washed three times with 0.05% Tween between incubations.

Membrane fractionation

Membranes recovery from plants was done according to Schaad *et al.* (56), with some modifications. *B. perviridis* plants (three-leaf stage) were infected with TuMV, or mock-inoculated with PBS. At ten days post-inoculation, the leaf that developed next to the inoculated one was harvested. Leaf tissue (2 g) was minced in 6 ml of homogenization buffer (50 mM Tris-HCl, pH 8.0, 10 mM KCl, 3 mM $MgCl_2$, 1mM EDTA, 1mM DTT, 0.1% BSA, 0.3% dextran, 13% sucrose, 5 µg/ml leupeptin and 2 µg/ml aprotinin). The homogenate was filtered through Miracloth and subjected to centrifugation at 3 700*g* for 10 min at 4°C. The supernatant (2.5 ml) was layered onto a 9 ml 20-45% sucrose gradient containing the respective homogenization buffer and subjected to centrifugation at 143 000*g*

in a Beckman SW41 Ti rotor for 4 h at 4°C. Fractions (0.7 ml) were collected. The fractions were diluted 1:1 in protein dissociation buffer and subjected to immunoblot analysis after 12.5% SDS polyacrylamide gel electrophoresis. Immunoreactions were detected using a chemiluminescence-based secondary antibody system.

Results

Expression profile of eIF4E isomers following TuMV infection

The functional state of eIF4E is altered following infection by certain animal viruses (8, 9, 12, 22). Furthermore, plants possess two isomeric forms of the initiation factor: namely eIF4E and eIF(iso)4E (7). These isomers share significant sequence identity, but their respective role in translation is not yet clearly defined. The consequence of TuMV infection on the expression state of the eIF4E isomers was then investigated. *B. perviridis* plants at the three-leaf stage were infected with TuMV or mock-inoculated. At ten days post-inoculation, the leaf that developed next to the inoculated one was harvested. Infection was confirmed by immunoblot analysis using a rabbit serum raised against the capsid protein of TuMV (data not shown). Proteins from a 3 700*g* leaf extract supernatant were separated by SDS-PAGE and subjected to immunoblot analysis using a rabbit serum that was raised against a recombinant form of eIF(iso)4E from *A. thaliana*. eIF4E and eIF(iso)4E of *A. thaliana* have a calculated size of 26.5 kDa and 22.5 kDa, respectively (51). Although the size for the *B. perviridis* isomers is not known, it is expected to be very similar since both plants belong to the same family. Fig. 1a shows that eIF(iso)4E was detected in mock-inoculated plants (lane 3). It migrated to the same gel distance as recombinant eIF(iso)4E of *A. thaliana* (lane 1) and it was retained on m^7GTP Sepharose, confirming its cap-binding property (data not shown). eIF(iso)4E was also detected in TuMV-infected plants (lane 4) but an additional signal was found that had the same molecular weight as recombinant eIF4E of *A. thaliana* (lane 2). Cross-reaction of the anti-eIF(iso)4E serum with eIF4E can be expected since the two proteins are highly homologous (51) and a rabbit serum raised

against wheat eIF(iso)4E recognized both eIF4E and eIF(iso)4E of *A. thaliana* (52). TuMV infection thus induced the production of eIF4E, while the expression level of eIF(iso)4E did not appear to change significantly. Interestingly, a protein of lower molecular weight than eIF(iso)4E was detected only in infected leaves, but the nature of this species is not known.

The phosphorylation state of eIF4E has been shown to be affected during animal virus infection: the consequence being de-phosphorylation and thus decreased activity of the translation initiation factor (12, 22). Wheat eIF4E isomers are also phosphorylated (18), and it may be possible that TuMV infection altered their phosphorylation state. Proteins from TuMV-infected or mock-inoculated leaves were separated by two-dimensional gel electrophoresis and subjected to immunoblot analysis using the anti-eIF(iso)4E rabbit serum. Fig. 1b shows that eIF(iso)4E possessed different isoelectric species, but these were the same whether they were extracted from mock-inoculated or TuMV-infected leaves. The reason why eIF(iso)4E migrated as two series of spots in 2D gels is unknown, but a similar situation has been noticed in (18). eIF4E, on the other hand, existed as one predominant isoelectric species. Consequently, TuMV infection did not alter the phosphorylation state of eIF(iso)4E.

Differential expression of eIF4E may be the result of transcriptional induction of the corresponding gene, or of translation de-repression of eIF4E-coding mRNA in infected leaves. To determine which event was the case, total RNA was purified from TuMV-infected and mock-inoculated leaves, separated by agarose gel electrophoresis, blotted to nitrocellulose and hybridized with ^{32}P-labelled eIF(iso)4E or eIF4E probes. The length of the mRNA coding for eIF4E of *A. thaliana* is 910 nucleotides (accession number Y10548), and 812 nucleotides

for the eIF(iso)4E mRNA (accession number Y10547). Again, the size for the *B. perviridis* mRNAs is not known but it is expected to be very similar. Because of the high sequence homology between the two isomers, the stringency of the washing conditions was chosen so that the radioactive probes hybridized to both mRNAs. Fig. 1c shows that the eIF(iso)4E and eIF4E mRNAs were detected in both mock-inoculated and TuMV-infected samples, irrespective of the probe used. Interestingly, the mRNA coding for eIF4E was more abundant than the one coding for eIF(iso)4E. This experiment then indicates that production of eIF4E in infected tissue was the result of post-transcriptional gene regulation.

Fig 1: Expression profile of eIF4E isomers following TuMV infection. (A) Recombinant *A. thaliana* eIF(iso)4E (lane1) and eIF4E (lane2) as well as proteins from mock-inoculated (lane 3) and TuMV-infected (lane 4) leaf extracts were separated by SDS-PAGE and electroblotted to nitrocellulose. Immunoblot analysis was performed using an anti-eIF(iso)4E serum. (B) Proteins from mock-inoculated (top panel) and TuMV-infected (bottom panel) leaf extracts were separated by two-dimensional gel electrophoresis and electroblotted to nitrocellulose. Immunoblot analysis was performed using an anti-eIF(iso)4E serum. (C) Total RNA was isolated from mock-inoculated (lanes 1 and 3) and TuMV-infected (lanes 2 and 4), separated by agarose gel electrophoresis and transferred to nitrocellulose. Lanes 1 and 2 were hybridized with a ^{32}P-labelled eIF(iso)4E cDNA probe and lanes 3 and 4 with a ^{32}P-labelled eIF4E cDNA probe.

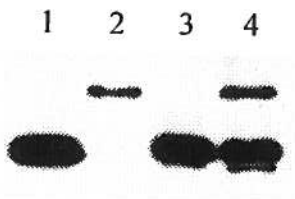

Fig. 1a

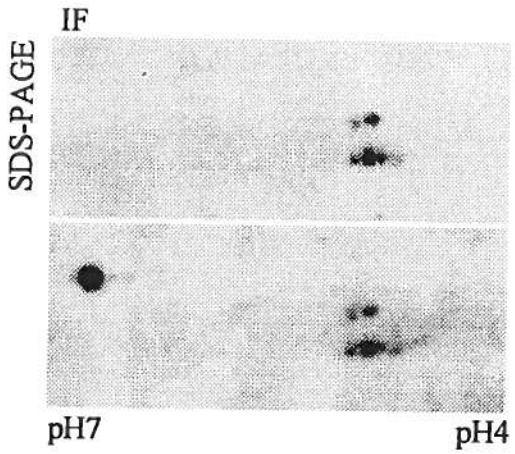

Fig. 1b

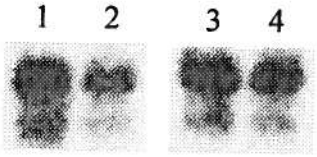

Fig. 1c

Membrane localization of VPgPro and eIF4E isomers

VPg has been shown to interact with eIF(iso)4E (65) and eIF4E *in vitro* (33), but one condition for *in planta* interaction is that the proteins must localize in the same cellular compartment. To determine whether VPg or VPg-containing precursor forms co-localize with any of the eIF4E isomers, sucrose gradients were used to separate membrane fractions extracted from TuMV-infected or mock-inoculated tissues. The gradients were carried out in the presence of 3 mM $MgCl_2$, which preserved the integrity of ribosomes associated with the rough endoplasmic reticulum (RER) (36). Fractions were collected, proteins separated by SDS-PAGE and analyzed by immunoblot assays using anti-eIF(iso)4E or anti-VPgPro rabbit sera. In mock-inoculated tissue, eIF(iso)4E was detected at the highest concentration in fraction 1 and in decreasing concentration thereafter up to fraction 12 (Fig. 2a). The initiation factor being associated with 48S ribosomal complexes (20), these fractions would indicate the position of the RER membranes in the sucrose gradient. eIF(iso)4E distribution in TuMV-infected plants was identical, the highest concentrations of eIF(iso)4E being found in the heavier fractions. eIF4E fractionated similarly as for eIF(iso)4E. Interestingly, the anti-eIF(iso)4E-reacting protein of lower molecular weight than eIF(iso)4E was found in the top fractions, presumably being localized in the cytosol. This protein may be a degradation, inactive, form of either eIF4E or eIF(iso)4E.

When the anti-VPgPro serum was used, no reactivity was noticed in the S3 extract recovered from mock-inoculated plants (Fig. 2c, lane N). On the other hand, several VPgPro-related species were detected in the S3 extract from TuMV-infected plants. These species had the expected molecular weight for

6KVPgPro, VPgPro and Pro. Recombinant forms of these species have been previously expressed in *E. coli* (28, 39) and migrated to the same position as for the proteins detected in TuMV-infected plants (data not shown). No species corresponding to the mature VPg was detected. In the sucrose gradient, the different VPgPro species localized to different fractions. A certain quantity of VPgPro, partially C-terminally processed VPgPro (39) and fully processed Pro were found on the top of the sucrose gradient (fractions 13 to 19). These species would presumably be localized in the cytosol. On the other hand, 6KVPgPro and the remaining portion of VPgPro were found in decreasing concentrations from fractions 1 to 10. The high 6KVPgPro/VPgPro concentration fractions were the same as those for eIF(iso)4E and eIF4E. The co-localisation of 6KVPgPro/VPgPro with eIF(iso)4E and eIF4E thus indicates that these proteins have the opportunity to interact *in planta*.

The sucrose gradient fractions were also analyzed by immunoblot assay using antibodies raised against the Bip protein, a marker of the endoplasmic reticulum. A relatively constant concentration of Bip was detected in fractions 1 to 14, but the concentration increased significantly in the remaining fractions. Presence of Bip in the heavy-membrane fractions is an indication for the presence of the ER (56).

Membrane separation in the presence of 0.1 mM $MgCl_2$ was also performed, and contrarily to what was observed by Schaad *et al.* (56) no shift of the proteins towards lighter fractions was noticed (data not shown). No explanation can be offered for this discrepancy. However, several fractionation experiments were done, each resulting in slight variations in the relative concentration peak position of the proteins being analyzed. In each case, the fractions containing the

highest concentration of the initiation factors corresponded to those containing the highest concentration of the 6KVPgPro/VPgPro pair.

Fig. 2. Detection of eIF4E isomers, VPgPro containing proteins and Bip in membrane fractions following centrifugation in sucrose gradient. Tissue extracts were prepared and centrifuged on 20-45% sucrose density gradients. The direction of sedimentation was from right to left, with fraction 19 representing the top of the gradient. Fractions were collected and proteins separated by SDS-PAGE and electrobotted to nitrocellulose. (A) Sucrose gradient using mock-inoculated extract and immunoblot analysis using anti-eIF(iso)4E serum. Recombinant eIF(iso)4E from A. thaliana (C) and non-fractionated extract (S) were also analyzed. (B) Sucrose gradient using TuMV-infected extract and immunoblot analysis using anti-eIF(iso)4E serum. Recombinant eIF4E from A. thaliana (C) and non-fractionated extract (S) were also analyzed. (C) Sucrose gradient using TuMV-infected extract and immunoblot analysis using anti-VPgPro serum. Recombinant VPg from TuMV (C), mock-inoculated (N) and TuMV-infected (S) non-fractionated extracts were also analyzed. (D) Sucrose gradient using TuMV-infected extract and immunoblot analysis using anti-Bip serum. Gel migration position of the different protein species is indicated to the left.

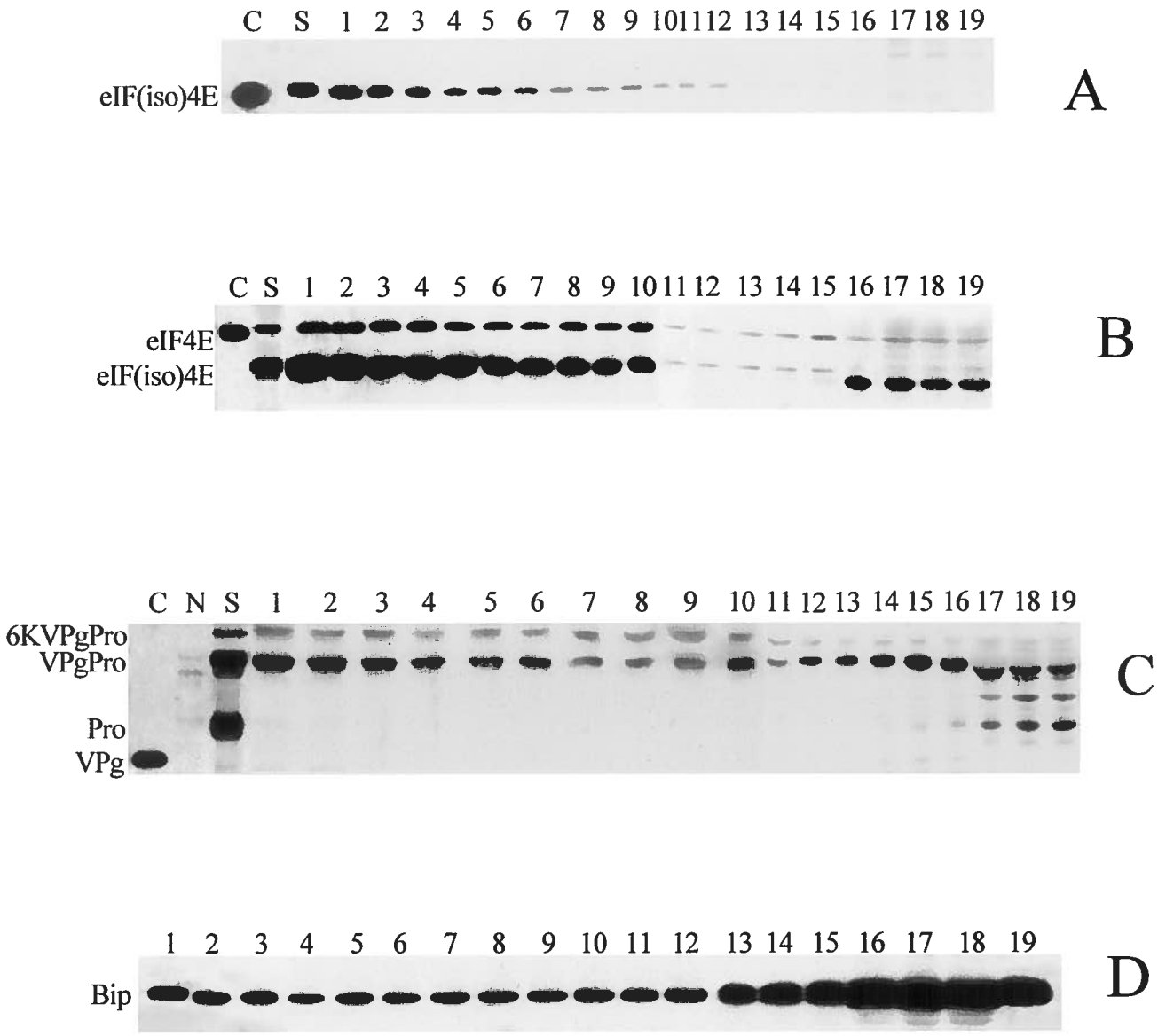
C S 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
eIF(iso)4E
A
C S 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
eIF4E
eIF(iso)4E
B
C N S 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
6KVPgPro
VPgPro
Pro
VPg
C
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Bip
D

Fig. 2

Membrane co-localization of RdRp and PABP

Potyvirus replication occurs in a membrane-associated cytoplasmic fraction (37), most likely the ER (56). Additionally, the RdRp is postulated to be recruited to the membrane via interaction with 6K-containing proteins, probably 6KVPgPro (50, 56). Consequently, membrane co-localization of the viral RdRp with 6KVPgPro was investigated. A rabbit serum that was raised against a recombinant form of the RdRp was used. A 59-kDa species, the expected molecular weight for the TuMV RdRp, was detected in fractions 1-15, with the highest concentration of RdRp found in fraction 1 (Fig. 3a). This reacting protein was not detected in the membrane fractions for mock-inoculated leaves (data not shown). The top fractions contained several anti-serum reactive species, but these were also observed for mock-inoculated leaf fractions. The membrane localization pattern for RdRp was thus identical to what was observed for 6KVPgPro/VPgPro, and indicated that these proteins have the opportunity to interact *in planta*.

An *in vitro* complex between VPg and eIF(iso)4G through the intermediary of eIF(iso)4E was observed (Plante *et al.*, submitted), and eIF(iso)4G interacts with PAPB (31). Additionally, the RdRp of *Zucchini yellow mosaic virus* was shown to *in vitro* interact with PABP (64). Consequently, a rabbit serum raised against PABP2 of *A. thaliana* was used to investigate the possibility that this protein could co-localize with VPgPro. A 69 kDa species, which corresponds to the molecular weight of PABP2, reacted with the antiserum and the highest concentrations were found in the heaviest fractions (Fig. 3b). Several reacting species were also found in fractions 16 to 19, and are likely other members of the

PABP family that cross-react with the anti-PABP2 serum. The membrane localization pattern for PABP2 was similar for mock-inoculated leaves (data not shown). The co-localization of PAPB2 with the other tested proteins is indicative that these proteins have the opportunity to interact *in planta*.

Fig. 3. Detection of RdRp and PABP in membrane fractions following centrifugation in sucrose gradient. Experimental conditions were as in Fig. 2. (A) Sucrose gradient using TuMV-infected extract and immunoblot analysis using anti-RdRp serum. (B) Sucrose gradient using TuMV-infected extract and immunoblot analysis using anti-PABP serum.

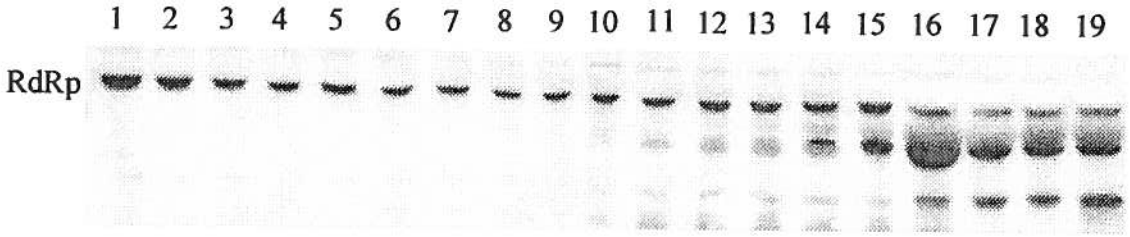
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
RdRp

Fig. 3a

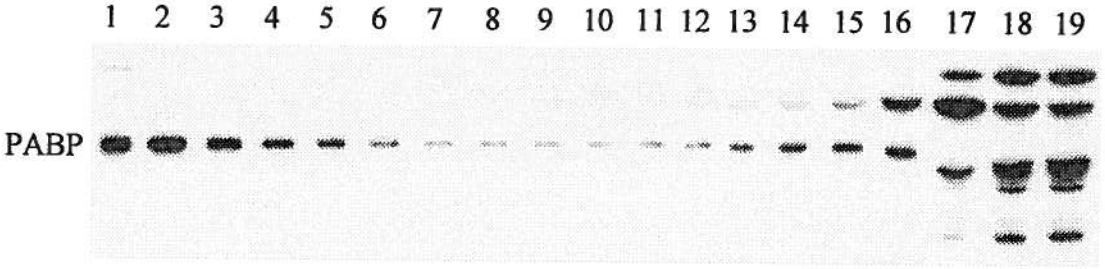
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
PABP

Fig. 3b

Co-purification of 6KVPgPro/VPgPro with RdRp, eIF(iso)4E/eIF4E and PABP2 from infected leaves

Membrane co-localization suggested that the tested proteins may interact with each other *in planta*. VPgPro has an intrinsic capacity to bind to nickel-agarose resin, the presence of an histidine tail being unnecessary (39). Purification of 6KVPgPro/VPgPro by metal chelation chromatography was thus attempted and co-purification of RdRp, eIF(iso)4E/eIF4E and PABP2 evaluated. For TuMV-infected and mock-inoculated tissues, fractions 1 to 10 from the membrane purification experiment were pooled and loaded onto a nickel-agarose column. After washing the resin, the bound proteins were eluted with 150 mM imidazole. Fig. 4 shows that both 6KVPgPro and VPgPro can be effectively purified from infected tissues. Similarly, RdRp, eIF4E, eIF(iso)4E and PABP were detected in the eluted protein fractions when the column was loaded with the membrane fractions from TuMV-infected leaves. They were not detected with mock-inoculated membrane fractions, even after prolonged film exposure. This experiment then indicates that eIF(iso)4E/eIF4E, PABP and RdRp form a complex with 6KVPgPro/VPgPro in infected cells.

Fig. 4. Co-purification of 6KVPgPro/VPgPro, RdRp, eIF(iso)4E/eIF4E and PABP by metal chelation chromatography. Fractions 1 to 10 from the sucrose gradient centrifugation experiment were pooled, membranes solubilized by the addition of 0.5% Tween-20 and loaded onto a column containing 0.4 ml of nickel-agarose resin. Proteins were eluted with imidazole, separated by SDS-PAGE and electrobotted to nitrocellulose. The membrane was probed using (A) anti-VPgPro serum, (B) anti-eIF(iso)4E serum, (C) anti-PABP serum and (D) anti-RdRp serum. Wells were loaded with (1) proteins derived from membrane fraction of mock-inoculated tissue, (2) proteins derived from membrane fraction of TuMV-infected tissue, (3) eluted proteins from mock-inoculated tissue and (4) eluted proteins from TuMV-infected tissue.

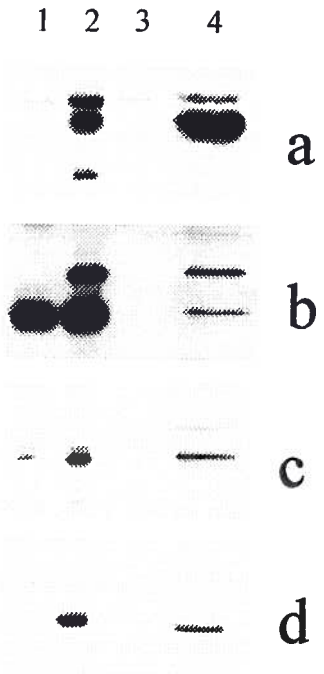

Fig. 4

Direct VPgPro interaction with RdRp and PABP

PABP and RdRp co-purification with 6KVPgPro/VPgPro may be the result of direct interaction with the viral protein, or through the intermediary of another protein that directly interacts with it. ELISA-based binding assays with recombinant proteins were done to investigate direct interaction with VPgPro. First, interaction between VPgPro and RdRp was investigated. ELISA plate wells were coated with VPg, VPgPro or with a nickel-agarose-purified *E. coli* lysate that did not contain VPg or VPgPro. The coated wells were then incubated with increasing concentrations of glutathione-S-transferase (GST)-tagged RdRp. The retention of RdRp was determined by using a rabbit anti-GST serum. A saturation binding curve was observed with both VPg and VPgPro, with OD values higher than those for the *E. coli* lysate control (Fig. 5a). To rule out the possibility that the GST domain was responsible for the interaction, binding to VPgPro by GST and GST-tagged RdRp was compared: no significant binding by GST was observed (Fig. 5b). These data indicate that the RdRp interacted directly with VPgPro through the VPg domain.

The ELISA-based binding assay was conducted to determine if VPgPro was also able to directly bind PABP. A recombinant form of PABP from wheat as described in Le *et al.* (30) was chosen. This isomer shares significant homology with PABP2 of *A. thaliana* with 58% identical amino acids. It should also be added that VPgPro interacted as effectively with eIF(iso)4E from *A. thaliana* as with eIF(iso)4E from wheat (33), suggesting that no major difference in binding exists between VPg-interacting proteins from different plant species. The PABP of wheat was produced in *E. coli* as a T7-tagged recombinant protein. Wells were coated either with VPgPro, VPg, the maltose binding protein (MBP) or

GST and incubated with an increasing volume of an *E. coli* lysate expressing T7-tagged PABP. Complex formation was detected using an anti-T7-tag antibody. Fig. 6 shows that VPg, MBP or GST did not interact with recombinant PABP. However, a saturation binding curve was observed for VPgPro. This experiment then indicates that the Pro domain of VPgPro interacted with PABP. Interaction with Pro cannot be demonstrated as this protein is rapidly degraded in *E. coli* (28). Binding conferred by the presence of the T7-tag moiety can be excluded as previous experiments have shown that eIF(iso)4E, whether possessing or not such a tag, bound similarly VPgPro (65).

Since VPgPro can bind PABP as well as RdRp, the influence of RdRp on PABP-VPgPro complex formation and vise-versa, was tested. ELISA plate wells were coated with VPgPro and incubated with increasing quantity PABP, in the absence or presence of a fixed quantity of RdRp. Retention of PABP was detected using an anti-T7 monoclonal antibody. As shown in Fig. 7a, a saturation binding curve between VPgPro and PABP was measured, whether RdRp was added or not. On the other hand, complex formation between VPgPro and RdRp was completely inhibited in the presence of PABP (Fig. 7b), suggesting that PABP competed with RdRp for VPgPro binding.

Fig. 5. VPg and VPgPro interaction with RdRp as demonstrated by ELISA-based binding assay. (A) Wells precoated with 1.0 μg of VPgPro (■), VPg(♦) and Ni-Sepharose purified *E. coli* proteins (▲) were incubated with increasing concentrations of GST-tagged RdRp. Complexes were detected using a rabbit anti-GST serum. (B) Wells precoated with 1.0 μg of VPgPro were incubated with 0.25, 0.5 and 1.0 μg of GST-tagged RdRp (filed column) or GST (empty column). Complexes were detected using anti-GST serum. Values are averages of three replicates from a typical experiment.

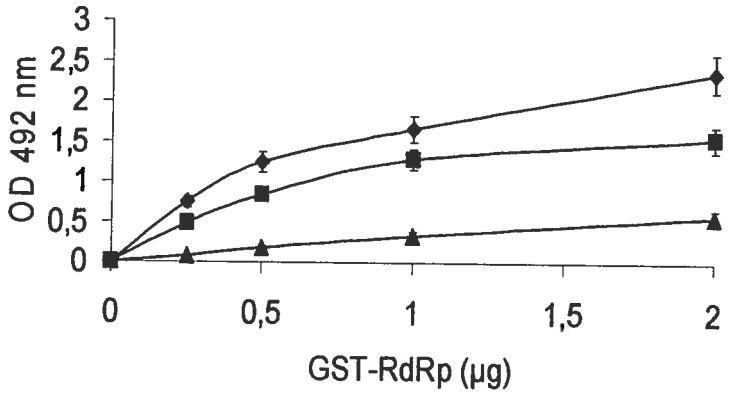
OD 492 nm
3
2,5
2
1,5
1
0,5
0
0
0,5
1
1,5
2
GST-RdRp (µg)

Fig. 5a

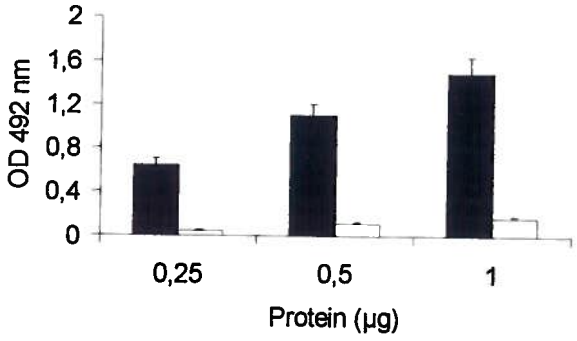
OD 492 nm
2
1,6
1,2
0,8
0,4
0
0,25
0,5
1
Protein (µg)

Fig. 5b

Fig.6. VPg and VPgPro interaction with T7-tagged PABP as demonstrated by ELISA-based binding assay. Wells precoated with 1.0 μg of VPgPro (■), VPg(●), the maltose binding protein (MBP)(♦) or GST(▲)and incubated with an increasing volume of an *E. coli* lysate expressing T7-tagged PABP. Complex formation was detected using an anti-T7-tag antibody. Values are averages of three replicates from a typical experiment.

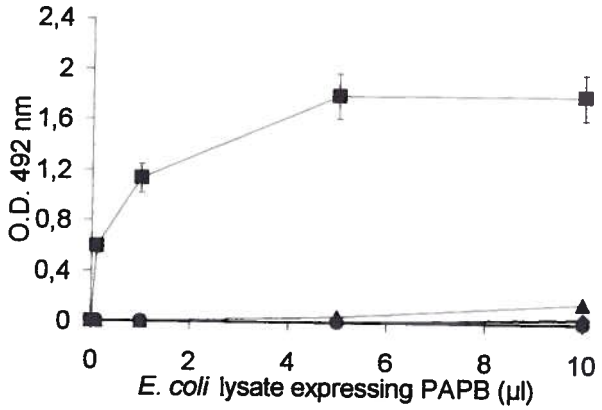
2,4
2
1,6
1,2
0,8
0,4
0
O.D. 492 nm
0
2
4
6
8
10
E. coli lysate expressing PAPB (µl)

Fig. 6

Fig. 7. Competitive interaction between PABP and RdRp for VPgPro binding. (A) Wells were coated with 1.0 μg of VPgPro and increasing volumes of an *E. coli* lysate expressing T7-tagged PABP were added in the absence (■) or presence (▲) of 1 μg RdRp. The capture of PABP was detected using an anti-T7 monoclonal antibody. (B) Wells were coated with 1.0 μg of VPgPro and increasing volumes GST-tagged RdRp (0.1 μg/μl) were added in the absence (■) or presence (▲) of a fixed volume (2 μl) of an *E. coli* lysate expressing T7-tagged PABP. Well were also incubated with increasing concentrations of GST instead of GST-RdRp (●). The capture of GST-tagged RdRp or GST was detected using an anti-GST rabbit serum. Values are averages of three replicates from typical experiments.

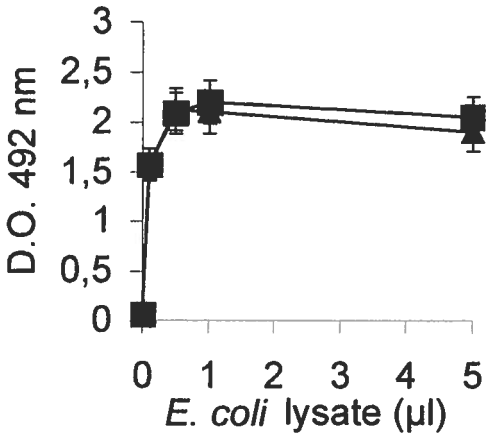
D.O. 492 nm
3
2,5
2
1,5
1
0,5
0
0 1 2 3 4 5
E. coli lysate (µl)

Fig. 7a

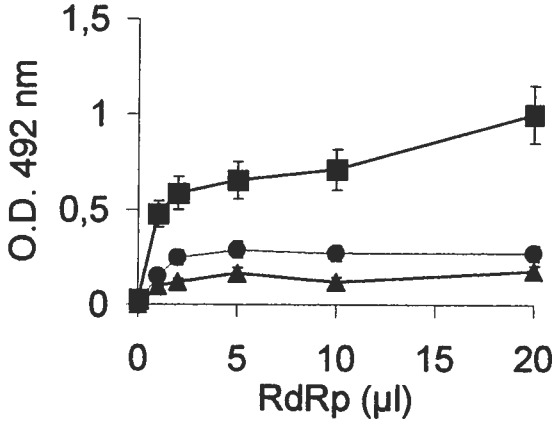
O.D. 492 nm
1,5
1
0,5
0
0 5 10 15 20
RdRp (µl)

Fig. 7b

Discussion

In this study, we found that infection of *B. perviridis* by TuMV modified the expression profile of the eIF4E isomers: only eIF(iso)4E was detected in mock-inoculated, healthy, leaves while both isomers were present in infected tissues. However, it is not known whether eIF4E has been produced in cells undergoing active virus replication, or in cells that have not yet been infected. A previous study demonstrated that the eIF4E and eIF(iso)4E mRNAs accumulated differentially in *A. thaliana* tissues (51). The eIF4E mRNA was expressed in flower, cauline leaf, leaf and stem tissues, but the relative abundance in root tissues was very low. On the other hand, the eIF(iso)4E mRNA, although detected in all tissues analyzed, was particularly abundant in floral organs and in young developing tissues. Our Northern experiment is in agreement with this study. Both mRNAs were detected in *B. perviridis* leaves and the eIF4E mRNA level was much higher than the eIF(iso)4E mRNA level. Interestingly, the mRNA intensity did not reflect the expression level of the corresponding proteins. This indicates that eIF4E expression was under post-transcriptional control, which has already been noticed in maize (11). Post-transcriptional control is adopted when a cell has to respond quickly to a particular stressful situation, without bringing into play nuclear pathways for mRNA synthesis. Considering the central role played by the eIF4E isomers in gene regulation, it is interesting to see such a response during a viral infection where important changes in gene expression are taking place quite rapidly. For example, during pea seed-borne mosaic potyvirus infection, there is inhibition of host gene

expression in cells undergoing active virus replication, and apparent normal gene expression resumes once replication is over (63). It should be noted that not all host cell gene expression was inhibited; on the contrary HSP70 and polyubiquitin mRNA level were increased in infected cells (3). Furthermore, our data support the notion that the eIF4E isoforms have distinct cellular functions (7). Recently, Gallie & Browning (17) found that eIF(iso)4F promoted translation preferentially from unstructured mRNAs, whereas eIF4F supported translation of mRNAs that contain a structured 5'-leader. The authors proposed that this ability would enable eIF4F to promote translation under cellular conditions (e.g. heat shock) in which cap-dependent translation is inhibited. This statement is appealing in the light that viral infection often leads to cap-dependent inhibition of host mRNA translation (14). It is then possible that induction of eIF4E production is linked to the cellular response needed to adjust to the pressure on gene expression caused by infection of TuMV.

The potyvirus RNA replication complex is associated with ER membranes (37, 56) and targeting of the replication complex to this site would involve interactions between the 6 kDa protein and the ER (56). Detection of the 6 kDa protein would thus be a marker of virus replication, and any 6 kDa-containing proteins, notably 6KVPgPro, would participate in replication (50). The membrane fractionation experiment showed that VPgPro fused to the 6 kDa protein was detected in infected tissue and co-localized with eIF(iso)4E/eIF4E. This information agrees with the finding that the potyvirus replication complex is associated with ER membranes, and more precisely with RER membranes. The membrane co-localization and the co-purification of 6KVPgPro/VPgPro with eIF(iso)4E/eIF4E and PABP also provide evidence that VPg-containing proteins

interact with the translation initiation factors *in planta*, possibly within the replication complex. This idea is in agreement with the finding that another translation initiation factor, eIF3, was present in highly purified replication complexes of both brome mosaic virus (48) and tobacco mosaic virus (44). In the case of the latter virus, there is *in vitro* evidence that the factor interacts with the methyltransferase-like domain of the 126 and 183 kDa replicase proteins (60).

Finally, this study showed that multiple and dynamic protein-protein interactions can take place and that VPgPro would serve as a focal point for complex assembly. Interestingly, the viral and cellular proteins could interact with each others in a multitude of ways, potentially increasing the overall stability of the complex. For example, VPgPro can bind to PABP in two different ways. The interaction can be direct, through the Pro domain, and indirect through the intermediary of the eIF(iso)4E/eIF(iso)4G dimer, since eIF(iso)4G have been shown to bind PABP (31). However, this latter possibility remains to be shown experimentally. Complex composition could also change depending on the concentration of each protein at a given time during viral replication. This would be exemplified by the inhibition of complex formation between RdRp and VPgPro by PABP. This dynamic equilibrium could be a key step in determining whether viral translation or replication is taking place (19). Moreover, the interaction among the potyvirus and host proteins can possibly promote RNA circularization, which has been shown to be necessary for efficient translation of cellular mRNAs (53) and also to be taking place for animal viral RNAs (40, 62). The TuMV RNA is linked at its 5' end to VPg and is polyadenylated. Additionally, VPgPro has been shown to be covalently linked to the viral RNA (41) and to possess RNA binding properties (10). Consequently, formation of the

VPgPro-PABP complex, and a likely VPgPro-eIF4E-eIF4G-PABP complex could bring both ends of the viral RNA in close proximity.

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Chapitre 4 :

Étude entre la VPgPro du Népovirus et le facteur eucaryotique iso 4E d'*Arabidopsis thaliana.*

Introduction à l'article III

Les népovirus sont similaires aux potyvirus et au picornavirus (virus de mammifères) lorsqu'on compare leur structure génomique et leur stratégie d'expression. Les népovirus font donc parties de la famille des virus "picorna-like" qui possèdent tous, en autre, une protéine VPg à l'extrémité 5' de leur ARN viral. Une des différences majeures qui réside entre les népovirus et les potyvirus est la taille de la protéine VPg. Celle des potyvirus varie entre 22 et 24 kDa tandis que celle des népovirus n'est constituée que de 27 acides aminés.
Nous avons montré lors de travaux antérieurs que la VPg du potyvirus interagit avec différentes formes et espèces du facteur eucaryotique d'initiation de la traduction 4E. Cette interaction est également correlée avec l'infectivité du virus. Dans le but de démontrer l'universalité de cette interaction, nous avons vérifié *in vitro* l'interaction entre une VPg d'un népovirus (le Tomato ringspot virus) et l'eIF(iso)4E d'*A thaliana*.

C'est à la suite de discussion avec le docteur Laliberté que j'ai eu l'idée de vérifier si une VPg différente des potyvirus pouvait interagir avec l'eIF4E. J'ai donc entrepris une collaboration avec le docteur Sansfaçon qui m'a refilé la protéine VPgPro du *Tomato ringspotvirus*. J'ai donc effectué les manipulations nécessaires à la réalisation des figures 1 (interaction de la VPgPro du Népovirus avec l'eIF4E en ELISA) et de la figure 3 de l'article (inhibition de l'interaction par ajout de m^7GTP).

Article III

Interaction *in vitro* between the proteinase of *Tomato ringspotvirus* (genus *Nepovirus*) and the eukaryotic translation initiation factor iso4E from *Arabidopsis thaliana*

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Résumé de l'article III

Le facteur eucaryotique d'initiation (iso) 4E lie la structure de la coiffe des ARNm et conduit à la formation du complexe d'inititation de la traduction. Ce facteur interagit également avec la VPg du potyvirus et cette liaison est correlée avec l'infectivité du virus. Dans cette étude, nous avons montré une interaction entre l'eIF(iso)4E et la protéinase (Pro) d'un népovirus (Tomato ringspot virus; ToRSV) *in vitro*. La VPg du ToRSV n'interagit pas avec l'eIF(iso)4E. Cependant, le précurseur VPgPro augmente l'affinité de la liaison du domaine pro pour le facteur d'initiation de la traduction. Un domaine de 93 acides aminés dans la Pro est un site déterminant pour cette interaction. La formation du complexe est inhibé par l'addition de m^7GTP (un analogue de la coiffe), suggérant que la protéine Pro peut compétionner avec les ARNm cellulaires pour la liaison à l'eIF(iso)4E. L'implication possible de cette interaction pour la traduction et/ou la réplication du génome viral est discutée.

Abstract

Eukaryotic initiation factor eIF(iso)4E binds to the cap structure of mRNAs leading to assembly of the translation complex. This factor also interacts with the potyvirus VPg and this interaction has been correlated with virus infectivity. In this study, we show an interaction between eIF(iso)4E and the proteinase (Pro) of a nepovirus *(Tomato ringspot virus*; ToRSV) *in vitro*. The ToRSV VPg did not interact with eIF(iso)4E although its presence on the VPg-Pro precursor increased the binding affinity of Pro for the initiation factor. A major determinant of the interaction was mapped to the first 93 residues of Pro. Formation of the complex was inhibited by addition of m^7GTP (a cap analogue), suggesting that Pro-containing molecules compete with cellular mRNAs for eIF(iso)4E binding. The possible implications of this interaction for translation and/or replication of the virus genome are discussed.

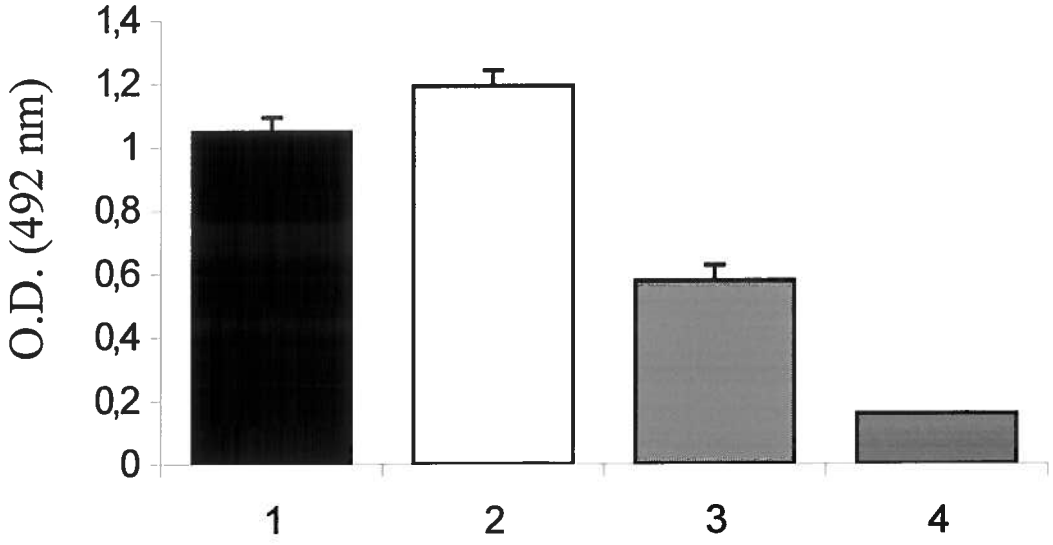
A key step in the replication cycle of viruses is translation of the viral genome. Optimal translation is achieved by recruiting, and in some cases selectively altering, host translation factors (Thompson & Sarnow, 2000; Gale *et al.*, 2000). This in turn often results in the inhibition of host mRNA translation. Most viral RNAs differ in structure from cellular mRNAs. This provides opportunities for viruses to redirect the host translation machinery in favour of viral protein synthesis. For example, the 5' end of the genomic RNA from picorna-like viruses (including animal and insect picornaviruses, and plant poty-, como- and nepoviruses) does not have a cap structure (m^7GpppN, where N is any nucleotide) as found in cellular mRNAs. Instead, the RNA is covalently linked to a virus-encoded protein termed VPg. Translation proceeds in a cap-independant manner through the use of an internal ribosome-entry site (IRES) (Martinez-Salas et al., 2001 ; Gallie, 2001)

Viral proteins are likely to participate in the regulation of viral genome translation (Thompson & Sarnow, 2000; Gale *et al.*, 2000). A case in point is the VPg of *Turnip mosaic virus* (TuMV; genus *Potyvirus*), which interacts with the eukaryotic initiation factor eIF(iso)4E of *Arabidopsis thaliana* (Wittmann *et al.*, 1997; Léonard *et al.*, 2000). eIF(iso)4E is a plant isomer of eIF4E (Rodriguez *et al.*, 1998) which binds the cap structure of cellular mRNAs and plays an important role in the regulation of translation initiation (Sonenberg & Gingras, 1998). The cap analogue m^7 GTP, but not GTP, inhibits VPg-eIF(iso)4E complex formation, suggesting that VPg and cellular mRNAs compete for eIF(iso)4E binding. Plants inoculated with TuMV infectious cDNA containing a mutation in the eIF(iso)4E binding domain of VPg remain symptomless and do not show

accumulation of virus coat protein, indicating that there is a correlation between VPg-eIF4E binding *in vitro* and virus viability *in planta* (Léonard *et al.*, 2000). The NIa protein (also called VPg-Pro) of *Tobacco etch virus* also interacts with eIF4E from tomato and tobacco, and the interaction was shown to enhance genome amplification (Schaad *et al.*, 2000). Although the precise biological function of the VPg (or VPg-Pro)-eIF4E interaction remains to be elucidated, it may either play a role in recruiting host factors for the translation and/or replication of the viral RNA or be involved in host translational shut-down, possibly through disruption of the interaction between cellular mRNAs and cap-binding translation initiation factors.

Nepoviruses are closely related to potyviruses in term of their genomic structure and genome expression strategy but differ from potyviruses in at least two significant aspects. First, the nepovirus genome is bipartite, with RNA1 encoding most of the proteins involved in virus replication (including VPg and Pro). Second, nepovirus VPgs are much smaller than potyvirus VPgs, which range from 22 to 24 kDa (Riechmann *et al.*, 1992). For example, the VPg of *Tomato ringspot virus* (ToRSV) is composed of 27 residues (Wang *et al.*, 1999). There is no amino acid sequence homology between potyvirus and nepovirus VPgs (Mayo & Fritsch, 1994).

Fig. 1. Demonstration of VPg-Pro interaction with eIF(iso)4E using an ELISA-based binding assay. Wells were coated with 1 ug of VPg-Pro from TuMV (lane 1), VPg-Pro (lane 2) or Pro (lane 3) from ToRSV and then incubated with 2 ug of eIF(iso)4E of *A. thaliana*. In lane 4, wells were coated with Blotto only and incubated with 2 ug of eIF(iso)4E. Retention of eIF(iso)4E was detected using anti-T7 tag antibodies. Values are averages of three replicates from a typical experiment. Error bars represent the standard deviation.



It was therefore of interest to determine if the nepovirus VPg, or larger precursor forms, interacts with eIF(iso)4E. One possible precursor of VPg is VPg-Pro. VPg-Pro (the functional equivalent of the potyvirus NIa) was found to accumulate during *in vitro* translation of larger precursors as a result of inefficient processing of the VPg-Pro cleavage site (Wang *et al.*, 1999; Wang & Sanfaçon, 2000), although accumulation of VPg-Pro in infected plants has not yet been demonstrated.

The interaction between VPg-Pro as well as Pro of ToRSV and eIF(iso)4E of A. *thaliana* was tested using an ELISA-based binding assay (Wittmann *et al.*, 1997; Léonard *et al.*, 2000). ToRSV has the ability to replicate in A. *thaliana* (R. I. Hamilton, personal communication). eIF(iso)4E was produced in E. *coli* and purified by m^7GTP-Sepharose chromatography (Wittmann *et al.*, 1997). The factor was fused to the N-terminal peptide of the T7 gene-10 protein (T7 tag), which allows its recognition by an anti-T7 tag monoclonal antibody (Novagen).

ToRSV proteins were produced in *E. coli*, purified (Chisholm *et al.*, 2001) and adsorbed to wells of ELISA plates (1.0 ug per well) by overnight incubation at 4 °C. Purified eIF(iso)4E (2 ug) diluted in 1% Blotto in PBS containing 0,2 % Tween was incubated for 1 h at 4 °C in the coated wells. Detection of bound initiation factor was achieved in an ELISA with the anti-T7 tag antibody and peroxidase-labelled goat anti-mouse immunoglobulin G (KPL). VPg-Pro from TuMV was purified as described previously (Wittmann *et al.*, 1997) and used as positive control. As shown previously (Wittmann *et al.*, 1997), the VPg-Pro of TuMV interacted with eIF(iso)4E (Fig. 1). VPg-Pro of ToRSV also interacted with eIF(iso)4E. The interaction was specific for the viral protein since the factor was not retained when wells were coated with an E. *coli* lysate not containing any VPg-Pro (Wittmann *et al.*, 1997). An interaction was also detected between the mature

Pro and eIF(iso)4E, although retention of the factor was less than for VPg-Pro (0.55 OD units for Pro vs 1.2 OD units for VPg-Pro). This suggests that the interacting domain resides within Pro, but that the presence of VPg increases the affinity of the viral protein for eIF(iso)4E. In contrast, the interacting domain of the TuMV VPg-Pro resides within VPg, and the TuMV VPg-Pro and VPg have the same binding affinity for eIF(iso)4E (Wittmann *et al.*, 1997).

The domain(s) of the ToRSV VPg-Pro involved in the interaction with eIF(iso)4E were mapped by Far-Western experiments. Mutants (Fig. 2a) were generated by amplifying cDNA fragments from plasmid pET21d-VPg-Pro (Chisholm *et al.*, 2001) using specific oligonucleotides. The PCR products were inserted into plasmids pET21d (Novagen) or pTrxFus (Invitrogen). Exceptions to this are plasmid pET15bVPg-Pro- de*lBam*HI, which was constructed by deleting a small *Ba*mHI fragment from plasmid pET15bVPg-Pro-N-Pol (Wang *et al.*, 1999), and plasmid pET21dVPg-Pro-de*lCla*I, which was constructed by inserting a *Nco*I-*Cla*I fragment from plasmid pET21d-VPg-Pro into plasmid pET21d. Protease mutants were expressed in E. *coli* as described by the supplier (Novagen). E. *coli* proteins were separated by SDS-PAGE and electroblotted to PVDF membranes (Bio-Rad). The membranes were probed with approximately 60 ug of the purified eIF(iso)4E essentially as described (Kao *et al.*, 1992). Binding of eIF(iso)4E was revealed by immunodetection using the anti-T7 tag monoclonal antibody and a goat anti-mouse secondary antibody linked to alkaline phosphatase (Sigma). The different proteinase forms were seen as a predominant band in E. *coli* extracts, as shown by Coomassie blue staining (Fig. 2b). The identity of these proteins was confirmed by immunodetection with polyclonal antibodies raised against the recombinant VPg-Pro (data not shown). Interaction of VPg-Pro with eIF(iso)4E was observed (Fig. 2b, c, lane

1) and no additional interactions were detected with E. *coli* proteins from the extract, indicating that the interaction was specific. An interaction was also detected with Pro (lane 7) but not with a protein containing the VPg domain fused to thioredoxin (lane 12).

Point mutations in the putative catalytic dyad ($H^{1283}D$) and in the putative substrate-binding pocket ($H^{1451}L$) that abolished the proteolytic activity of the proteinase (Hans & Sanfaçon, 1995) did not affect the binding affinity for the factor (Fig. 2c, lanes 2 and 3), indicating that the determinants for the interaction were distinct from those for proteolytic activity. All proteins containing the first 93 amino acids of the Pro domain were shown to interact with eIF(iso)4E (lanes 1-7 and 11). Truncated proteinases containing a deletion of the N-terminal 93 residues could interact with the factor (lanes 8 and 10), but a truncated protein harbouring the last 81 amino acids of Pro did not (lane 9). Taken together these results indicate that a major determinant of the interaction resides within the first 93 amino acids of the Pro domain, although an additional domain within the C-terminal two-thirds of Pro may contribute to the binding.

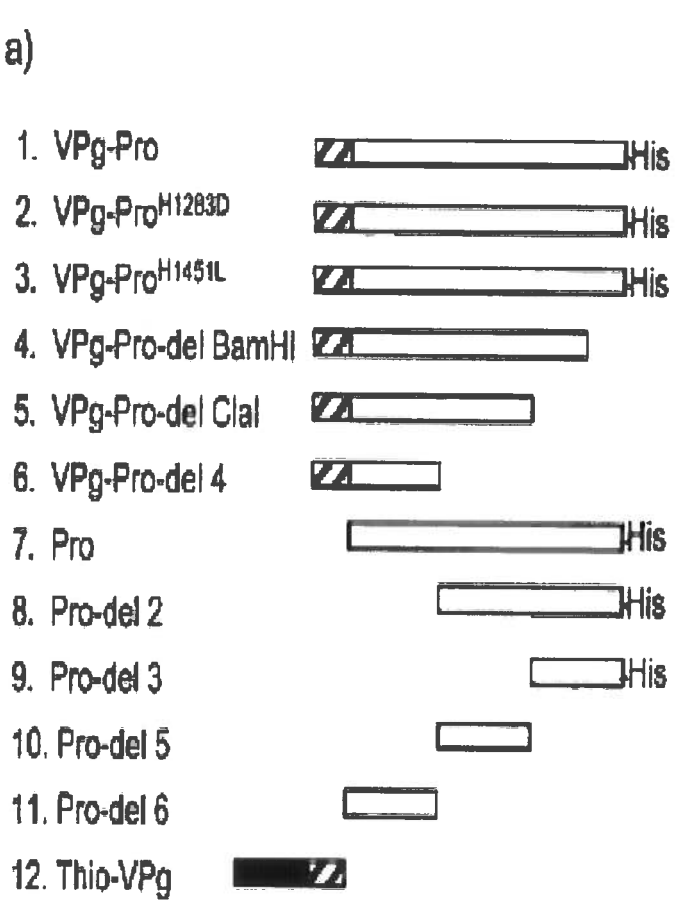
To test whether ToRSV proteins and the cap structure of cellular mRNAs compete for eIF(iso)4E binding, the influence of the cap analogue m^7GTP on the formation of the VPg-Pro-eIF(iso)4E and Pro-eIF(iso)4E complexes was tested. ELISA plate wells were coated with 1.0 ug of viral proteins and incubated with 2.0 ug of eIF(iso)4E and either 10 or 20 mM m^7GTP. The cap analogue inhibited the formation of the complexes by approximately 30% at a concentration of 20 uM (Fig. 3). No inhibition was observed with 20 uM of GTP, indicating that the inhibition was cap-related. As previously shown, 20 uM of m^7GTP inhibited the interaction of eIF(iso)4E with the VPg-Pro of TuMV by 60% (Léonard *et al.*, 2000).

One possible role for the interaction between the nepovirus Pro and eIF(iso)4E is that it could promote assembly of the translation complex on the viral RNA. In the circular translation model, efficient translation of cellular mRNAs requires interactions between initiation factors bound to the 5' cap structure and the poly(A)-binding protein (PABP) bound to the 3' poly(A) tail (Sachs, 2000).

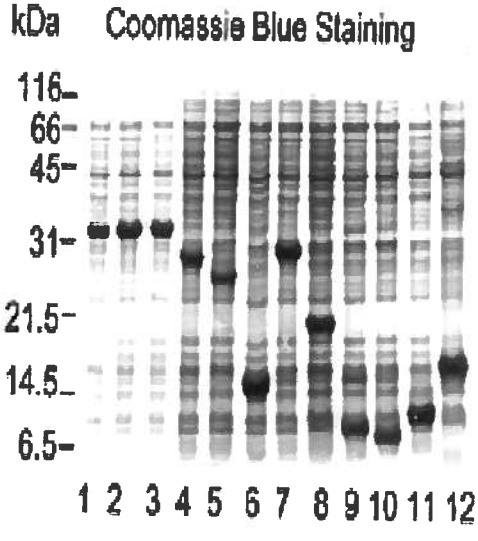
Fig. 2 : Definition of the domains in ToRSV VPg-Pro involved in the interaction with eIF(iso)4E using a Far-Western assay.

(a) Schematic representation of the ToRSV protease mutants. The white boxes represent regions of the Pro domain, the hatched boxes represent the VPg domain and the black box represents the thioredoxin protein (from vector pTrxFus; Invitrogen) fused in-frame with the VPg coding region. A histidine tail (His) at the C termini of the proteins is shown when present. The amino acids of the ToRSV RNA1-encoded polyprotein (P1) present in each recombinant protein are indicated [numbering from the first amino acid of the polyprotein according to Rott *et al.* (1995)]. (b and c) Analysis of the interaction of the ToRSV protease mutants with eIF(iso)4E using the Far-Western assay. After induction of the expression of viral proteins, *E. coli* cells were resuspended in 50 mM NaH 2 PO 4 ,pH 8.0, 0.3 M NaCl, 0.1% Triton X-100. After sonication the extracts were separated by SDS–PAGE and either stained by Coomassie blue staining (b) or electroblotted to PVDF membranes (Bio-Rad). Interaction with eIF(iso)4E was tested using the Far-Western method as described in the text (c). The migration of molecular mass standards is indicated on the left of the gels. The following proteins were tested in each lane. Lane1, VPg-Pro; lane 2, VPg-Pro H1283D ; lane 3, VPg-Pro H1451L ; lane 4, VPg-Pro-del *Bam*HI; lane 5, VPg-Pro-del *Cla*I ; lane 6, VPg-Pro-del 4; lane 7, Pro; lane 8, Pro-del 2; lane 9, Pro-del 3; lane 10, Pro-del 5; lane 11, Pro-del 6; lane 12, Thio-VPg.

a)


a. a. (ToRSV P1)
eIF(iso)4E binding
1. VPg-Pro
His
1213-1486
+
2. VPg-Pro^{H1283D}
His
1213-1486
+
3. VPg-Pro^{H1451L}
His
1213-1486
+
4. VPg-Pro-del BamHI
1213-1455
+
5. VPg-Pro-del ClaI
1213-1405
+
6. VPg-Pro-del 4
1213-1332
++
7. Pro
His
1240-1486
+
8. Pro-del 2
His
1299-1486
+
9. Pro-del 3
His
1406-1486
-
10. Pro-del 5
1332-1404
+/-
11. Pro-del 6
1240-1332
+
12. Thio-VPg
1213-1239
-

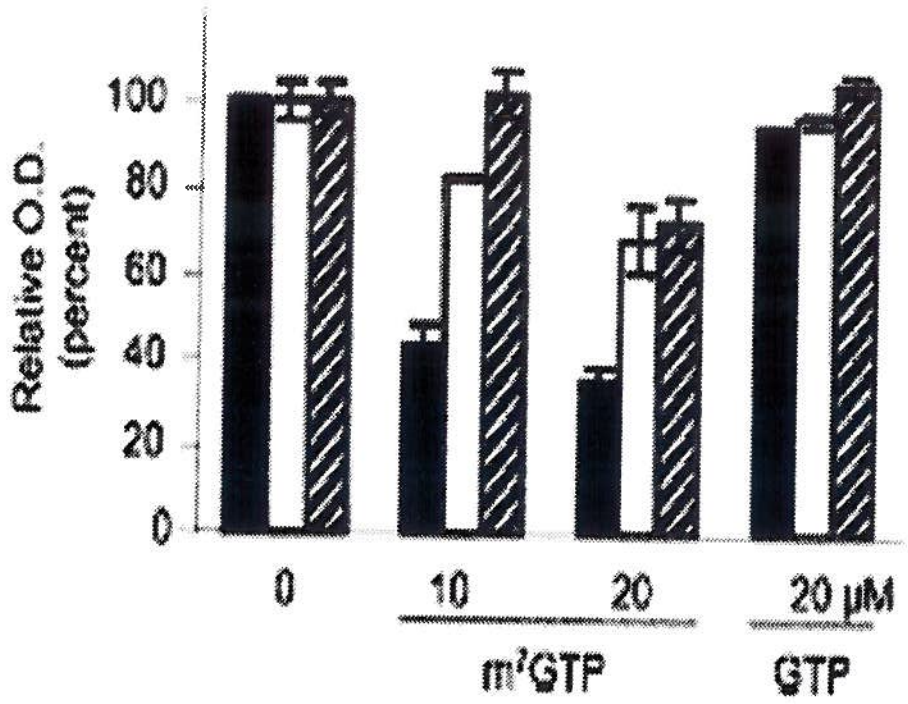
b)


kDa
Coomassie Blue Staining
116-
66-
45-
31-
21.5-
14.5-
6.5-
1 2 3 4 5 6 7 8 9 10 11 12

c)


Far-Western with eIF(iso)4E
1 2 3 4 5 6 7 8 9 10 11 12

Fig. 3 : Inhibition by m^7 GTP of VPg-Pro-eIF(iso)4E complex formation as determined by ELISA-based binding assay. Wells were coated with 1 ug of VPg-Pro from TuMV (black column), VPg-Pro (white column) and Pro (hatched column) from ToRSV and were incubated with 2 ug of eIF(iso)4E of A. *thaliana*, with no cap analogue, 10 and 20 uM m^7GTP or 20 uM GTP. Retention of eIF(iso)4E was detected using anti-T7 tag antibodies. Values are averages of three replicates from a typical experiment and error bars represent the standard deviation. Values in the absence of m^7GTP were arbitrarily adjusted to 100% for each protein.



Similarly, translation of several viruses has been shown to be mediated by protein bridges between the 5' and 3' termini of the RNA which involve viral proteins and cellular translation initiation factors. For instance, the rotavirus NSP3 protein promotes genome circularization and efficient translation of the viral RNA by simultaneously binding to the 3' untranslated region of the non-polyadenylated viral RNA and to eIF4G, which in turn binds to eIF4E (Piron *et al.*, 1998, 1999; Vende *et al.*, 2000). In the case of picornaviruses, the IRES interacts directly with eIF4E, eIF4G and eIF3 (Ali *et al.*, 2001; Borman *et al.*, 2001; Lopez de Quinto *et al.*, 2001). Given that eIF4G interacts with PABP and picornavirus RNA is polyadenylated, circularization was expected. Indeed, eIF4G-PABP interaction was required for poly(A) tail-mediated stimulation of IRES translation (Michel *et al.*, 2001).

The formation of protein bridges leading to genome circularization may also be an important feature of the translation of plant virus genomes. Recently, the coat protein of *Alfalfa mosaic virus* was shown to stimulate translation of viral RNAs, presumably by acting as a functional analogue of PABP (Neeleman *et al.*, 2001). The nepovirus Pro could similarly participate in genome circularization by acting as a bridging element between host initiation factors and the viral RNAs. Pro is a member of the 3C-like proteinase family and is likely to have RNA-binding properties (Blair *et al.*, 1998). It could thus interact with the 5' end of the viral genome, as shown for the picornavirus 3C (Gamarnik & Andino, 2000; Kusov *et al.*, 1997; Kusov & Gauss-Muller, 1997; Harris *et al.*, 1994; Walker *et al.*, 1995) and the potyvirus Nia proteinases (Daros & Carrington, 1997). Alternatively, Pro as a precursor protein with VPg may be covalently linked to

viral RNAs. In addition to possibly improving translation of the viral RNAs, genome circularization could provide several advantages for virus replication, including the coordination of translation and RNA synthesis, the localization of the viral polymerase at the appropriate start site and a control mechanism for the integrity of the viral genome (Herold & Andino, 2001). Finally, the interaction of the ToRSV Pro with eIF(iso)4E may also have other biological functions, one of which may be a direct or indirect role in a possible shut-down of host translation.

The presence of the VPg domain on a precursor of the ToRSV Pro regulates the different activity of the proteinase as it enhances its ability to interact with eIF(iso)4E (this study) and decreases its ability to release the movement protein and coat protein from RNA2-derived substrates (Chisholm *et al.*, 2001). The results presented here provide additional support for our previous suggestion that the inefficient cleavage at the VPg-Pro site may play an important role in the biology of ToRSV (Chisholm *et al.*, 2001).

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Interaction of the viral protein genome linked of turnip mosaic potyvirus with the translational eukaryotic initiation factor (iso) 4E of *Arabidopsis thaliana* using the yeast two-hybrid system. *Virology* 234, 84-92. Received 11 February 2002; Accepted 5 April 2002

Discussion

Universalité de l'interaction VPg-eIF(iso)4E

Nous avons poursuivi les travaux de l'interaction entre la protéine VPg du TuMV et le facteur initiant la traduction cellulaire eIF4E. Il existe chez les plantes, deux isomères de l'eIF4E: soit l'eIF4E et l'eIF(iso)4E. Ces deux protéines montrent un haut pourcentage d'identité soit de 70 pourcent. Des études comparatives sur d'autres espèces de plantes comme le blé et le riz montrent également un pourcentage similaire (Rodriguez et al., 1998). Par conséquent, nous avons vérifié si la VPgPro du TuMV pouvait intéragir avec différentes formes d'eIF4E. Nous avons montré, par la technique ELISA, que la VPg peut non seulement lier l'eIF(iso)4E d' *A.thaliana*, mais également l'eIF4E d' *A.thaliana* et l'eIF(iso)4E de blé. Ces résultats indiquent que l'interaction VPg-eIF4E peut s'effectuer chez différentes espèces de plantes avec les deux isomères d'eIF4E. De plus des expériences préliminaires ont indiqué que la VPg du TVMV (tobacco vein mottling potyvirus) et du PPV (plum pox potyvirus) lient l'eIF(iso)4E d' *A. thaliana* (M.G. Fortin, communication personnelle). Un autre groupe a montré que la VPgPro du TEV (tobacco etch virus) liait l'eIF4E provenant de la tomate et du tabac (Schaad et al., 2000). Ce qui indique que l'interaction VPg-eIF4E semble un mécanisme retrouvé lors de l'infection de d'autres potyvirus. Ces résultats nous ont incité à faire une étude comparative avec d'autres VPg de virus n'appartenant pas au groupe des potyvirus. Aucune identité d'acides aminés, de nucléotides ou de structure conformationnelle de la protéine n'a été détectée que ce soit pour les VPg de virus de mammifère (par exemple le poliovirus) ou de virus de plante (comme les népovirus).

Dans le but de démontrer l'universalité de l'interaction VPg–eIF4E, nous avons vérifié si la VPg du *tomato ringspot virus* (genre népovirus) pouvait lier le

facteur eucaryotique initiant la traduction cellulaire. Ceci malgré l'absence d'identité ou de motif structural avec la VPg du TuMV. La VPg du *tomato ringspot virus* ne possède qu'une masse moléculaire de 3 kDa. Par la technique ELISA, nous avons montré que la VPgPro du népovirus interagit avec l'eIF(iso)4E de blé ou d'*A.thaliana* de façon similaire à la VPgPro du potyvirus (Léonard et al., 2002). Nous avons aussi observé que pour ce virus, la protéase Pro peut lier l'eIF4E, mais non la VPg seule. Cependant la protéine VPgPro interagit plus fortement avec l'eIF4E que la Pro seule. Ces résultats sont différents de ceux auxquels nous nous attendions car pour le potyvirus la protéine VPg et non la Pro était responsable de l'interaction avec l'eIF4E. Ces résultats ont été confirmés par nos collaborateurs qui ont caractérisé le domaine d'interaction de la Pro pour l'eIF4E en utilisant la technique du Far Western. Ils ont identifié les 93 premiers acides aminés de la protéine Pro comme étant responsables de l'interaction avec l'eIF4E. Ils semblent donc que le népovirus opte pour une stratégie différente du potyvirus lorsqu'il interagit avec la protéine initiant la traduction cellulaire 4E.

Dans le même ordre d'idée, nous avons entrepris une collaboration avec l'équipe de L. Robert qui effectue des recherches sur les calicivirus. Notre équipe a montrée en ELISA que la VPg de calicivirus interagit avec l'eIF(iso)4E (J-F Laliberté, communication personnelle). Les membres du laboratoire de L. Robert ont également démontré (par co-purification) que cette interaction s'effectue dans les cellules infectées (L Robert, communication personnelle). Conséquemment, l'interaction VPg-eIF(iso)4E s'effectue non seulement chez les différentes espèces de virus de plante, mais se produit également chez les virus de mammifères. Ces résultats ne sont pas vraiment surprenant puisque dans la

littérature, on retrouve plusieurs exemples de virus qui vont cibler directement ou indirectement l'eIF4E pour générer une infection virale (voir revue bibliographique section 3). Ceci est notamment le cas pour virus de mammifère, le LCMV (lymphocytic choriomeningitis virus). Par le biais de sa protéine "ring Z", ce virus interagit avec l'eIF4E pour inhiber la traduction cellulaire et générer l'infection virale.

Caractérisation de l'interaction VPg-eIF(iso)4E

Nous avons également caractérisé le domaine d'interaction de la VPg pour l'eIF(iso)4E. Une gamme de gènes tronqués de la VPg ont été produites et insérées dans les plasmides du système double-hybride. Lorsque nous avons regardé l'interaction de ces VPg pour l'eIF(iso)4E, une VPg tronquée dans sa partie centrale interagissait très peu avec l'eIF(iso)4E. Ces travaux nous ont permis de cibler, une région centrale de 35 aminés responsables de la liaison avec l'eIF4E. Nous avons vérifié si cette perte d'interaction pour l'eIF(iso)4E n'était pas tout simplement attribuable à un erreur de clonage, à un problème de conformation ou d'expression de la protéine dans la levure. Cette VPgPro tronquée a donc été sous-clonée dans un vecteur d'expression et produite dans *E.coli.* Nous avons par la suite purifié cette protéine sur une colonne Ni^{2+}-NTA agarose selon les mêmes conditions que la VPgPro. À l'aide d'un test ELISA, nous avons montré que le domaine de 35 acides aminés était nécessaire pour que la VPgPro interagisse avec l'eIF(iso)4E. Nous avons également vérifié si cette région était suffisante pour l'interaction avec l'eIF(iso)4E. Un peptide d'une vingtaine d'acides aminés dans cette région fut synthétisé et testé contre l'eIF(iso)4E avec l'aide d'un test de type ELISA. Cependant, aucune interaction

entre ces deux protéines n'a été observée (résultat non présenté). Un problème de conformation du peptide, la faible masse moléculaire du peptide ou encore un problème technique sont des hypothèses plausibles pour expliquer le résultat négatif observé. Une étude comparative avec d'autres potyvirus nous a montré que cette région centrale dans la VPg est très conservé chez les VPg de potyvirus.

Des études de mutagenèses dirigées dans le domaine de 35 acides aminés (nécessaire à la liaison de l'eIF4E) de la VPgPro du TuMV ont été entreprises. Nous avons ciblé, pour effectuer la mutagenèse, des acides aminés convervés chez huit VPg de potyvirus. Trois acides aminés ont été sélectionnés pour effectuer la mutagenèse. La phénylalanine en position 59, la tyrosine en position 63 et l'acide aspartique en position 77 ont été mutés et insérés dans les plasmides du système double-hybride. Seule une mutation de l'acide aminé en position 77 affectait de façon significative la liaison de la VPg pour l'eIF(iso)4E. Le remplacement de cet acide aminé par l'alanine, l'acide glutamique ou encore par l'asparagine abolissait complètement l'interaction avec l'eIF(iso)4E. Ces expériences indiquent que l'acide aspartique en position 77 de la VPg est nécessaire pour l'interaction avec l'eIF(iso)4E. Ce résultat n'exclue pas la possibilié que d'autres résidus ou d'autres domaines soient impliqués pour la liaison avec l'eIF4E. De plus, il serait intéressant, de connaître la structure tridimensionnelle de la VPg. Nous pourrions ainsi regarder où se situe l'acide aspartique 77 dans la VPg et de quelle façon cet acide aminé pourrait interagir avec la protéine eIF(iso)4E. Nous pourrions également voir si d'autres acides aminés de la VPg sont susceptibles d'interagir avec l'eIF(iso)4E. Ces informations seraient importantes pour notre compréhension de l'interaction

entre la VPg et l'eIF4E. Il pourrait également être fort utile lorsque viendra le moment d'expliquer l'action de la VPg sur l'eIF4E.

Compétition entre la VPgPro et le m^7GTP pour la laison au facteur eucaryotique initiant la traduction 4E.

L'eIF4E est une CBP ("cap-binding protein"), c'est-à-dire qu'elle reconnaît la coiffe des ARNm cellulaires pour ensuite enclencher le processus de traduction cellulaire. Nous voulions voir si la VPg, située à l'extrémité 5' de l'ARN viral, compétitionnait avec la coiffe située à l'extrémité 5' de tous les ARNm cellulaires pour la liaison à l'eIF4E. Pour ce faire, nous avons effectué une expérience *in vitro* qui simulait ce processus. Nous avons montré à l'aide d'un test de type ELISA que l'ajout de faible quantité (10μM) de m^7GTP (un analogue de la coiffe) diminuait grandement l'interaction entre l'eIF(iso)4E et la VPgPro (60%). Le GTP à une même concentration n'affectait pas l'interaction VPgPro-eIF(iso)4E. Ceci suggère que l'inhibition observée est bel et bien due à la structure imitant la coiffe et non à l'ajout d'un produit qui aurait pu interférer dans lors de l'expérience. Le traitement des données nous indique également que l'inhibition est non compétitive de type mixte signifiant que la VPgPro et le m^7GTP peuvent simultanément lier l'eIF(iso)4E. Cependant la liaison de l'un des deux constituants affecte la liaison de l'autre. Conséquemment, ces résultats suggèrent que lors de l'infection du TuMV, la VPgPro pourrait lier l'eIF(iso)4E et faire obstacle au recrutement des ARNm cellulaires lors de l'initiation de la traduction cellulaire.

Le même genre d'expérience a été effectuée avec la VPgPro et la Pro du népovirus. Nous avons montré que l'interaction entre la VPgPro (ou la Pro) du

népovirus et l'eIF4E de blé est sensible à l'ajout de m^7GTP mais de façon moins drastique que pour la VPgPro du potyvirus. On pourrait donc croire que l'affinité entre la VPgPro (ou la Pro) du népovirus et l'eIF4E est plus forte que la liaison de la VPgPro du potyvirus pour l'eIF4E. En effet, pour obtenir un pourcentage d'inhibition similaire à celui observé pour le potyvirus (soit d'environ 60%), les quantités d'analogues de la coiffe à ajouter sont beaucoup plus grande (résultats non présentés). Conséquemment, on peut penser que lors de l'infection du *tomato ringspot virus*, tout comme TuMV, la VPgPro ou plutôt la Pro pourrait lier l'eIF(iso)4E et faire obstacle au recrutement des ARNm cellulaires lors de l'initiation de la traduction cellulaire.

Relation entre l'interaction VPg-eIF(iso)4E et l'infectivité du virus

Les expériences sur l'interaction VPg-eIF4E que nous avions démontrées jusqu'à présent avaient été réalisées grâce à des systèmes « artificiels », c'est-à-dire *in vitro*. Nous ne savions pas, *in vivo* ou plutôt chez l'hôte du TuMV, quel était l'effet de l'association de la VPg à l'eIF-4E pour l'établissement de l'infection du TuMV. Nous avons donc acquis, par l'intermédiaire de Fernando Ponz, un plasmide infectieux pour le TuMV. Une construction d'ADN de ce genre est un outil extraordinaire pour l'étude de l'expression et de la réplication des virus à ARN(+). Avec ce plasmide nous pouvons muter n'importe quelle région du TuMV et vérifier ensuite *in vivo* l'effet de ces mutations chez l'hôte du TuMV. Par une stratégie de clonage, nous avons remplacé la VPg native du p35Tunos par une VPg mutée qui ne liait pas l'eIF4E (VPgD77N). Nous avons infecté, par la technique de bombardement, des plantes sensibles au TuMV en utilisant le plasmide natif et le plasmide muté pour la VPg. Après 20 jours

d'infection, seules les plantes infectées avec le plasmide infectieux natif montraient des symptômes d'une infection virale caractéristique du TuMV. Ces résultats suggèrent que l'interaction VPg-eIF4E est importante pour l'établissement de l'infection virale. D'autres expériences sont en accord avec cette hypothèse, notamment celles effectuées par l'équipe de J.C. Carrington. Leurs travaux ont consisté à effectuer une banque de mutants d'*A.thaliana.* Ils ont identifié 3 trois lignées de plantes résistantes au TuMV. En analysant ces lignées, ils ont identifié que le locus qui conferrait l'immunité au TuMV était celui du gène codant pour l'eIF(iso)4E (Lellis et al., 2002). Les auteurs expliquent la résistance probable de ces plants mutés à une incapacité de la VPg du TuMV à lier l'eIF(iso)4E. Comme nos travaux, ces expériences démontrent l'importance de l'interaction VPg-eIF(iso)4E pour l'infectivité du TuMV.

Nous voulons également, dans un avenir rapproché, introduire dans le plasmide infectieux du TuMV des mutants VPg ayant plus ou moins d'affinité pour l'eIF4E. Il sera intéressant d'observer l'effet de ces mutations lors de l'infection des plantes avec le plasmide infectieux. Des mutants VPg ayant moins d'affinité pour l'eIF4E pourraient avoir du mal à générer une infection virale, par exemple, un retard lors de l'apparition des symptômes. À l'opposé une VPg ayant une affinité plus forte pour l'eIF4E pourrait augmenter la virulence du virus et montrer, en autre, des phénotypes sévères de l'infection du potyvirus. Ces expériences nous confirmeraient (en plus de nous donner des informations supplémentaires sur le mode d'infection de ce virus) qu'il y a une relation entre la force de l'interaction VPg-eIF4E et la virulence du TuMV.

Stratégie utilisée par le TuMV pour générer l'infection virale

Des virus d'animaux ciblent les facteurs initiant la traduction cellulaire pour établir une infection virale. L'eIF4E est, dans plusieurs cas, directement ou indirectement affectée par ces virus, permettant ainsi la traduction préférentielle de leur ARNm (voir revue bibliographique sur le contrôle traductionnel). Par analogie avec les virus d'animaux et à la lumière de nos résultats obtenus, nous croyons que le TuMV peut modifier l'activité de l'eIF4E et empêcher la traduction des ARNm cellulaires. Des travaux effectués par nos collaborateurs de l'Université McGill ont montré que le TuMV induisait une inhibition de la traduction cellulaire dans les protoplastes (Plante et al., soumis 2002). En effet, la tranfection de protoplastes par le plasmide infectieux du TuMV montrait une inhibition de la traduction cellulaire d'environ 70 pour cent. Les protoplastes infectés par le plasmide comportant une mutation dans la VPg n'ayant plus d'affinité pour l'eIF4E ne montraient pas, quant à eux, d'inhibition de la traduction. Ces résultats suggèrent que l'inhibition de la traduction observée dans les protoplastes est dépendante de l'interaction VPg-eIF4E.

D'autres expériences réalisées dans notre laboratoire, indiquent que la VPg induit un changement du phénotype de croissance lorsqu'elle est produite dans des plantes sensibles au TuMV. En effet, des plantes transgéniques pour la VPg montraient des retards importants de croissance et des phénotypes différents par rapport aux plantes contrôles (Henriette Chatel, communication personnelle, résultat non présenté). Cette expérience solidifie notre hypothèse que la VPg modifie l'activité de l'eIF4E lors de l'infection. En liant l'eIF4E, la VPg produite dans les plantes transgéniques empêche probablement certains ARNm cellulaires coiffe-dépendant d'être traduits efficacement. Ce qui expliquerait les phénotypes

observés pour les plants transgéniques VPg. Des travaux sont présentement en cours pour répéter cette expérience, mais cette fois ci en introduisant une ligné de plants transgéniques pour la VPgD77N. En principe ces plantes transgéniques comportant une VPg mutée ne possédant pas d'affinitée pour l'eIF4E, ne devraient pas montrer d'anomalies. Ces résultats démontreraient l'hypothèse que la protéine VPg est vraiment responsable du changement de phénotype observé pour les plants transgéniques. Il se peut que les symptômes précédemment observés soient dus à une insertion du gène de la VPg dans un endroit du génome qui inactive un autre gène important pour le développement de la plante. D'autres travaux se poursuivent dans notre laboratoire pour détecter la présence de la protéine VPg dans les plants transgéniques. Les expériences qui ont été réalisées jusqu'à présent avec un anticorps polyclonal anti-VPgPro n'ont pas démontré la présence de la protéine dans les plants transgéniques pour la VPg. L'utilisation d'un anticorps monoclonal serait beaucoup plus efficace pour la détection de cette protéine.

L'action de la VPg sur l'eIF4E pourrait bien expliquer d'autres travaux réalisés sur d'autres potyvirus. L'équipe de Maule ont examiné le front d'une infection par le PSbMV (pea seed-borne mosaic potyvirus) sur des embryons de pois. Ils ont observé que certains ARNm cellulaires, mais pas tous, présentaient un taux d'expression moins élevé qu'à la normale (Aranda et al., 1996 ; Wang et al., 1995). Aucune explication n'a encore été proposée pour ce phénomène. Cependant, l'action de la VPg sur l'eIF4E empêcherait peut être certains ARNm d'être traduit efficacement. Ces derniers ARNm seraient alors plus susceptibles à la dégradation (par des exonucléase, "decapping enzymes", etc), corrélant avec

leur taux d'expression plus bas qu'à la normale. Cette hypothèse demeure toutefois à être démontrée expérimentalement.

Lors de l'infection virale, le génome du TuMV est traduit en une seule polyprotéine qui est par la suite clivée en une dizaine d'autres protéines dont la VPg. La VPg ainsi que son précurseur protéique (la VPgPro) peuvent toutes deux soit, se retrouver libre dans le cytoplasme ou bien, être liées de façon covalente à l'extrémité 5' de l'ARN viral. Nous avons donc tenté pour une meilleure compréhension du mode de fonctionnement des potyvirus de vérifier si la VPg liée à l'ARN viral peut interagir avec l'eIF4E *in vitro*. Des expériences ont débuté en ce sens, cependant après avoir purifié l'ARN viral du TuMV, nous avons été incapable de montrer que la VPg était effectivement liée à l'ARN viral (résultat non présenté). Le processus pour libérer l'ARN viral de sa capside pourrait avoir provoqué un changement de conformation de la VPg et expliquer pourquoi nous sommes incapable de la détecter avec un anticorps polyclonal anti-VPgPro. Pour les futures expériences, il serait préférable d'utiliser un moyen moins drastique pour la protéine VPg lors de la purification de l'ARN viral. Des travaux dans notre laboratoire devraient normalement être poursuivis pour réaliser cet objectif.

Dans cette étude, nous avons été les premiers à étudier l'effet d'une mutation virale contre une protéine cellulaire bien ciblée. Nous avons observé une corrélation entre l'affinité de l'interaction VPgPro-eIF4E et l'infection par le TuMV pour son hôte. Cette étude aide à mieux comprendre les mécanismes d'infections de ces groupes de virus.

Effet de l'infection du TuMV sur l'expression des isomères d'eIF4E.

Certains virus de mammifères altèrent la fonction de l'eIF4E pour établir l'infection virale (voir section 3, revue de littérature). Pour ce qui est du TuMV, nos travaux démontrent une corrélation entre l'interaction VPg-eIF4E *in vitro* et l'infection virale. Bien que nous ayons montré qu'il y a formation d'un complexe VPg-eIF4E *in vitro*, nous ignorons qu'elle est l'effet de la VPgPro sur l'eIF4E *in vivo*. Nos résultats démontrent que l'eIF(iso)4E présente un profil d'expression similaire tant dans les plantes infectées que dans les plantes non infectées. Par contre, lorsque nous avons comparé les plantes infectées aux plantes non infectées, un signal correspondant au poids moléculaire de l'eIF4E fut retrouvé dans les plantes infectées. Ce résultat suggère que l'eIF4E est induite lors de l'infection par le TuMV. Nous croyons que l'interaction de la VPg aux isomères d'eIF4E est directement responsable de cette augmentation du taux de la protéine eIF4E. En effet, nos travaux antérieurs nous incitent à croire que la VPg peut non seulement interagir mais également affecter le rôle de l'eIF4E lors de la traduction cellulaire. Par conséquent, pour assurer une traduction efficace de ses ARNm, la cellule se doit de maintenir un taux acceptable d'eIF4E fonctionnelle dans la cellule. Nous croyons donc que lors de l'infection du TuMV, l'induction de l'eIF4E est peut être un mécanisme compensatoire réalisé par la cellule pour assurer une traduction efficace de ses ARNm cellulaires. L'eIF4E et l'eIF(iso)4E sont exprimées différemment dans la plante, mais ont une fonction similaire pour l'initiation de la traduction cellulaire (Rodriguez et al., 1998). Cependant, des expériences biophysiques ont démontré que l'eIF4E et l'eIF(iso)4E ont une

affinités différentes lors de leur liaison aux ARNm. L'eIF(iso)4E possède une affinité plus forte pour les ARNm ayant une coiffe hyperméthylé (Carbery et al., 1991). L'eIF4E possède un pouvoir de liaison plus grand pour les ARNm munis de structure secondaire en 5' (Carberi et Goss, 1991). Conséquemment, l'expression d'eIF4E et non d'eIF(iso)4E après infection du TuMV est peut être une façon pour la cellule d'effectuer la traduction d'ARNm bien spécifique (et dépendant de l'eIF4E) important pour la survie de la cellule.

Par ailleurs, la cellule exerce un contrôle sur la traduction cellulaire en modulant l'activité de l'eIF4E principalement par phosphorylation (Raught et Gingras, 1999). Certains virus affectent également l'état de phosphorylation de l'eIF4E pour générer l'infection virale. Nous avons donc observé si le TuMV modifiait l'état de phosphorylation de l'eIF4E lors de l'infection. Nos résultats montrent cependant qu'après électrophorèse des protéines en gel 2-dimensions suivie d'un immunobuvardage qu'aucun changement de mobilité électrophorétique entre l'eIF4E et l'eIF(iso)4E de plantes infectées ou non infectées n'a été observé. Conséquemment, le TuMV ne semble pas phosphoryler les isomères d'eIF4E lors de l'infection. Le TuMV utilise donc un moyen différent du virus de l'infuenza et de l'adénovirus pour mobiliser le facteur 4E et générer l'infection virale.

Interaction entre la VPgPro et la PABP

Chez les mammifères, il est maintenant reconnu que la PABP est impliquée lors de l'initiation de la traduction cellulaire (voir section 2.3 de la revue bibliographique). La PABP effectue la jonction entre l'extrémité 5' et 3' des ARNm cellulaires grâce à une interaction entre la queue poly(A) et le facteur d'initiation de la traduction 4G. Étant donné que la PABP se retrouve associée au facteur d'initiation de la traduction, nous nous sommes demandé si la VPgPro qui interagit avec l'eIF4E ne pourrait pas aussi lier la PABP. Avec l'aide d'un test de type ELISA, nous avons montré que la VPgPro est effectivement capable de lier la PABP, alors que la VPg seule ne montrait aucune affinité pour la PABP. Ce résultat suggère que le domaine Pro est requis pour l'interaction avec la PABP. L'incapacité de purifier *in vitro* la Pro nous empêche cependant de déterminer si la protéase peut lier la PABP sans être associée à la VPg. Il serait pertinent de déterminer le domaine d'interaction de la Pro pour la PABP comme nous l'avons fait précédemment entre la VPgPro et l'eIF4E. Des mutants dans la partie Pro pourraient être effectués et introduits dans les plasmides du système du double-hybride. Nous pourrions ainsi en connaître d'avantage sur le site de liaison de la Pro à la PABP.

D'autres virus de mammifères clivent des facteurs d'initiation de la traduction cellulaires pour produire une infection virale. Ceci est notamment le cas pour le picornavirus dont l'eIF4G et la PABP sont dégradées par des protéases virales (Joachims et al., 1999; Michel et al., 2001). En s'inspirant de la stratégie utilisée par ces virus, nous avons tenté, en vain, de démontrer l'hydrolyse directe de la PABP par la VPgPro. Nous avons effectué une série de tests *in vitro* en mettant

en présence les protéines recombinantes (résultats non-présentés) sans toutefois observer la moindre dégradation de la PABP. Il se peut donc que la VPgPro n'ait pas d'activité protéolytique sur la PABP. Il se peut aussi que les conditions utilisées pour l'hydrolyse ne soient pas adéquates pour effectuer une dégradation protéique. De plus, il se peut qu'un cofacteur protéique soit requis pour le clivage de la PABP. Cependant nos travaux de co-localisation de la PABP et de la VPgPro suite à une infection par le TuMV ne montrent aucune dégradation de la PABP (Léonard et al., 2002., voir figure 3). Suite à ces résultats, il est donc peu probable que le TuMV clive la PABP lors de l'infection virale.

Interaction multiple avec la VPgPro dans les cellules infectées

Plusieurs fonctions sont attribuées à la VPgPro des potyvirus, notamment celle de participer à la réplication du virus. Le site de réplication des potyvirus est situé dans les membranes du réticulum endoplasmique rugueux et la 6KVPgPro est identifiée comme étant un marqueur de la réplication virale. Dans le but de déterminer des protéines (virales ou non) participant au complexe de réplication du virus, un gradient de sucrose a été utilisé pour séparer les membranes récoltées de plantes infectées ou non par le TuMV. Des immunobuvardages ont montré que la 6KVPgPro/VPgPro, l'ARN dépendante ARN polymérase (RdRp), l'eIF(iso)4E/eIF4E et la poly(A) binding protein (PABP) sont co-localisées dans les mêmes fractions, suggérant que ces protéines ont l'opportunité d'interagir. Ces protéines sont donc fortement susceptible d'intervenir dans la multiplication virale. *In planta* l'interaction a été confirmé par purification de la 6KVPgPro/VPgPro par chromatographie d'affinité par chelation de métaux: la RdRp, l'eIF(iso)4E et la PABP2 ont été co-purifié avec la VPgPro. Des

interactions directes entre la VPgPro, l'eIF(iso)4E, la RdRp ou la PABP ont également été montré (discutées plus loin) en utilisant un test de type ELISA. Ces expériences suggèrent qu'un complexe multi-protéine peut se former autour de la VPgPro du TuMV.

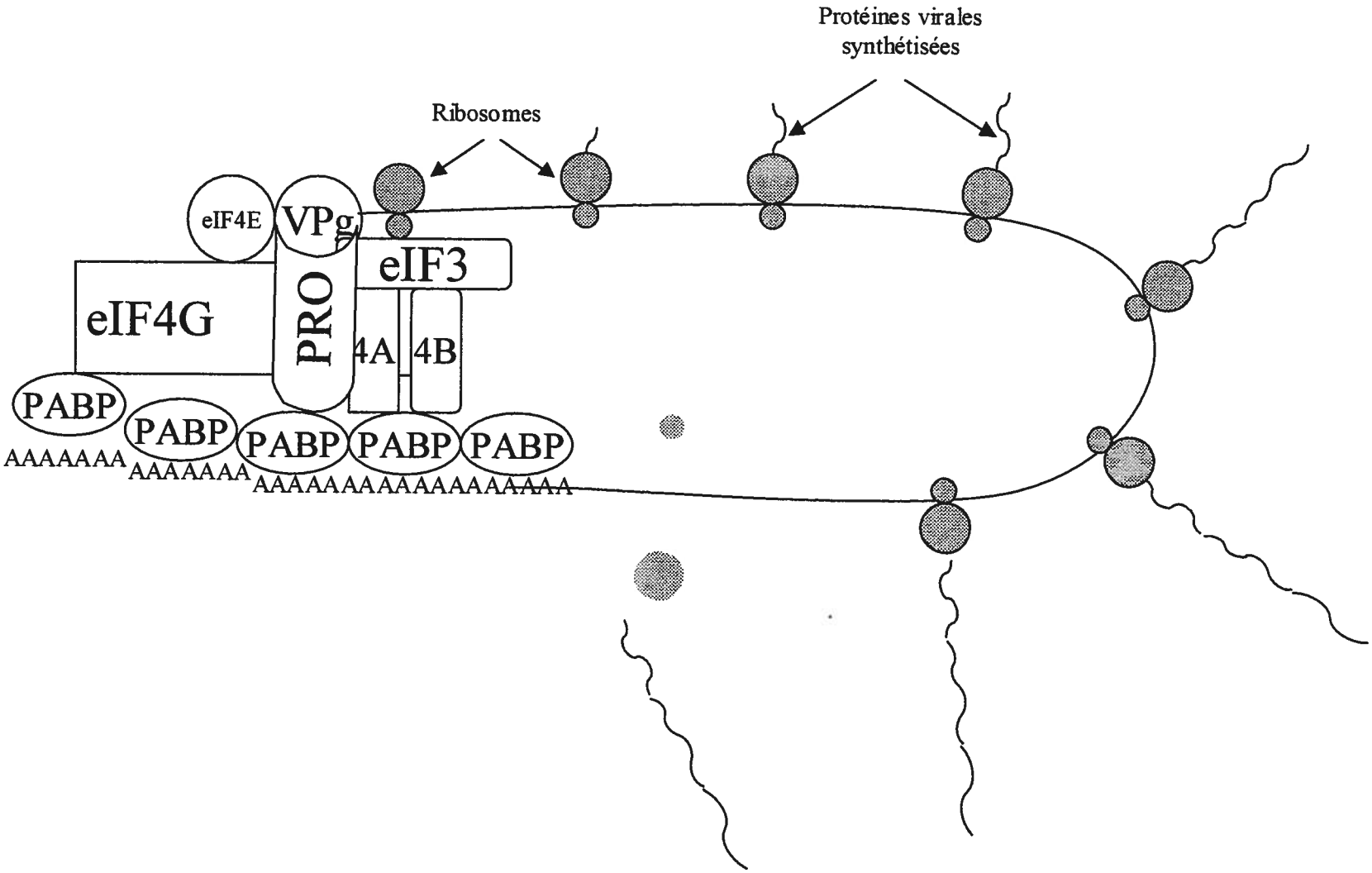
Traduction de l'ARN viral du potyvirus

Nous ne savons pas comment les potyvirus effectuent la traduction de leur ARN viral. Cependant les résultats que nous avons cumulés nous ont permis d'établir un modèle de traduction circulaire pour les potyvirus (voir figure 1). Ce modèle a été présenté et accueilli avec enthousiasme lors du sixième congrès international sur les virus à ARN(+) en juin 2001 à Paris. Dans ce modèle, la VPg liée de façon covalente à l'ARN viral compétionnerait avec les ARNm cellulaires et s'approprierait le complexe eIF4F par une liaison avec l'eIF4E. Nos travaux antérieurs ont montré que la VPg du TuMV interagit avec différentes eIF4E de plantes et que l'ajout d'un analogue de la coiffe (m^7GTP) inhibe la formation de ce complexe (Wittmann et al., 1997 ; Léonard et al., 2000). De plus, nous avons montré (collaboration avec l'équipe de M.G.Fortin) qu'un complexe entre la VPg, l'eIF4E et l'eIF4G peut s'effectuer *in vitro* (Plante et al., voir annexe 1). Ce complexe est également sensible à l'ajout de m^7GTP. Conséquemment, dans notre modèle la VPg mimerait en quelques sortes le rôle de la coiffe des ARNm pour s'emparer des facteurs initiant la traduction cellulaire. Deuxièmement, l'association que nous avons montrée entre la VPgPro et la PABP pourrait très bien résulter en la circularisation de l'ARN viral. Tout comme les ARNm cellulaires, l'extrémité 3' de l'ARN du potyvirus est constituée d'une queue polyadénylée. La PABP pourrait donc lier la queue

poly(A) et effectuer la jonction entre l'extrémité 5' et 3' de l'ARN viral par le biais d'interaction avec l'eIF4G et la VPgPro. Si le domaine VPg avait comme fonction de s'approprier l'eIF4F, le domaine Pro lui, pourrait recruter prioritairement la PABP au dépend de la traduction des ARNm cellulaires. Des études ont montré que la VPg ainsi que la VPgPro se retrouvent associées de façon covalente à l'ARN viral, appuyant la fonctionnalité de notre modèle. D'autres facteurs, comme l'eIF4B et l'eIF4A, seraient également nécessaires pour assurer une traduction efficace de l'ARN viral. Chez les potyvirus, l'ARN viral doit être traduit au moins 2000 fois pour la synthèse d'une seule particule virale (Riechmann et al., 1992). Le processus de traduction de l'ARN viral se doit donc d'être extrêmement efficace pour générer une infection virale. Le modèle de traduction circulaire des potyvirus que nous proposons comportent donc de nombreux avantages. Premièrement, à cause de la proximité de l'extrémité 5' et 3' de l'ARN viral, le complexe ribosomique serait en bonne position pour initier de nouveau la traduction cellulaire une fois sa traduction terminée. L'efficacité de la synthèse des protéines virales serait ainsi grandement maximisée. Deuxièmement, une fois circularisé, l'ARN viral serait protégé contre les attaques d'enzymes de dégradation. Le modèle de traduction circulaire proposé pour le TuMV est similaire au mécanisme utilisé par d'autres virus de mammifères. Le rotavirus utilisent vraisemblablement un mécanisme circulaire pour la traduction de leurs ARN viraux (Poncet et al., 1994 ; Piron et al., 1998, Vende et al., 2000). Des études ont démontré qu'une traduction efficace d'un ARN viral requiert simultanément l'interaction entre la protéine virale NSP3 liant l'extrémité 3' de l'ARN et l'eIF4G (Vende et al., 2000). La circularisation des ARN viraux du poliovirus serait également requis pour la traduction de ce

virus (Michel et al., 2001). Maintenant en ce qui à trait au virus de plantes, la circularisation du génome a aussi été évoquée pour ces types de parasites. La protéine de la capside (CP) de l'*alfalfa mosaic virus* augmente le taux de traduction de l'ARN de ce virus en agissant comme un analogue de la PABP (Neeleman et al., 2001).

Figure 1 : Modèle circulaire de traduction de l'ARN du TuMV



Réplication de l'ARN viral du potyvirus

Le processus de réplication du potyvirus s'effectue en synthétisant un brin complémentaire (ARN de polarité négative) à partir de l'ARN génomique qui lui est de polarité positive. Cet ARN intermédiaire nouvellement fabriqué est ensuite utilisé comme matrice pour la synthèse d'un ARN de polarité positive. Très peu de donnés expérimentales nous renseignent sur le mécanisme de réplication du potyvirus. Des expériences ont indiqué que le complexe de réplication s'effectue dans le cytoplasme, associé aux membranes du réticulum endoplasmique via une interaction avec la protéine 6K et ses protéines associées comme la 6KVPgPro (Wang et al., 2000; Schaad et al., 1997). Parmi les protéines du potyvirus qui sont susceptibles de participer à la réplication du génome viral, la VPgPro et, évidemment, la polymérase ont fait l'objet de quelques études. La VPgPro du TEV ainsi que celle du TVMV interagissent toutes deux avec la polymérase de ces virus *in vitro* (Hong et al., 1995 ; Li et al., 1997 ; Fellers et al, 1998 ; Daros et al., 1999). L'équipe de Fellers à également montré que la VPg et la VPgPro stimulaient l'activité de la polymérase virale *in vitro*, suggérant une participation active de la VPg lors de la réplication virale. Nos travaux ont montré que la VPg ou la VPgPro du TuMV liait aussi la polymérase de ce virus *in vitro*. Nos résultats indiquent que la région VPg serait responsable de l'interaction, ce qui concorde avec les résultats obtenus par le groupe de Fellers et al., 1998 et par celui de Hong et al., 1995 . Cependant pour le TEV le domaine Pro serait responsable de l'interaction avec la polymérase, suggérant que ces virus pourraient effectuer leur réplication différemment (Li et al., 1997).

L'interaction *in vitro* entre la VPgPro et l'eIF4E évoque également que le facteur initiant la traduction 4E peut être impliqué lors du processus de réplication du potyvirus. D'ailleurs d'autres virus de plantes utilisent des facteurs d'initiation de la traduction pour stimuler la synthèse de leur génome viral (Quadt et al., 1993). Chez le BMV et le TMV, l'eIF3 a été co-purifié avec le complexe de réplication de ces virus (Quadt et al., 1993; Osman et Buck, 1997; Blumenthal et Carmichael 1979; Kajitani et Ishihama, 1991). Lors de nos travaux, nous avons montré que l'eIF4E et la 6KVPgPro étaient colocalisées aux membranes du réticulum endoplasmique, endroit ou la réplication du virus s'effectue. Nous avons aussi montré avec l'aide d'un test de type ELISA que la VPgPro peut lier à la fois l'eIF4E et la polymérase du TuMV. Par analogie à ce qu'on observe chez d'autres virus de plantes, nous croyons que l'eIF4E est une des composants du processus de réplication des potyvirus. Il serait très interessant de vérifier, par exemple, si l'ajout d'eIF4E stimule l'activité de la polymérase *in vitro* comme c'est le cas pour la polymérase du BMV et l'eIF3.

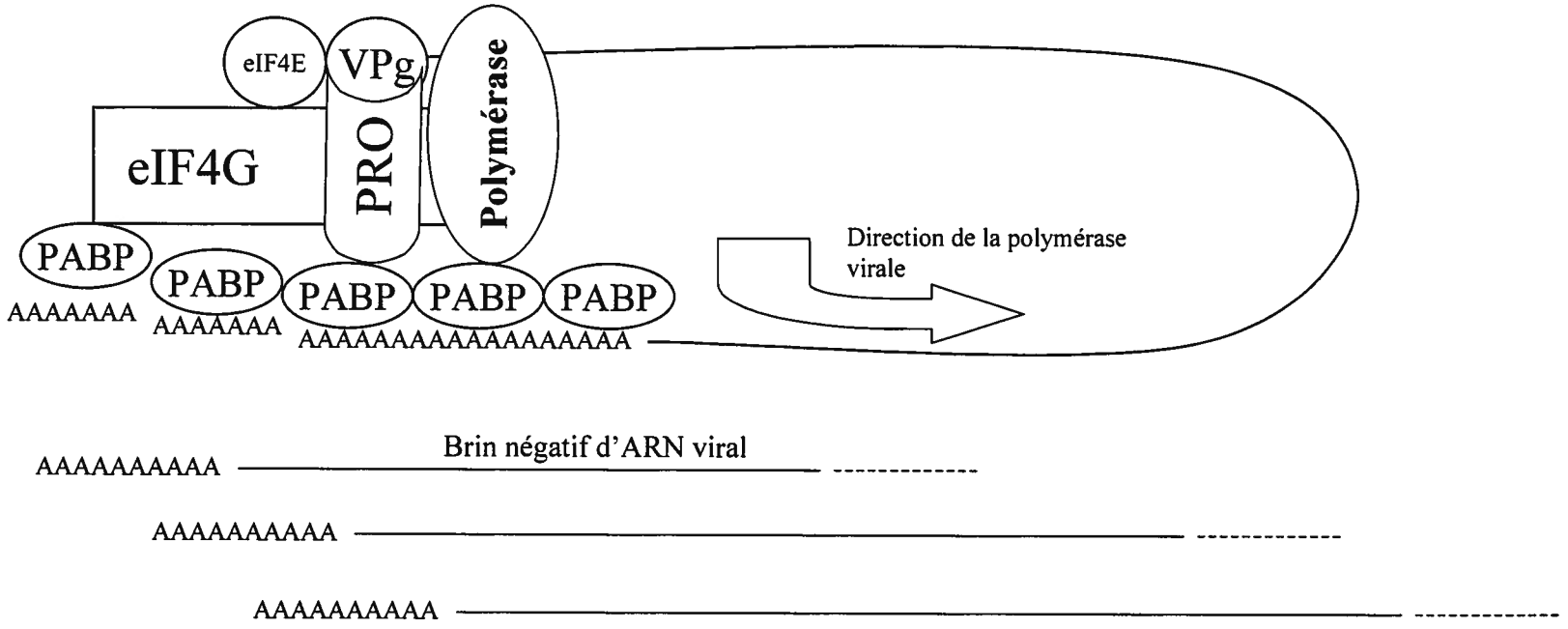
D'autre part, la polymérase RdRp (RNA dependant RNA polymerase) du Zucchini Yellow Mosaic potyvirus interagit avec la PABP du concombre (Wang et al., 2000). Les auteurs émettent la possibilité d'une inhibition de la traduction et/ou le recrutement de la polymérase sur la queue de poly(A) pour faciliter la réplication virale. Étant donné que nous avons montré que la VPgPro liait aussi bien la RdRp que la PABP, nous avons vérifié l'effet de l'ajout de la polymérase virale sur la formation du complexe VPgPro-PABP. Nos résultats démontrent que l'addition de la polymérase n'affecte pas l'interaction entre la PABP et la VPgPro. Cependant, à l'inverse, l'addition de PABP empêche la polymérase de lier la VPgPro, suggérant que la PABP compétitionne avec la polymérase pour la

liaison à la VPgPro. Différentes hypothèses ont été émises pour expliquer ces résultats. Premierement, l'affinité de la PABP pour la VPgPro est peut-être beaucoup plus grande que celle de la polymérase pour la VPgPro. Deuxièment, une fois liée à la VPgPro, la PABP qui possède une grande taille (environ 70 kDa) empêche peut-être la reconnaisance du domaine d'interaction de la polymérase pour la VPgPro.

Dans le même ordre d'idée, la circularisation des ARN viraux serait également requise pour la réplication de certains virus. Des travaux effectués notamment chez le poliovirus, ont montré qu'une interaction entre l'extrémité 5' et 3' de l'ARN est nécessaire pour la synthèse du brin négatif d'ARN (Herold et al., 2001). À la lumière des résultats que nous avons obtenus *in vitro*, nous proposons, tout comme nous l'avons fait pour la traduction, un modèle de réplication circulaire du potyvirus (figure 2). Comme illustré, la VPgPro à l'extrémité de l'ARN viral pourrait d'abord s'approprier le complexe eIF4F en liant l'eIF4E. La circularisation de l'ARN viral serait assurée par l'interaction entre l'eIF4G ainsi que par le domaine Pro pour la PABP fixée sur la queue poly(A). La polymérase pourrait ensuite être recrutée par soit la VPg ou bien par la PABP et elle serait alors à proximité de l'extrémité 3' de l'ARN viral pour initier la synthèse du brin négatif d'ARN en direction de l'extrémité 5'. Une fois le cycle de réplication terminé, la polymérase serait bien positionnée pour enclencher un autre cycle de réplication, maximisant l'efficacité de reproduction du virus. La circularisation de l'ARN viral aurait également l'avantage d'éviter la synthèse d'ARN viraux incomplets.

Ce mécanisme est complémentaire au modèle de traduction viral du potyvirus précédemment proposé. Nous croyons que la traduction et la réplication du virus devraient être considérées comme des événements étroitement liées. Ainsi une fois l'ARN viral circularisé, la traduction et la réplication pourrait avoir lieu alternativement : la traduction débutant à l'extrémité 5' et allant vers l'extrémité 3', la réplication s'initiant à l'extrémité 3' pour se diriger vers l'extrémité 5'. Nos travaux futurs pourront éclaircir et mieux définir ces mécanismes régissant la synthèse des potyvirus.

Figure 2: Modèle circulaire de réplication de l'ARN du TuMV



Conclusion

Nous avons poursuivi les travaux impliquant la VPg (viral protein genome linked) du virus de la mosaïque du navet (TuMV) et le facteur eucaryotique d'initiation de la traduction 4E (eIF4E) (cap binding protein) d'*Arabidopsis thaliana* (Wittmann et al., 1997). En plus de caractériser le domaine d'interaction de la VPg pour l'eIF(iso)4E, nous avons montré que cette interaction était corrélée avec l'infectivité du virus. Ces résultats suggèrent que l'interaction VPg-eIF(iso)4E est un événement critique pour la réplication du virus. Nous avons vérifié l'effet d'une infection par le TuMV sur l'expression des isomères d'eIF4E, seule l'eIF4E est induite lors de l'infection par le TuMV. Un gradient de sucrose a été utilisé pour séparer les membranes récoltées de plants infectés ou non par le TuMV. Des immunobuvardages ont montré que la 6KVPgPro/VPgPro, l'ARN dépendante ARN polymérase (RdRp), l'eIF(iso)4E/eIF4E et la poly(A) binding protein (PABP) sont co-localisées dans les mêmes fractions, suggérant que ces protéines ont l'opportunité d'interagir. *In planta* l'interaction a été confirmé par purification de la 6KVPgPro/VPgPro par chromatographie d'affinité par chelation de métaux: la RdRp, l'eIF(iso)4E et la PABP2 ont été co-purifié avec la VPgPro. Des études de liaison *in vitro* ont montré que de multiples interactions entre la VPgPro, la RdRp, l'eIF(iso)4E et la PABP peuvent se produire, la VPgPro agissant comme point de central pour la formation de ces complexes. Ces résultats suggèrent que la traduction et la réplication du TuMV peuvent être étroitement liées et que l'ARN viral peut être circularisé. Ainsi, en se basant sur nos résultats et en s'inspirant de d'autres virus, nous avons proposé un modèle circulaire pour la réplication et la traduction des potyvirus. Nous avons également vérifié si la VPg d'un autre type de virus que le potyvirus pouvait interagir avec le facteur initiant la traduction

4E. Nous avons montré une interaction entre l'eIF(iso)4E et la protéinase (Pro) d'un népovirus (*Tomato ringspot virus*; ToRSV) *in vitro*. La formation du complexe est inhibé par l'addition de m^7GTP (un analogue de la coiffe), suggérant que la protéine Pro peut compétionner avec les ARNm cellulaires pour la liaison à l'eIF(iso)4E. Ces résultats démontrent donc l'universalité de l'interaction VPgPro-eIF(iso)4E, cependant la façon dont le ToRSV procède pour lier le facteur d'initiation de la traduction diffère du TuMV.

Nous sommes innovateurs en ce qui concerne l'étude du processus d'infection des virus de plante de polarité positive. L'avancement des connaissances, depuis la découverte de l'interaction VPg-eIF(iso)4E à aujourd'hui, est considérable pour la compréhension du mécanisme d'infection des potyvirus. Nos résultats et notre modèle de réplication/traduction des potyvirus nous permettent maintenant d'effectuer une comparaison entre le processus d'infection des virus de plantes et ceux de divers virus de mammifères.

Les travaux futurs dans le laboratoire permettont d'établir une dynamique entre les protéines virales et non-virales retrouvées dans le modèle de réplication/traduction proposé. Nous espérons donc mieux connaître la fonction de ces protéines nécessaires à la multiplication virale.

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Annexes

Annexe I

Interaction of *Turnip Mosaic Virus* VPgPro with the Translation Initiation Factor 4E and the Poly(A) Binding Protein *in Planta*

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Running title: TuMV and translation initiation factor 4E

Abstract

The viral protein linked to the genome (VPg) of *Turnip mosaic virus* (TuMV) interacts *in vitro* with the translation eukaryotic initiation factor (eIF) 4E. In the present study, we investigated the consequence of TuMV infection on eIF4E expression. Two isomers are present in plants, namely eIF4E and eIF(iso)4E. The latter was detected in both TuMV infected and mock inoculated *Brassica perviridis* plants, but eIF4E was found only in infected plants. Membranes from TuMV infected or mock inoculated tissues were separated by sucrose gradient centrifugation and fractions were collected. Immunoblot analyses showed that 6KVPgPro/VPgPro were associated with endoplasmic reticulum membranes and were the viral forms likely to interact with eIF(iso)4E and eIF4E. *In planta* interaction between 6KVPgPro/VPgPro and eIF(iso)4E/eIF4E was confirmed by co-purification by metal chelation chromatography. The poly(A) binding protein (PABP) was also found to co-purify with VPgPro. Direct interaction between VPgPro and PABP was shown by an ELISA-based binding assay. These experiments suggest that a multi-protein complex may form around VPgPro of TuMV.

Key words: Plant RNA virus; potyvirus; turnip mosaic virus; viral genome linked protein; VPg; eukaryotic initiation factor; eIF4E; poly(A) binding protein; PABP; protein interaction

Introduction

Translation of viral RNAs by the host machinery is a crucial event in the virus cell cycle and proceeds essentially as for cellular mRNAs (15;60). One of the first steps in translation is the recruitment of mRNAs by the translation eukaryotic initiation factor (eIF)4F complex. eIF4E is a member of this complex and recognizes the cap structure (m^7GpppN, where N is any nucleotide) at the 5' end of mRNAs. The other members of the eIF4F complex are eIF4G and eIF4A. In conjunction with additional proteins, the eIF4F complex links mRNAs to ribosomes and promotes the search of the translation start site. Among the proteins that interact with components of eIF4F is the poly(A) binding protein (PABP) (26;58). A functional consequence of this interaction is an increase in affinity of PABP for the poly(A) tail and of eIF4F for the 5' cap structure of mRNAs (16). In addition, association of PABP with eIF4F results in the circularization of mRNAs and promotes the re-initiation of translation (53).

Animal viruses have elaborated strategies that allow the preferential translation of viral mRNAs (7), with the consequence that host protein synthesis is usually blocked. This phenomenon is known as host translation shutdown. Plant virus infection and its impact on the cell protein synthesis has however not been as extensively studied as for animal virus infections (2). The most studied example of a block in translation involves the picornaviruses. The inhibition is mediated by the cleavage of eIF4G by viral or cellular proteinases. Hydrolysis of eIF4G prevents eIF4E from binding to the 43S ribosome subunit complex and thus precludes the recruitment of capped cellular mRNAs (25;30). However, this presumption has recently been challenged (1). Equally, adeno- and influenza

viruses inhibit host cell protein synthesis by inactivating eIF4E through de-phosphorylation (13;22). Finally, the ring Z protein of the lymphocytic choriomeningitis virus was shown to interact with eIF4E and to repress host mRNA translation (8).

RNA viruses may also recruit translation factors in order to further increase their translation efficiency. A case in point is the rotavirus NSP3 protein. It interacts with the 3' end of rotavirus mRNAs, which are capped but not polyadenylated (45;46). NSP3 also interacts with eIF4G (44). Further studies showed that enhancement of rotavirus mRNA translation requires the simultaneous interaction of NSP3 with eIF4G and the mRNA 3' end (61). During rotavirus infection, PABP is probably evicted from eIF4G, impairing the translation of polyadenylated host mRNAs (44). Finally, the VPg (viral protein genome linked) of the Norwalk virus has been shown to bind eIF3, suggesting a role of the viral protein in translation initiation complex recruitment (10).

Turnip mosaic virus (TuMV) is a member of the potyvirus family (49). Potyviruses have a plus sense, single-stranded RNA genome of about 10 kb in length, a poly(A) tail at the 3' end and a VPg covalently linked to the 5' end. The genome codes for one large polyprotein, which is processed into at least ten mature proteins by three viral proteinases (Pro) (49). Several functions have been attributed to the potyvirus VPg. First, the viral protein and its precursor form VPgPro interact with and have a stimulating effect on the activity of the viral RNA-dependent RNA polymerase (RdRp), suggesting a participation in viral replication (9;14;21;29). Additionally, interaction between the VPg and eIF4E has been reported for *Tobacco etch virus* (TEV) (55) and TuMV (28;62). The

importance of this factor for TuMV infection has been shown in mutant *Arabidopsis thaliana* plants that do not express eIF(iso)4E (12;27). Although these plants had a normal phenotype, they were immune to TuMV. Finally, VPg has also been shown to have a role in overcoming viral resistance in plants (5;23;24;33;39;40;48;57). In the case of pepper and lettuce, the recessive resistance gene against *Potato virus Y* and *Lettuce mosaic virus* has been identified as coding for eIF4E (38;51).

In the present study, we investigated whether eIF4E expression is affected following TuMV infection and if VPg and its precursor form VPgPro could act as a recruitment focal point for other proteins of cellular origins. Our data show that indeed the eIF4E isomers expression profile was modified, and that, in addition to eIF(iso)4E and eIF4E, PABP can interact with VPgPro *in planta*.

Materials and methods

Microorganisms and media

Manipulations of bacteria as well as nucleic acids and proteins were done by standard methods (54). *E. coli* XL1-Blue was used for subcloning, and *E. coli* BL21 (DE3) (Novagen) was used for protein expression.

PABP expression in *E. coli*

Plasmid pETtagPABPhis codes for PABP2 of *A. thaliana* (accession number NM_119572). RT-PCR was performed on total RNA of *A. thaliana* using the forward

(5' TATATACATATGGCTAGCCCGAATTCGATGGCGCAGGTTCAACTT 3'; *Nhe*I and *EcoR*I sites are underlined) and the reverse primer

(5' TATATACTCGAGAGAGAGGTTCAAGGAAGC 3'; *Xho*I site is underlined). The amplified fragment was digested with *EcoR*I and *Xho*I and was cloned in similarly restricted pET21b (Novagen). The resulting PABP was fused at its N-terminal end to the 11-amino-acid N-terminal peptide of the T7 gene 10 protein (T7 tag), which is recognized by the anti-T7 tag monoclonal antibody (Novagen). It also had a His tail at its C-terminal end. pETtagPABPhis was digested with *Xho*I and ends were blunted with Klenow fragment giving PABP2 without the His-tail. An overnight culture of *E. coli* BL21 (DE3) was diluted 1:100 in fresh medium and incubated at 37°C until A_{600} of 0.6. Protein production was then induced at room temperature with 0.4 mM isopropylthio-β-galactoside (IPTG) for three hours. Purification by metal chelation was performed as in (35).

ELISA-based binding assay

The specified proteins were absorbed to wells of an ELISA plate (1.0 µg/well) by overnight incubation at 4 °C and wells were blocked with 5% Blotto in phosphate-buffered saline (PBS). Appropriate proteins were diluted in PBS with 1% Blotto and 0.2% Tween and incubated for 1.5 h at 4°C with the previously coated wells. Detection of retained protein was achieved as in ELISA with the anti-T7 tag antibody and peroxydase-labelled goat anti-mouse immunoglobulin G (KPL). Wells were washed three times with 0.05% Tween between incubations.

Membrane fractionation

Membranes recovery from plants was done according to (56). *Brassica perviridis* plants (three-leaf stage) were infected with TuMV, or mock inoculated with PBS. At ten days post-inoculation, the leaf that developed next to the inoculated one was harvested. Leaf tissue (2 g) was minced in 6 ml of homogenization buffer (50 mM Tris-HCl, pH 8.0, 10 mM KCl, 3 mM $MgCl_2$, 1mM EDTA, 1mM DTT, 0.1% BSA, 0.3% dextran, 13% sucrose and one tablet of Complete Mini EDTA-free (Roche) per 10 ml of buffer was added). The homogenate was filtered through Miracloth and subjected to centrifugation at 3 700*g* for 10 min at 4°C. The supernatant (2.5 ml) was layered onto a 9 ml 20-45% sucrose gradient containing the respective homogenization buffer and subjected to centrifugation at 143 000*g* in a Beckman SW41 Ti rotor for 4 h at 4°C. Fractions (0.9 ml) were collected, diluted 1:1 in protein dissociation buffer and subjected to immunoblot analysis after 12.5% SDS polyacrylamide gel electrophoresis. Immunoreactions

were detected using the ECL chemiluminescence-based secondary antibody system (Amersham).

Results

Expression of eIF4E isomers following TuMV infection

There are at least two isomers of initiation factor 4E in plants, namely eIF4E and eIF(iso)4E (6). They share significant sequence homology, but their respective role in translation initiation is unknown. Furthermore, the functional state of eIF4E is altered following infection by certain animal viruses (8;13;22). The consequence of TuMV infection on the expression state of the eIF4E isomers was then investigated. *B. perviridis* plants at the three-leaf stage were mock inoculated or TuMV infected. After ten days following inoculation, the leaf that developed next to the inoculated one was harvested. Infection was confirmed by immunoblot analysis using a rabbit serum raised against the capsid protein of TuMV (data not shown). Proteins from a 3 700*g* leaf extract supernatant were separated by SDS-PAGE and subjected to immunoblot analysis using a rabbit serum that was raised against a recombinant form of eIF(iso)4E from *A. thaliana*. eIF4E and eIF(iso)4E of *A. thaliana* have a calculated size of 26.5 kDa and 22.5 kDa, respectively (50). Although the size for the *B. perviridis* isomers is not known, it is expected to be very similar since both plants belong to the same family. Fig. 1 shows that eIF(iso)4E was detected in mock inoculated plants. It migrated to the same gel distance as recombinant eIF(iso)4E of *A. thaliana*. This protein was retained on m^7GTP Sepharose, which confirmed its cap-binding property (data not shown). eIF(iso)4E was also detected in TuMV infected plants along with an additional protein that had the same molecular weight as recombinant eIF4E of *A. thaliana*. Cross-reaction of the anti-eIF(iso)4E serum with eIF4E can be expected since the two proteins are highly homologous (50) and a rabbit serum raised against wheat eIF(iso)4E recognized both eIF4E and

eIF(iso)4E of *A. thaliana* (52). TuMV infection thus induced the production of eIF4E, while the expression level of eIF(iso)4E did not appear to change significantly compared to its level in mock inoculated plants. Interestingly, a protein migrating ahead of eIF(iso)4E was detected in infected leaves, but the nature of this species was not investigated further.

Membrane localization of VPgPro

eIF4E is associated with 48S ribosomal complexes (20). To determine which VPg-containing protein type is found in ribosome-containing structures, cellular membranes from mock inoculated and TuMV infected leaves were fractionated on 20-45% sucrose gradients. The gradients were either carried out in the presence or in absence of 3 mM $MgCl_2$. The presence of $MgCl_2$ preserves the integrity of ribosomes associated with the endoplasmic reticulum (ER). On the other hand, the absence of $MgCl_2$ promotes the dissociation of ribosomes from ER, which results in a shift of the ER membranes toward the top of the gradient (31). Fractions were collected and first analyzed by immunoblot assay using antibodies raised against BiP (a marker of the ER), proteins containing xylose-β1→2-mannose modifications (a marker of the medial- and trans-Golgi), and the tonoplast H^+-ATPase. In the presence of 3 mM $MgCl_2$, two BiP-containing peaks were resolved, one near the bottom and one near the top of the gradient (Fig. 2a). This result has previously been described by others (18), but no explanation was provided concerning the nature of the light BiP-containing peak (56). This may reflect the tendency of BiP to be released from the ER membranes and to float in the gradient. The heavy BiP-containing fractions contained rRNA (data not shown). The peak was moved 2-3 fractions up the gradient in the absence of $MgCl_2$, which suggests that fractions 1-5 contain ER, with a proportion of the

membranes associated with ribosomes. The proteins containing β-xylosyl were detected near the top of the gradient (Fig. 2b; fractions 11 and 12). Likewise, the tonoplast H^+-ATPase was located in fractions 10-12 (Fig. 2c). This indicates that the Golgi apparatus and vacuolar membranes were well resolved from the ER in the sucrose gradient.

In mock inoculated tissue, eIF(iso)4E sedimented to a position near the bottom of the gradient in the presence of 3 mM $MgCl_2$ (Fig. 2d; fractions 1-5). eIF(iso)4E distribution in TuMV infected plants was similar. On the other hand, two eIF4E-containing peaks were resolved, one near the bottom of the gradient (fractions 1 and 2) and one in the middle (fractions 5-8). These last fractions also contained rRNA (data not shown). This dual distribution may reflect the position of eIF4E in polysomes and monosomes in the sucrose gradient (11). No shift of the initiation factors to lighter fractions was observed in the absence of $MgCl_2$ (data not shown), which was expected since they are associated with ribosomes and not with ER membranes. Localization of eIF(iso)4E in fractions 1-5 supports the notion that these fractions contained ribosomes. Interestingly, the anti-eIF(iso)4E-reacting protein of low molecular weight was found in the top fractions where no rRNA was detected.

When the anti-VPgPro serum was used, no reactivity was noticed in the 3 700g supernatant (S3) recovered from mock inoculated plants (Fig. 2e, lane N). On the other hand, several VPgPro-related polypeptides were detected in the S3 extract from TuMV infected plants (Fig. 2e, lane S). These species had the expected molecular weight for 6KVPgPro, VPgPro and Pro. Recombinant forms of these proteins have been previously expressed in *E. coli* (35;62) and migrated to the same position as for the proteins detected in TuMV infected plants (data not

shown). No polypeptide corresponding to the mature VPg was however detected. The different viral proteins were localized to different fractions in the 3 mM $MgCl_2$ sucrose gradient. A sub-population of VPgPro, C-terminally processed VPgPro (35) and Pro were found in the lighter fractions of the sucrose gradient. On the other hand, 6KVPgPro and the remaining portion of VPgPro were found near the bottom of the gradient (fractions 1-5). In the 0 mM $MgCl_2$ sucrose gradient, the 6KVPgPro/VPgPro peak was moved up the gradient by ~2-3 fractions, which was similar to what had been observed for the bottom BiP-containing fractions. This shift then suggests that 6KVPgPro/VPgPro are present with ribosome associated ER membranes and are likely to be the protein type to interact with the translation initiation factor(s) *in planta*.

Co-purification of 6KVPgPro/VPgPro with eIF(iso)4E/eIF4E

Detection of 6KVPgPro and VPgPro in the same sucrose gradient fractions as the initiation factors opens the question whether the two viral proteins interact with eIF(iso)4E, eIF4E or both *in planta*. VPgPro has an intrinsic capacity to bind to nickel-agarose resin, the presence of a histidine tail being unnecessary (35). The binding to the resin is mediated by the VPg domain (data not shown). Purification of 6KVPgPro and VPgPro by metal chelation chromatography was thus attempted and co-purification of eIF(iso)4E as well as eIF4E evaluated. For TuMV infected and mock inoculated tissues, fractions 1 to 5 from a 3 mM $MgCl_2$ membrane fractionation experiment were pooled and loaded onto a nickel-agarose column. After washing the resin, the bound proteins were eluted with 100 mM imidazole and analyzed by immunoblot assay following SDS-PAGE. Fig. 3a shows that both 6KVPgPro and VPgPro were effectively purified from infected tissues. Similarly, eIF4E and eIF(iso)4E were detected in the

eluted protein fraction when the column was loaded with the membrane fractions from TuMV infected leaves (Fig. 3b). They were not detected with mock inoculated membrane fractions, even after prolonged film exposure. This experiment then indicates that 6KVPgPro and/or VPgPro interacts with both eIF(iso)4E and eIF4E in infected cells.

VPgPro interaction with PABP

Interaction of 6KVPgPro/VPgPro with eIF(iso)4E and eIF4E *in planta* evokes the possibility that other factors involved in the initiation of translation might be, directly or indirectly, associated with the viral protein. One candidate protein is PABP. A rabbit serum raised against PABP2 of *A. thaliana* (42) was used to detect PABP co-purification with 6KVPgPro/VPgPro. Fig. 4 shows that a *B. perviridis* 69-kDa PABP2-like isomer can be co-purified from infected tissues. This protein was not purified by metal chelation chromatography from mock inoculated membrane fractions. This experiment then indicates that 6KVPgPro and/or VPgPro interacts with PABP *in planta.*

PABP co-purification with 6KVPgPro/VPgPro may be the result of direct interaction with the viral protein, or through the intermediary of another protein that directly interacts with VPgPro. ELISA-based binding assays with recombinant proteins were done to investigate direct interaction. PABP2 of *A. thaliana* was produced in *E. coli* as a T7-tagged, His-tailed fusion protein and purified by metal chelation chromatography. ELISA plate wells were coated either with VPgPro or metal chelation chromatography purified proteins from a control *E. coli* lysate containing pET21b. The coated wells were then incubated

with increasing concentrations of T7-tagged PABP2 His-tailed. Complex retention was detected by using an anti-T7-tag antibody. Fig. 5a shows that proteins from the *E. coli* lysate did not interact with PABP2. However, a saturation binding curve was observed for VPgPro. This experiment then indicates that VPgPro interacts directly with PABP2. Binding conferred by the presence of the His tail was excluded as complex formation between VPgPro and PABP2 was equally observed with PABP whether His-tailed or not (Fig. 5b). The T7 tag was shown previously not to interact with VPgPro (62).

Discussion

Infection of *B. perviridis* by TuMV modified the expression profile of the eIF4E isomers. Only eIF(iso)4E was detected in mock inoculated, healthy, leaves while both isomers were present in infected tissues. An additional eIF(iso)4E related protein was also detected in infected leaves and may correspond to another isomer of the initiation factor (52), or may be a cleavage product of the initiation factor. This protein does not appear to be associated with ribosomes and it is not known if it has any cap binding activity. Differential expression supports the notion that eIF(iso)4E and eIF4E carry out distinct cellular functions (6). For instance, Gallie & Browning (17) proposed that eIF4F [a higher protein complex containing eIF4E] may promote translation under cellular conditions in which cap-dependent translation is inhibited. This statement is appealing in the light that viral infection often leads to cap-dependent inhibition of host mRNA translation (7;15). Other proteins have been shown to increase during plant viral infections (3;19). Although the mechanism and purpose of induction remain unknown, it was proposed that this increase in expression could prepare the cell for the biosynthetic demands of virus replication (34). It is thus possible that eIF4E production in *B. perviridis* is linked to the cellular response needed to adjust to the pressure on protein synthesis caused by TuMV infection. This is supported by the work of Lellis *et al.* (27) and Duprat *et al.* (12) who have isolated *A. thaliana* lines bearing mutations in the gene coding for eIF(iso)4E. These lines had a normal phenotype even though they did not produce eIF(iso)4E. It was noted that the amount of eIF4E had increased significantly in the transposon mutated line (12). The mutant *A. thaliana* may then have compensated for the lack of eIF(iso)4E by an increased synthesis of eIF4E in

order to keep up with the demand of protein synthesis associated with normal plant development. Additionally, absence of eIF(iso)4E rendered these lines resistant to TuMV. We may then think that TuMV infection of *B. perviridis* lead to the inactivation of eIF(iso)4E, or to its monopolization for viral protein synthesis. The pressure by the virus on the host protein synthesis machinery then had to be relieved by *de novo* synthesis of eIF4E to fulfil the needs of the plant.

The binding domain to eIF(iso)4E is located within VPg (28;62). We showed by membrane co-localization and co-purification experiments that it was 6KVPgPro and/or VPgPro, precursor forms of VPg, that do the actual binding in plants. Curiously, we did not detect fully processed VPg in infected leaves. Fully processed VPg would at the very least be found within virions. Detection of this form would however require significant loading of material on SDS gels (37). Our membrane fractionation experiment also indicated that the interaction is likely to take place in ribosome-associated ER membranes. In the case of TEV, Schaad *et al.* (56) showed an association of 6KVPgPro with these sub-cellular membranes and demonstrated that the 6K domain is an integral protein of ER membranes. Furthermore, the potyvirus RNA replication complex is associated with ER membranes (32;56) and it has been proposed that 6 kDa-containing proteins, notably 6KVPgPro, would participate in replication (49). It appears then that potyvirus replication and translation are closely linked phenomena involving a common set of proteins and found in the same sub-cellular compartment. This close link between replication and translation is in agreement with the finding that another translation initiation factor, eIF3, was present in highly purified replication complexes of both *Brome mosaic virus* (47) and *Tobacco mosaic virus* (41). In the case of the latter virus, there is *in vitro*

evidence that the factor interacts with the methyltransferase-like domain of the 126 and 183 kDa replicase proteins (59).

Furthermore, we showed that VPgPro can also interact with PABP *in planta*. PAPB is ubiquitous in eukaryotes and participates in at least three major post-transcriptional processes: initiation of protein synthesis, mRNA turnover and mRNA biogenesis. *A. thaliana* PABPs are encoded by a very diverse gene family (4). The isomer PABP2 is highly expressed in all organs of *A. thaliana* (43) and was shown to function in yeast translational processes (42). This isomer was thus the first choice to test for interaction with the viral protein. It remains to be seen if the other isomers of PABP are also capable of interacting with VPgPro. VPgPro and PABP could interact with each other in two separate ways, potentially increasing the overall stability of the complex. First, the interaction can be direct as shown in this study. The other way would be through the connection of the eIF(iso)4E/eIF(iso)4G dimer. Indeed, eIF(iso)4G has been shown to bind PABP (26) and can interact with VPgPro through the intermediary of eIF(iso)4E (in preparation). This last possibility requires however to be experimentally demonstrated. Moreover, the interaction between VPgPro and eIF(iso)4E/eIF4E as well as PABP could possibly promote RNA circularization during translation. Circularization has been shown to be necessary for efficient translation of cellular mRNAs (16) and also to be taking place for animal viral RNAs (36). The TuMV RNA is linked at its 5' end to VPg and is polyadenylated. Additionally, VPgPro could also be linked to the viral RNA (37). Consequently, formation of the VPgPro-PABP complex, and a likely VPgPro-eIF4E-eIF4G-PABP complex, could bring both ends of the viral RNA in close proximity. It now remains to be demonstrated, through a similar approach

used for eIF(iso)4E (12;27), if *A. thaliana* PABP2 knockouts are immune to TuMV infection.

Finally, interaction with eIF(iso)4E, eIF4E and PABP suggests that VPgPro may serve as a focal point for translation initiation complex assembly. Preliminary experiments also showed

that other proteins interact with the viral protein (in preparation). This concept of VPg as a protein recruitment factor has recently been suggested for the Norwalk virus VPg (10). This VPg was shown to interact with eIF3 and pull-down experiments showed that other translation initiation factors were co-purified, notably eIF4GI, eIF4E and eIF2α. The extent to which these interactions are direct with VPg, or mediated through eIF3, is not yet known. Future experiments will investigate what other proteins are found associated with VPgPro of TuMV and if these proteins are the same whether the viral protein is localized in ER, the cytoplasm or the nucleus.

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Figure legend

Fig. 1 : Expression profile of eIF4E isomers following TuMV infection. Recombinant *A. thaliana* eIF(iso)4E and eIF4E as well as proteins from mock inoculated and TuMV infected leaf extracts were separated by SDS-PAGE and electroblotted to nitrocellulose. Membrane was probe with a rabbit serum against eIF(iso)4E of *A. thaliana*.

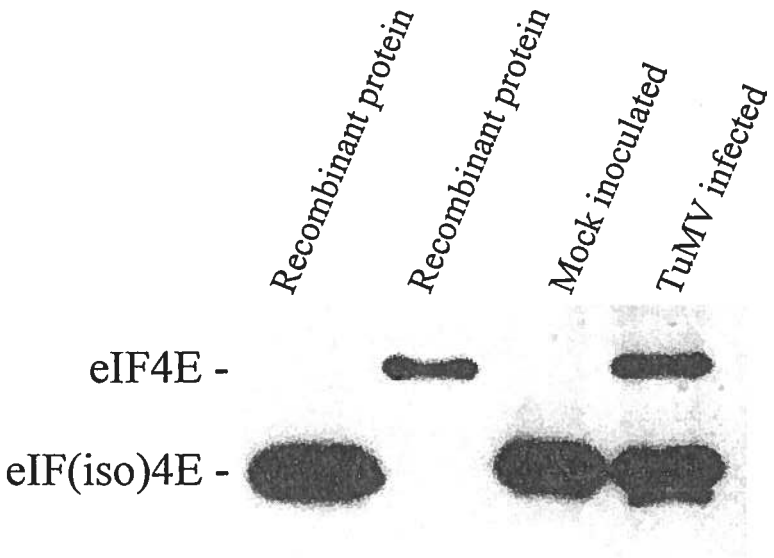
Recombinant protein
Recombinant protein
Mock inoculated
TuMV infected
eIF4E -
eIF(iso)4E -

Fig. 1

Fig. 2 : Detection of proteins in membrane fractions following centrifugation in sucrose gradient. Tissue extracts were prepared and centrifuged on 20-45% sucrose density gradients in the presence or absence of $MgCl_2$, as indicated. The direction of sedimentation was from right to left, with fraction 12 representing the top of the gradient. Fractions were collected and proteins separated by SDS-PAGE and electrobotted to nitrocellulose. Sucrose gradient in (**a**) 3 mM and 0 mM $MgCl_2$ of infected extract and immunoblot analysis using anti-BiP serum; (**b**) 3 mM $MgCl_2$ of infected extract and immunoblot analysis using anti-β-xylosyl serum; (**c**) 3 mM $MgCl_2$ of infected extract and immunoblot analysis using anti-tonoplast H^+-ATPase serum; (**d**) 3 mM $MgCl_2$ of mock inoculated and TuMV infected extract and immunoblot analysis using anti-eIF(iso)4E serum. Recombinant eIF(iso)4E from *A. thaliana* (C) and non-fractionnated extracts (S) were analysed. Gel migration position of eIF4E and eIF(iso)4E is indicated to the left; (**e**) 3 mM and 0 mM $MgCl_2$ of infected extract and immunoblot analysis using anti-VPgPro serum. Recombinant VPgPro from TuMV (C), mock inoculated (N) and TuMV infected non-fractionated extracts (S) were also analyzed. Gel migration position of the 6KVPgPro, VPgPro and Pro is indicated to the left.

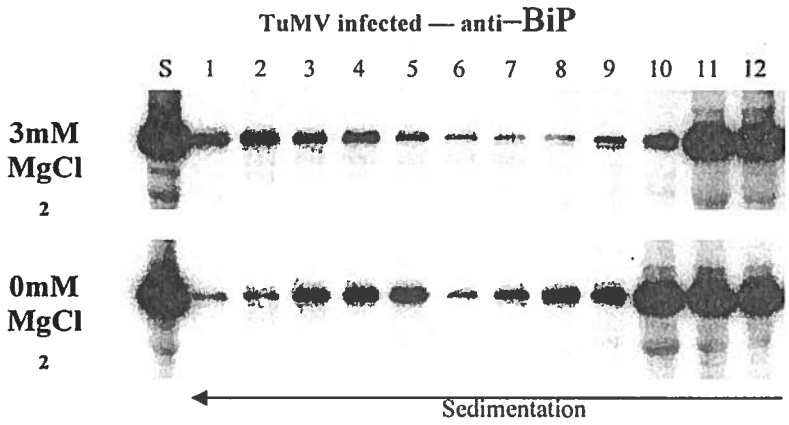
TuMV infected — anti–BiP
S 1 2 3 4 5 6 7 8 9 10 11 12
3mM MgCl2
0mM MgCl2
Sedimentation

Fig. 2a

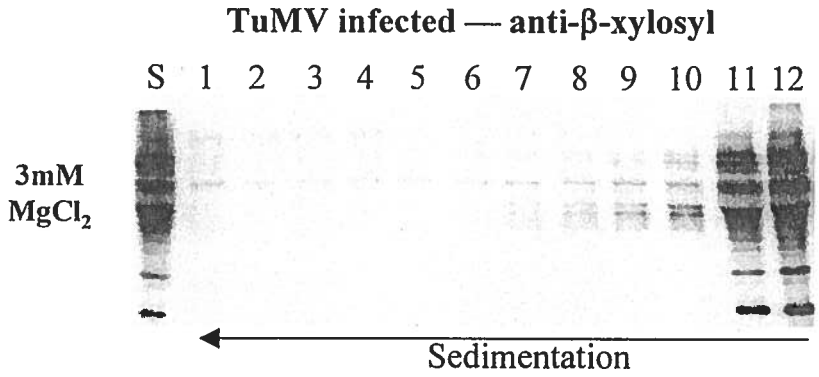
TuMV infected — anti-β-xylosyl
S 1 2 3 4 5 6 7 8 9 10 11 12
3mM
MgCl2
Sedimentation

Fig. 2b

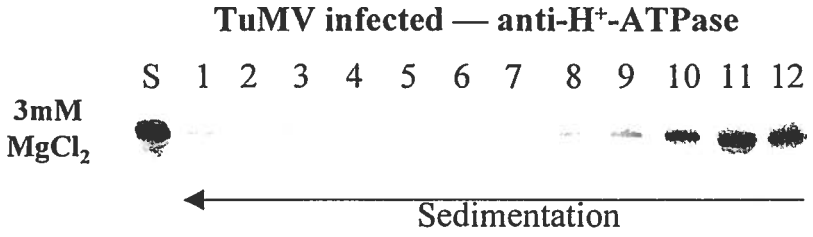
TuMV infected — anti-H+-ATPase
S 1 2 3 4 5 6 7 8 9 10 11 12
3mM
MgCl2
Sedimentation

Fig. 2c

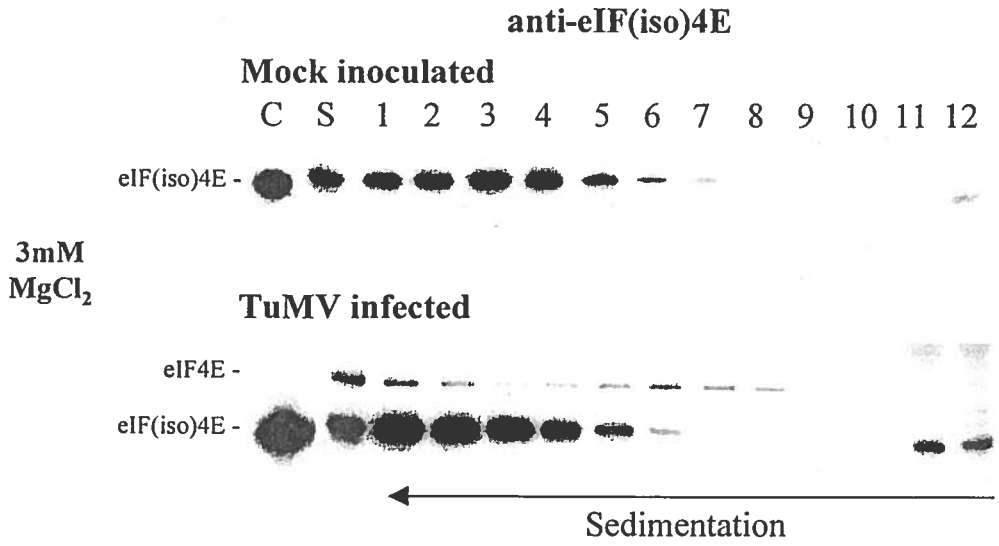
anti-eIF(iso)4E
Mock inoculated
C S 1 2 3 4 5 6 7 8 9 10 11 12
eIF(iso)4E -
3mM
MgCl2
TuMV infected
eIF4E -
eIF(iso)4E -
Sedimentation

Fig. 2d

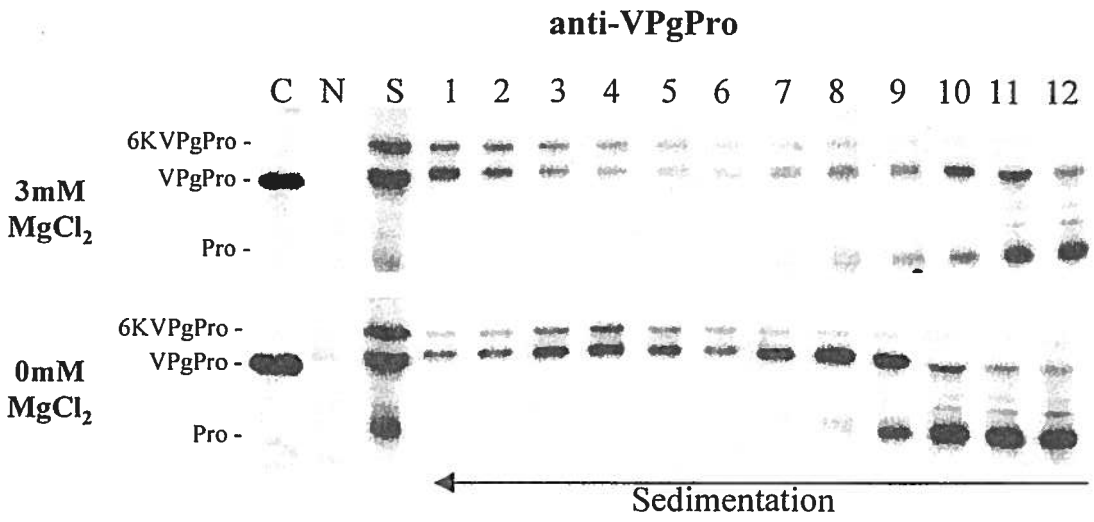
anti-VPgPro
C N S 1 2 3 4 5 6 7 8 9 10 11 12
3mM MgCl2
6KVPgPro -
VPgPro -
Pro -
0mM MgCl2
6KVPgPro -
VPgPro -
Pro -
Sedimentation

Fig. 2e

Fig. 3 : Co-purification of 6KVPgPro/VPgPro with eIF(iso)4E and eIF4E by metal chelation chromatography. Fractions 1 to 5 from a sucrose gradient centrifugation experiment were pooled, membranes solubilized by the addition of 0.5% Tween-20 and loaded onto a column containing 0.4 ml of nickel-agarose resin. Proteins were eluted with 100 mM imidazole, separated by SDS-PAGE and electrobotted to nitrocellulose. The membrane was probed using **(a)** anti-VPgPro serum and **(b)** anti-eIF(iso)4E serum. Wells were loaded with proteins derived from membrane fractions of mock inoculated tissue, proteins derived from membrane fractions of TuMV infected tissue, eluted proteins from mock inoculated tissue and eluted proteins from TuMV infected tissue. Gel migration position of the different proteins is indicated to the left.

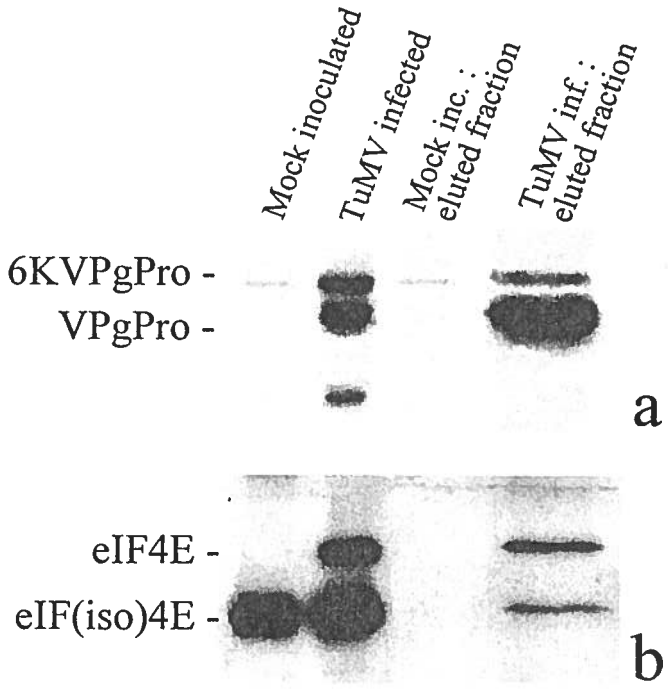
Mock inoculated
TuMV infected
Mock inc.: eluted fraction
TuMV inf.: eluted fraction
6KVPgPro -
VPgPro -
a
eIF4E -
eIF(iso)4E -
b

Fig. 3

Fig. 4 : Co-purification of PABP2 by metal chelation chromatography. Experimental approach was as in Fig. 3. The membrane was probed using anti-PABP2 serum. Wells were loaded with proteins derived from membrane fraction of mock inoculated tissue, proteins derived from membrane fraction of TuMV infected tissue, eluted proteins from mock inoculated tissue and eluted proteins from TuMV infected tissue. Gel migration position of PABP2 is indicated to the left.

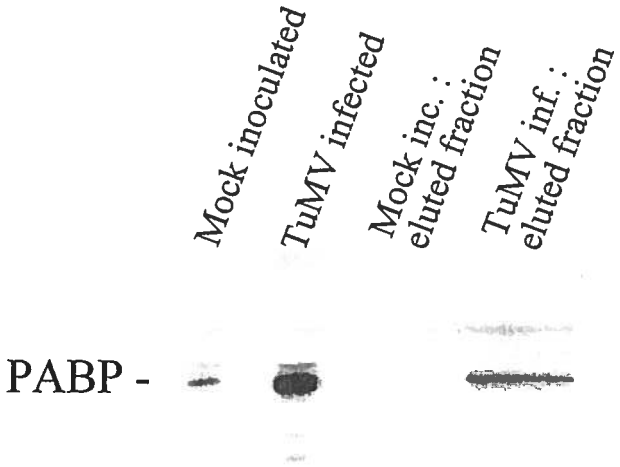
Mock inoculated
TuMV infected
Mock inc. : eluted fraction
TuMV inf. : eluted fraction
PABP -

Fig. 4

Fig. 5 : VPgPro interaction with PABP of *A. thaliana* as demonstrated by ELISA-based binding assay. (**a**) Wells were coated with 1.0 µg of purified VPgPro (●), metal chelation-purified *E.coli* lysate containing pET21b vector (■) or with Blotto only (▲) and then incubated with increasing concentration of purified T7-tagged PAPB His-tailed. Retention of complex was detected using anti-T7 tag antibodies. (**b**) Wells were coated with 1.0 µg of metal chelation-purified *E.coli* lysate containing pET21b only (lanes 1 and 3) or purified VPgPro (lanes 2 and 4) and incubated with 50 µg of an *E. coli* lysate expressing T7 tagged PABP His-tailed (lanes 1 and 2) or expressing T7 tagged PABP (lanes 3 and 4). Retention of complex was detected using anti-T7 tag antibodies. Values are averages of four replicates from a typical experiment. Error bars represent the standard deviation.

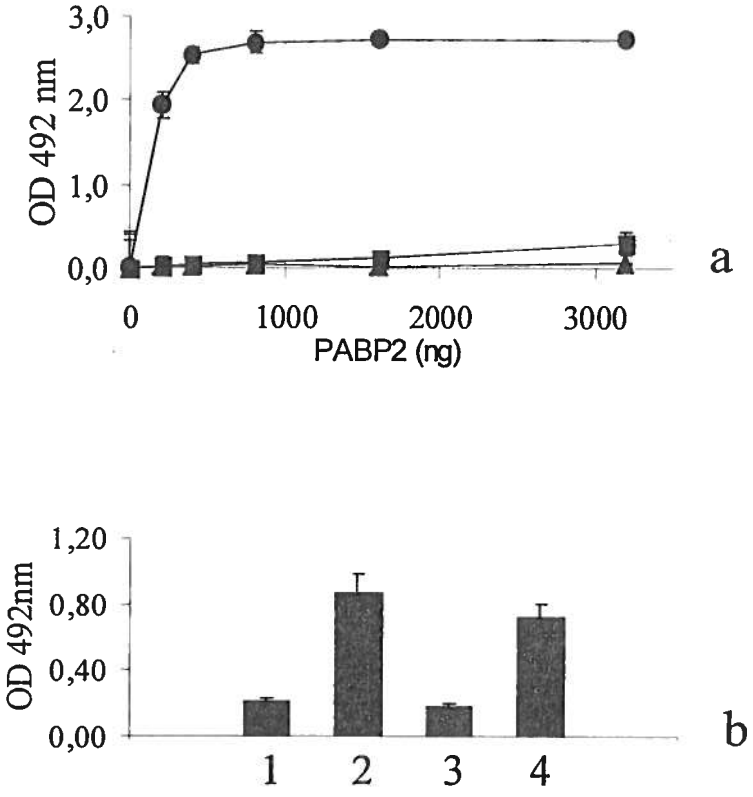
3,0
2,0
1,0
0,0
OD 492 nm
0
1000
2000
3000
PABP2 (ng)
a
1,20
0,80
0,40
0,00
OD 492nm
1
2
3
4
b

Fig. 5

Annexe II

Interaction of Turnip mosaic virus VPg protein with translation initiation factor eIF4F and inhibition of host protein synthesis during infection.

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Running title: VPg interaction with host cell translation

Summary = 194 words Figures = 7 total Text = 4998 words

Summary

Plant potyvirus RNAs have a virus protein covalently attached at their 5'-end: the VPg (virus protein genome-linked). In part because of its position at the 5'end of the virus RNA, the VPg has been speculated to play a role in virus translation and replication. We report that the VPg of *Turnip mosaic virus* (TuMV) interacts with the plant translation initiation factor eIF(iso)4F. VPg bound to the eIF(iso)4E-eIF(iso)4G complex without promoting its dissociation. The cap analogue m^7GTP competed with the binding of VPg to both eIF(iso)4E and eIF(iso)4F. This suggested a role for the VPg in initiating translation of the virus. Plant protoplasts were transfected with an infectious clone of TuMV to examine the impact of virus infection on host protein synthesis. Expression of the virus genome led to a transient inhibition of protein synthesis. On the other hand, a cloned copy of the virus carrying a mutant VPg unable to bind eIF(iso)4E *in vitro* did not inhibit protein synthesis when used to transfect plant cells. These results suggest that VPg may play a regulatory role in modulating host protein synthesis to favor virus genome translation over host cell mRNA translation.

Introduction

The potyvirus group, of which *Turnip mosaic virus* (TuMV) is a member, has the unique feature of a virus protein (VPg) covalently linked to the 5' end of the virus genome that consists of a single RNA molecule of positive polarity (Riechmann *et al.*, 1992; Siaw *et al.*, 1985). This RNA molecule features only one open reading frame encoding a large polyprotein, which is autoproteolytically cleaved to yield at least 10 functional viral products. Large amounts of virus protein accumulate in the nucleus of infected plant cells where they form crystalline structures called nuclear inclusions (Riechmann *et al.*, 1992; Riedel *et al.*, 1998). One is the so called Nuclear Inclusion a protein (NIa or VPgPro) which is formed of two domains: the N-terminal portion consists of the VPg; the C-terminal portion is a proteinase with homology to the picornaviral 3C protease (Schaad *et al.*, 1996).

Although the role of VPg during infection remains unknown, interactions with other proteins suggest potential functions. The most documented of these interactions occurs with the viral RNA-dependent RNA-polymerase (Hong *et al.*, 1995; Li *et al.*, 1997). Fellers *et al.* (1998) also demonstrated that both VPg and its precursor form VPgPro had a stimulatory effect on the activity of the polymerase, which raises the possibility that VPg participates in virus replication. Other evidence for involvement in replication comes from the observation that the protein has non-specific RNA-binding activity (Daros and Carrington, 1997; Merits *et al.*, 1998). Finally, several groups have identified VPgPro as a virulence determinant for host infection (Johansen *et al.*, 1996; Keller *et al.*, 1998; Nicolas *et al.*, 1997; Chu *et al.*, 1997).

We have previously reported an interaction between VPg and the *Arabidopsis thaliana* eukaryotic translation initiation factor (iso)4E [eIF(iso)4E] (Witmann *et al.*, 1997). The interaction was identified using the yeast two-hybrid system and was confirmed by an in vitro binding assay using purified proteins. A clone of the virus carrying a mutant VPg unable to bind eIF(iso)4E did not produce symptoms after inoculation onto plants (Léonard et al., 2000). It was also shown recently that eIF(iso)4E was essential for virus infection using *Arabidopsis* mutants that lack eIF(iso)4E; mutants with mutations at the

eIF(iso)4E locus showed increased resistance to TuMV infection (Lellis *et al.*, 2002). This supports the hypothesis for a central role *in planta* for eIF(iso)4E in virus multiplication. The binding of eIF(iso)4E by a plant virus protein suggests possible interference with the host translation apparatus and also a mechanism for the virus to efficiently recruit the host components required for translation of cellular mRNAs.

Translation initiation is a conserved mechanism among eukaryotes; the first step is the recruitment of the translation apparatus to the 5' end of the mRNA via the interaction of eIF4F with the m^7GpppN residue at the 5' end of polymerase II-transcribed mRNAs. In mammals, this factor is a complex of three different proteins: eIF4G, eIF4E and eIF4A. eIF4E is the 26 kDa protein that directly binds the mRNA cap structure (Bushell and Sarnow, 2002). It appears to be the least abundant of the eIF4F proteins and for this reason is thought to be a key factor for the regulation of translation rates. For instance, eIF4E plays a role in progression through the cell cycle and cellular proliferation (Altmann and Trachsel, 1989; De Benedetti and Rhoads, 1990; Lazaris-Karatzas *et al.*, 1990). eIF4G serves as the backbone of the complex and also has RNA binding activity. The N-terminal portion of eIF4G binds eIF4E, and the middle and C-terminal thirds of eIF4G each have a binding site for eIF4A. eIF4A is an RNA helicase which melts the secondary structure of mRNAs prior to (or during) translation. In plants, eIF4F contains 2 subunits: eIF4E and eIF4G. Plants also have a second isoform of eIF4F (Browning *et al.*, 1987; Browning *et al.*, 1992; Browning, 1996): this second isoform [eIF(iso)4F] is composed of two proteins: the 28 kDa eIF(iso)4E and the 86 kDa eIF(iso)4G. eIF(iso)4F is a functional homolog of eIF4F but the two isoforms are antigenically distinct (Browning *et al.*, 1992). They also have different expression patterns: eIF4E mRNA is expressed in all tissues except the root tips, whereas eIF(iso)4E mRNA is particularly abundant in flowers and young developing organs (Rodriguez *et al.*, 1998).

In order to achieve translation of their genomes in cells they infect, some picornaviruses, namely poliovirus, coxsackievirus and rhinovirus, have been known to induce cleavage of eIF4G which results in host translation shutdown. Thus, upon cleavage by picornaviruses, the N-terminal third of eIF4G (containing the eIF4E binding

site) is released from eIF4F which then becomes unable to recruit capped mRNAs; the rest of eIF4F remains functional for cap-independent internal ribosome entry site-driven virus translation (Bushell and Sarnow, 2002). The result is a general shutdown of host mRNA translation leaving only virus RNA to be translated.

Given the generalized interference of picornaviruses with host translation and the fact that TuMV is a member of that family, and given the interaction between TuMV-VPg and eIF(iso)4E, we hypothesized that plant virus VPg could interfere in some fashion with host translation. In this report, we show that VPg interacted not only with eIF(iso)4E but also with eIF(iso)4F. Presence of the m^7GTP cap analogue prevented formation of the complexes. We also show that TuMV infection inhibited host protein synthesis and provide evidence that this translation inhibition is related to the interaction of VPg with eIF(iso)4E. These observations suggest a role for VPg in promoting virus translation at the expense of host mRNA translation.

Methods

Plasmids and bacterial strains

Plasmids pET-eIF(iso)4G, encoding the wheat eIF(iso)4G (van Heerden and Browning, 1994), and pET-eIF(iso)4E, encoding wheat eIF(iso)4E (Wittmann *et al.*, 1997), were obtained from Dr. K. Browning (University of Texas at Austin, USA). Plasmid pET-NIa/24, encoding TuMV VPgPro, has been described in Léonard *et al.* (2000). All plasmids were transformed into the *E. coli* strain BL21(DE3) (F^- *ompT* *hsd*S_B ($r_B^-m_B^-$) *gal dcm* (DE3); Invitrogen Inc., USA) for T7-based expression and purification of the recombinant proteins. Plasmid pMON-GUS was constructed by subcloning the XbaI/EcoRI fragment of pBI121 (Clonetech Inc., USA) into the corresponding sites of pMON999 (Monsanto Inc., USA) which contains an enhanced 35S promoter followed by a multiple cloning site and a nopaline synthase terminator.

Purification of eIF(iso)4G.

The eIF(iso)4G protein was purified according to van Heerden and Browning (1994) with minor modifications. A freshly streaked single colony of pET-eIF(iso)4G in *E. coli* BL21(DE3) was grown overnight at 37°C in LB ampicilin (1% yeast extract, 0.5% tryptone, 1% NaCl, 75 □g/ml ampicilin). 50 ul of this overnight culture was transferred to 50 ml of prewarmed fresh LB ampicilin and grown to OD_{600}= 0.8. The cells were collected by centrifugation at 6,000 g for 10 min at 4□C and suspended in 50 ml of fresh LB ampicilin. This culture was used to inoculate five 1-liter flasks, each containing 250 ml of prewarmed LB ampicilin and grown at 37 °C with agitation until OD_{600}= 0.6. The cells were then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated at 30°C with agitation for an additional 3 hours. The cells were collected by centrifugation and stored at -70 °C.

For protein purification, the pellet was thawed on ice and resuspended in 9 ml of ice-cold buffer B600 (20 mM HEPES pH 7.6, 0.1 mM EDTA, 10% glycerol, 600 mM KCl) supplemented with 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 100 ug/ml of soybean trypsin inhibitor. This suspension was sonicated and cell debris was collected by centrifugation at 13,000 g at 4°C for 30 min. The supernatant was decanted into a fresh

centrifuge tube and the ribosomal fraction was pelleted at 100,000 g at 4°C for 30 min. The supernatant (S175) was poured into a clean tube and sufficient buffer B0 (20 mM HEPESs pH 7.6, 0.1 mM EDTA, 10% glycerol) was added to bring the final KCl concentration to 80 mM. The resulting solution was applied to a Whatman P11 phosphocellulose resin at a flow rate of 400 ul/min. The column was washed for two hours with buffer B100 (20 mM HEPES pH 7.6, 0.1 mM EDTA, 10% glycerol, 100 mM KCl) at the same flow rate followed by elution with a linear gradient of 100-250 mM KCl. The purified protein was analyzed by SDS-PAGE, quantified by a standard Bradford assay (BioRad, USA), and stored in small aliquots at -70°C.

Purification of eIF(iso)4E.

A freshly streaked colony of *E. coli* BL21(DE3) harboring pET21a/eIF(iso)4E was inoculated into 25 ml of LB ampicillin and grown overnight. 2.5 ml of that culture were used to inoculate four 250 ml flasks of fresh LB ampicillin and grown at 37°C until OD_{600} = 0.4-0.5; cells where induced by addition of IPTG to a final concentration of 0.4 mM. The culture was incubated at 25°C for an additional 2.5 hours. The cultures were then centrifuged at 5,000 g and the drained pellets were kept at -70°C.

All purification steps for eIF(iso)4E were performed at 4°C. The pellet from 1 l of culture was thawed and resuspended in 4 ml of buffer B500 (20 mM HEPES pH 7.6, 0.1 mM EDTA, 10% glycerol, 500 mM KCl) supplemented with 1 mM dithiothreitol (DTT). The cells were disrupted by three passages through a French pressure cell set at 16,000 kPa; cell debris was removed by two centrifugations at 30,000 g for 15 min. PMSF was added to the supernatant at a final concentration of 0.5 mM after the first round of centrifugation. Following the second centrifugation, the supernatant was diluted 5-fold with buffer B0 (20 mM HEPES pH 7.6, 0.1 mM EDTA, 10% glycerol), supplemented with DTT at a final concentration of 1 mM and loaded onto 1 ml of m^7GTP-Sepharose 4B (Pharmacia Biotech) previously equilibrated in buffer B100 (20 mM HEPES pH 7.6, 0.1 mM EDTA, 10% glycerol, 100 mM KCl). The resin was then washed with 50 ml of buffer B100 supplemented with 1 mM DTT. The bound proteins were eluted with 2 ml of 0.1 mM m^7GTP in buffer B100 supplemented with 1 mM DTT. The eluted protein

was analyzed by SDS-PAGE, quantified with a Bradford assay (BioRad) and stored at -70°C.

Expression and purification of VPgPro.

Plasmid pET-NIa/24 (Laliberte *et al.*, 1992) was introduced into *E. coli* BL21 (DE3) and protein expression was induced as described above. The cell pellet from 1 liter of induced *E. coli* was resuspended in 5 ml of buffer A (100 mM Tris-Cl pH 8.0, 300 mM NaCl, 10 mM 2-mercaptoethanol, 0.01% Nonidet P-40). After sonication, the cell lysate was centrifuged for 30 min at 10,000 g at 4°C, transferred to a fresh tube and stored at -20°C. The concentration of VPgPro in the lysate was estimated by Western blot analysis of serial dilutions and comparison with purified VPgPro samples of known concentration.

SDS-PAGE and Western blots.

Electrophoresis of proteins in denaturing polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed essentially as described in Ausubel *et al.* (1989). For Western blot analysis, proteins were transferred to nitrocellulose. For chromogenic detection, the membrane was washed as above and developed in the dark in 10 ml of development buffer (0.1M Tris-Cl pH9.5, 0.5 mM $MgCl_2$) supplemented with 15 mg/ml of 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt and 30 mg/ml of p-nitro blue tetrazolium chloride. For chemiluminescent detection, the membrane was washed as above, incubated 5 min with 1 ml of Immun-star substrate with enhancer (BioRad), drained, wrapped in Saran-Wrap and exposed to Kodak BioMax MR film.

ELISA assays.

Enzyme-linked immunosorbent assays (ELISAs) were performed as described in Wittmann *et al.* (1997) with some modifications. The first protein was diluted in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4) and adsorbed onto the ELISA plate wells. The wells were washed three times with PBST (0.05% Tween 20 in PBS) and blocked with PBS/MILK (5% non-fat dry milk in PBS). The second (and third, if required) proteins were diluted in PBSMT (1% non-fat dry milk, 0.2% Tween 20 in PBS) and incubated in the wells for 90 min at 4°C. The wells were washed again three times with PBST and incubated with the appropriate primary

antibody diluted in conjugate buffer (0.2% non-fat dry milk, 2% polyvinyl pyrolidone in PBST). The eIF(iso)4E protein was detected using its T7 tag and a monoclonal anti-T7 tag antibody (Novagen) at a 1:10,000 dilution. The eIF(iso)4G protein was detected with a rabbit polyclonal anti-wheat eIF(iso)4G serum (kindly provided by Dr. K. Browning) at a 1:7500 dilution. After overnight incubation at 4°C, the wells were washed with PBST and incubated with monoclonal goat anti-mouse IgG antibodies coupled to alkaline phosphatase at a 1:5000 dilution in conjugate buffer. The wells were then washed with PBST and filled with PNP buffer (9.7% diethanolamine-Cl pH 9.8) containing 1 mg/ml of p-nitrophenyl phosphate.

Co-immunoprecipitation

Proteins (2□g VPgPro, 0.5ug eIF(iso)4E and 1.5ug eIF(iso)4G) were mixed in a final volume of 100 ul of buffer B100 (20 mM HEPES pH 7.6, 0.1 mM EDTA, 10% glycerol, 100 mM KCl) supplemented with 1 mM DTT, 0.5 mM PMSF and 0.1 mg/ml soybean trypsin inhibitor and incubated at 4□C for 60 min. Then 0.2 □g of purified mouse anti-tetra-his monoclonal antibodies (Qiagen, USA) were added and incubated for two hours at 4°C. The protein-antibody complexes were recovered by addition of 100 ul of agarose-protein A (Pharmacia Biotech) and further incubated at 4□C for two hours. The agarose suspension was then transferred to a chromatography micro-column and washed with 35 ml of buffer B100 supplemented with 1 mM DTT at 4°C. The washed agarose was recovered from the column and resuspended in 30 ul of SDS-PAGE running buffer for Western blot analysis.

Protoplast transfection

Nicotiana tabacum var. White Burley protoplasts were prepared essentially as described by Abel and Theologis (1994). Leaves from 8-12 week old plants were harvested, rinsed 15 min under tap water and surface-sterilised with 70% ethanol. Sliced leaves were pre-plasmolysed for 60 min in 0.55 M mannitol and incubated overnight in enzyme solution (1% cellulase Onozuka R-10, 0.25% macerozyme Onozuka R-10, 0.55 M mannitol, 8 mM $CaCl_2$, 0.1% MES pH 5.8). The resulting protoplast suspension was filtered through an 88 um sieve, layered onto 0.55 M sucrose and centrifuged for 5

min at 30 *g*. The green band at the interphase was collected, swirled into 0.55 M mannitol and centrifuged for 5 min at 70 *g*. The pellet was gently resuspended in W5 (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, 0.1% MES pH 5.8) and centrifuged as previously. The pellet was resuspended in fresh W5 and cell density was determined. Protoplasts were kept on ice for 45 min before proceeding to transfection. Protoplasts were transfected according to the procedure described by Negrutiu *et al.* (1987). The protoplasts were then washed with 5 ml W5, resuspended in MS at a final density of 100,000-150,000 protoplasts/ml and kept in sealed petri dishes at room temperature in the dark. For *in vivo* labeling, aliquots corresponding to 20,000 protoplasts were collected and incubated for 45 min in 200 ul of MS supplemented with [^{35}S]-methionine (Amersham) at a final concentration of 50 uCi/ml. Protoplasts were washed twice in ice-cold W5, resuspended in 10 □l of 10% SDS and kept at -70°C. TCA precipitation of radiolabelled proteins and scintillation counting were performed as described (Mans and Novelli, 1961). All labelings were done in triplicate. *In vivo* GUS staining was performed at 48 hours post-transformation, as described in VanBokhoven *et al.* (1993).

Results

Purification of recombinant VPg, eIF(iso)4E and eIF(iso)4G

The interaction of VPg with eIF(iso)4E (Wittman *et al.*, 1997; Léonard *et al.*, 2000) suggests that the viral protein could act as a negative regulator of host mRNA translation. This could happen in two different ways. VPg could hamper translation by disrupting or preventing the formation of eIF(iso)4F complexes. This hypothesis assumes that eIF(iso)4G and VPg binding to eIF(iso)4E are mutually antagonistic. On the other hand, VPg could bind to eIF(iso)4F but prevent binding of the host mRNA to eIF(iso)4E. This would imply that VPg and eIF(iso)4G binding to eIF(iso)4E are not antagonistic.

To address these hypotheses, we conducted *in vitro* experiments to determine if VPg could bind the eIF(iso)4F complex or if it would promote its dissociation. Recombinant VPgPro (including a C-terminal histidine tail), a precursor form of VPg, was used instead of recombinant VPg because the former was more readily produced in recombinant *E. coli (*Ménard *et al.*, 1995). Wittmann *et al.* (1997) showed that the Pro domain had no effect on the interaction of VPg with eIF(iso)4E. Purification of recombinant wheat eIF(iso)4G was performed by cation exchange chromatography (van Heerden and Browning, 1994) (Figure 1). Wheat eIF(iso)4G was used as we have previously shown that VPg interacts equally well with wheat and Arabidopsis factors (Léonard *et al.*, 2000). Recombinant wheat eIF(iso)4E (with a T7 tag fused at the N terminus) was affinity-purified on a m^7GTP-Sepharose resin. Protein identity was verified using antibodies specific to each protein (Figure 1B).

In vitro co-immunoprecipitation

Co-immunoprecipitation experiments were used to test for the formation of a complex between VPgPro, eIF(iso)4E, and eIF(iso)4G. Recombinant VPgPro (2 □g), eIF(iso)4E (0.5 □g) and eIF(iso)4G (1.5 □g) were added together in phosphate buffer. After a one-hour incubation at 4□C, mouse monoclonal antibodies specific for the 6x His tag of recombinant VPgPro were added and the mixture was left to incubate at 4□C for two more hours (only recombinant VPgPro had a 6x His-tagged C-terminus). The antibody-protein complexes were precipitated with agarose-protein A and analyzed by Western blot using a mixture of antibodies recognizing VPgPro, eIF(iso)4E and eIF(iso)4G. Control experiments showed the absence of cross-reactivity between the antibodies used and either eIF(iso)4E or eIF(iso)4G. Figure 2 (lane 4) shows that all three proteins were immuno-precipitated from solution even though the antibodies used were specific for the His-tag of VPgPro. When VPgPro was omitted from the initial mixture, only traces of eIF(iso)4E and eIF(iso)4G were detected (lane 5). These results showed that VPgPro can form an *in vitro* protein complex containing eIF(iso)4G and eIF(iso)4E.

ELISA-based binding assays

In order to confirm the results obtained using co-immunoprecipitation assays and to obtain quantitative data on the interaction of VPg with the eIF(iso)4F complex, binding experiments were performed using a modified ELISA procedure. The reliability of the ELISA used to study tripartite interactions was tested by demonstrating that we could detect two previously published interactions: between eIF(iso)4E and VPgPro (Wittmann *et al.*,1997) and between eIF(iso)4E and eIF(iso)4G (Browning *et al.*,1987). To test the VPgPro-eIF(iso)4E interaction, 1 □g of recombinant viral protein was coated onto the wells of an ELISA plate, followed by addition of a gradient of 0 to 2.5 □g of purified recombinant eIF(iso)4E. Retention of eIF(iso)4E in the wells was detected with anti-T7 tag monoclonal antibodies. As shown in Figure 3a, up to 1 □g of eIF(iso)4E was efficiently retained in the wells. Addition of 2.5 times more eIF(iso)4E resulted in only a

marginal increase of OD_{405}. To confirm that the capture of eIF(iso)4E in the wells was due to a specific interaction with VPgPro, an eIF(iso)4E gradient was applied to wells not coated with VPgPro. In these wells, only minimal retention of eIF(iso)4E was observed (Figure 3a) which confirmed the specificity of the VPgPro-eIF(iso)4E interaction. To test the interaction between eIF(iso)4E and eIF(iso)4G, purified recombinant eIF(iso)4E was coated onto microtitre plate wells, followed by incubation with a gradient of 0 to 1 □g of purified recombinant eIF(iso)4G. The capture of eIF(iso)4G in the wells was detected with anti-eIF(iso)4G rabbit polyclonal antibodies. When an eIF(iso)4G gradient was applied to the wells (Figure 3b), absorbance increased rapidly at first and reached a plateau with the addition of 300 ng of eIF(iso)4G. Addition of more eIF(iso)4G resulted in only a marginal increase in absorbance. To verify that the capture of eIF(iso)4G into the wells was due to a specific interaction with eIF(iso)4E, an eIF(iso)4G gradient was applied to wells not coated with eIF(iso)4E (Figure 3b). In these wells, a constant background was observed, regardless of the amount of eIF(iso)4G added. This background was due to unspecific binding of the polyclonal antibody used as it was also observed even when no eIF(iso)4G was added to the wells (data not shown).

Interaction of VPgPro with eIF(iso)4F

We investigated the formation of the tripartite complex between VPgPro, eIF(iso)4E and eIF(iso)4G in the ELISA-based binding assay. We first tested the possibility that VPgPro could directly interact with eIF(iso)4G. Two micrograms of VPgPro were coated onto ELISA plate wells and a gradient of 0 to 0.5 μg of eIF(iso)4G was added to the wells. No significant binding was observed for all eIF(iso)4G concentrations (Figure 4). To test whether complex formation of VPg with eIF(iso)4G is mediated by eIF(iso)4E, 2 μg of VPgPro were coated onto the plate wells and 2 μg of eIF(iso)4E were added to all wells along with a gradient of eIF(iso)4G ranging from 0 to 0.5 μg. The capture of eIF(iso)4G was detected with a rabbit polyclonal anti-eIF(iso)4G serum. As shown in Figure 4, the absorbance raised rapidly with the addition of increasing amounts

of eIF(iso)4G up to 0.1 μg, then reached a plateau at 0.5 μg of eIF(iso)4G. When eIF(iso)4G was omitted, the background caused by the polyclonal antibodies was found to be constant. Presence of eIF(iso)4E is thus necessary for the retention of eIF(iso)4G onto VPgPro-coated wells.

To test the interaction of virus VPg with the translation initiation factor (iso)4F, the fate of the VPgPro-eIF(iso)4E complex formation following addition of eIF(iso)4G was investigated. Two micrograms of VPgPro were coated onto the plate wells and then 2 μg of eIF(iso)4E were added to all wells along with a gradient of eIF(iso)4G ranging from 0 to 0.5 μg. Retention of eIF(iso)4E was measured with mouse monoclonal anti-T7 tag antibodies, whereas eIF(iso)4G was monitored using rabbit polyclonal anti-eIF(iso)4G antibodies. As shown in Figure 4, increasing amounts of eIF(iso)4G were found in the wells, and reached a plateau after addition of 100 ng of eIF(iso)4G. The background level is indicated in the wells where no eIF(iso)4G was added. The addition of small amounts of eIF(iso)4G (less than 100 ng) initially led to a displacement of up to 25% of eIF(iso)4E from VPgPro. Taken together, these experiments demonstrated that VPgPro formed a complex with eIF(iso)4E and eIF(iso)4G *in vitro* and that the VPgPro binding to eIF(iso)4F was mediated by the interaction with eIF(iso)4E.

eIF(iso)4E binds the cap analogue m^7GTP and we have previously shown that m^7GTP (but not GTP) inhibits formation of the VPg-eIF(iso)4E complex (Léonard et al., 2000). To test whether the presence of eIF(iso)4G could alter the inhibition of m^7GTP on VPg-eIF(iso)4F complex formation, ELISA wells were coated with VPgPro and incubated with eIF(iso)4E only or with eIF(iso)4E and eIF(iso)4G in the presence of increasing concentrations of m^7GTP. Figure 5 shows that inhibition of VPg-eIF(iso)4E complex formation by m^7GTP occurred whether eIF(iso)4G was present or not. These results indicated that the presence of eIF(iso)4G did not affect the binding of eIF(iso)4E to VPg.

TuMV transfections in plant protoplasts

The above results suggested that VPg could affect cellular translation by interfering with mRNA binding to eIF(iso)4E. Interference with the formation of the translation initiation complex could lead to a reduction in the amount of cap-dependent protein

synthesis. This inhibition of translation would be reflected in a decreased rate of incorporation of [^{35}S]-methionine into proteins when VPg is expressed in plant cells. Attempts to show inhibition of *in vitro* translation in wheat germ lysate by VPg were inconclusive since KCl concentrations required for recombinant VPgPro solubility were incompatible with those required for protein synthesis. To test the effect of TuMV infection on protein synthesis in host cells, protoplasts from *N. tabacum* were transfected with p35Tunos, which contains an infectious DNA copy of TuMV under the control of the *Cauliflower mosaic virus* 35S promoter (Sanchez *et al.*, 1998). As a control, protoplasts were transfected with pMON-GUS, which contains the coding region for ß-glucuronidase also under the control of the 35S promoter. At selected time points, protoplasts were incubated with [^{35}S]-methionine and collected after 45 minutes to determine the amount of radioactivity incorporated into polypeptides. We observed a reduction in protein synthesis of as much as 30% in treated protoplasts (Figure 6a). However, this data is an underestimate of the extent of translation inhibition in cells since only a subset of the protoplasts in the samples were actually transfected. Since it was not possible to determine the proportion of transfected protoplasts in each sample, we estimated the proportion of transfected cells by transfecting in parallel with pMON-GUS, a plasmid that expresses β-glucuronidase. Staining and microscopic examination of these protoplasts revealed that 30% to 40% were transfected, as evidenced by their blue color. If this estimate of transfection efficiency is applied to p35Tunos-transfected protoplasts, the extent of protein synthesis inhibition is approximately 70%.

The role of the VPg-eIF(iso)4E interaction in host translation inhibition was determined using similar experiments with p35D77N, a TuMV cDNA clone derived from p35Tunos but with a Asp to Asn mutation in the VPg portion of VPgPro. This particular mutant was used since the VPg of p35D77N was previously showed to have no affinity for eIF(iso)4E *in vitro* (Léonard *et al.*, 2000). Plants transfected with the D77N mutant do not accumulate virus over a three-week period. When this mutant clone was used to transfect tobacco protoplasts, no inhibition of protein synthesis was observed (Figure 6b).

Viral products accumulated in cells to similar levels, whether they were transfected with either p35TuD77N or p35Tunos, as monitored by the amount of virus coat protein detected by Western blot analysis 48 hrs post-transfection (Figure 7), indicating normal translation and processing of the viral proteins with both cDNA clones. Additionally, the lower incorporation of methionine was not the result of differential expression of a few proteins; SDS-PAGE and autoradiography analysis showed that the entire protein profile was equally affected, with no single protein being reduced or enhanced in expression (data not shown). The subsequent recovery in methionine incorporation was also associated with a general recovery of translation rather than differential expression of a few protein species. This general decrease in protein expression profiles was also observed following infection of whole leaves (results not shown).

Discussion

We have investigated the interaction between the TuMV VPgPro and eIF(iso)4F. VPgPro was previously shown to bind to eIF(iso)4E, a subunit of eIF(iso)4F that is also composed of eIF(iso)4G. Investigation of the fate of the eIF(iso)4F multi-subunit complex in the presence of VPgPro was performed using two different *in vitro* assays. Two potential outcomes were predicted: either 1) VPgPro joins the two subunits of eIF(iso)4F to form a tripartite complex, or 2) VPgPro promotes the dissociation of the complex or prevents its formation. These two different outcomes allow predictions on how the virus is translated in the host cell: either the VPg-eIF(iso)4E promotes translation initiation of the virus RNA, or it inhibits translation initiation of host cell mRNAs.

To test these hypotheses, eIF(iso)4E, eIF(iso)4G and VPgPro recombinant proteins were prepared and used in ELISA-based binding assays. We found that eIF(iso)4G could be efficiently captured in wells previously coated with VPgPro when added simultaneously with eIF(iso)4E. When eIF(iso)4E was omitted, little or no eIF(iso)4G was captured, indicating the absence of direct interaction between VPgPro and eIF(iso)4G. Similar observations were made using co-immunoprecipitation assays. In these experiments, the three proteins were mixed and could be co-immunoprecipitated from solution with rabbit polyclonal antibodies directed against only one of the three protein, VPgPro. These results indicate that the three proteins can bind together to form a tripartite complex *in vitro* in which eIF(iso)4E acts as a bridge between the other two proteins. Taken together, these observations indicate that VPgPro can bind to eIF(iso)4E even if the latter is complexed with eIF(iso)4G.

Léonard *et al.* (2000) reported that interaction of VPgPro with eIF(iso)4E was inhibited by addition of the cap analogue m^7GTP; no effect was observed after addition of GTP. These observations indicate that the virus VPg protein binds to the translation initiation complex and suggest two modes of action: the VPg could interfere (competitively) with binding of eIF4F to capped host mRNAs, or the VPg could

sequester eIF4F complexes and reduce their availability for translation initiation. We report here that the presence of eIF(iso)4G, in addition to eIF(iso)4E, does not alter the ability of VPgPro to compete with cap analogues for binding to eIF(iso)4E. This reinforces the hypothesis that a complete translation initiation complex could form around the VPg protein attached to the virus RNA, and that complex formation would compete for translation with host mRNAs.

These observations suggest that the VPgPro protein in infected plant cells could act as a negative regulator of cap-dependent translation initiation, and therefore lead to reduced translation of host cell mRNAs. Wang and Maule (1995) have previously observed a significant reduction in the amount of protein expressed in pea embryos infected with pea seed-borne mosaic virus; only a few stress-related proteins were not observed to be down-regulated during virus infection. We have shown that tobacco protoplasts transfected with an infectious clone of TuMV (p35Tunos) exhibited a significant but transient reduction in protein synthesis as measured by ^{35}S labelling, similar to that observed by Wang and Maule (1995).

These results support previous observations by Wang and Maule (1995) and Aranda *et al* (1996) on a few measured proteins and extend their observations to protein synthesis in general. We investigated the role of VPgPro in this phenomenon by using a TuMV cDNA clone harboring a point mutation (D77N) in the VPgPro coding sequence which abolished its interaction with eIF(iso)4E. Protoplasts transfected with the D77N mutant TuMV clone showed no detectable reduction in protein synthesis. We can not rule out that undetected pleiotropic effects of the D77N mutation may be responsible for the absence of protein synthesis inhibition. However, taken together, these observations suggest a role for the VPgPro binding to eIF(iso)4E in protein synthesis inhibition. This hypothesis is further supported by recent observations that *Arabidopsis* mutants lacking eIF(iso)4E are resistant to TuMV infection (Lellis *et al*, 2002), pointing at the essential role of eIF(iso)4E for virus infection.

In conclusion, we have shown that VPgPro can bind to eIF(iso)4F. We have also shown that TuMV induces inhibition of protein synthesis and that VPgPro is likely

involved in this phenomenon since a mutation that abolished interaction with eIF(iso)4E also abolished host translation inhibition.

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Figure legends

Figure 1. Purification of recombinant proteins. A) Coomassie blue staining of purified recombinant proteins run on 12.5% SDS-PAGE. B) Western blot analysis of purified recombinant proteins probed with mouse monoclonal anti-T7 tag and rabbit polyclonal anti-eIF(iso)4G antibodies. Lane 1: purified wheat eIF(iso)4E; lane 2: purified wheat eIF(iso)4G.

Figure 1

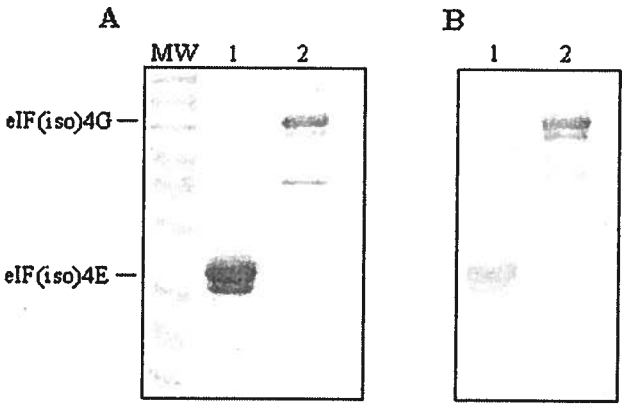


Figure 2. Co-immunoprecipitation of VPgPro, eIF(iso)4E and eIF(iso)4G. Proteins were mixed in phosphate buffer and immunoprecipitated with mouse monoclonal antibodies directed against the histidine tag of the recombinant VPgPro. The immunoprecipitated proteins were analyzed by Western blot, simultaneously using rabbit polyclonal antibodies directed against VPgPro, eIF(iso)4E and eIF(iso)4G. Lanes 1, 2 and 3 were purified eIF(iso)4E, VPgPro and purified eIF(iso)4G, respectively. Lane 4: VPgPro, eIF(iso)4E and eIF(iso)4G were mixed together before immunoprecipitation. Lane 5: VPgPro was omitted from the protein mixture.

Figure 2

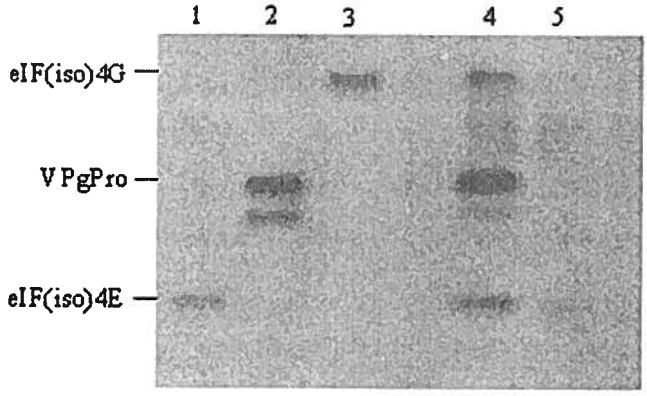
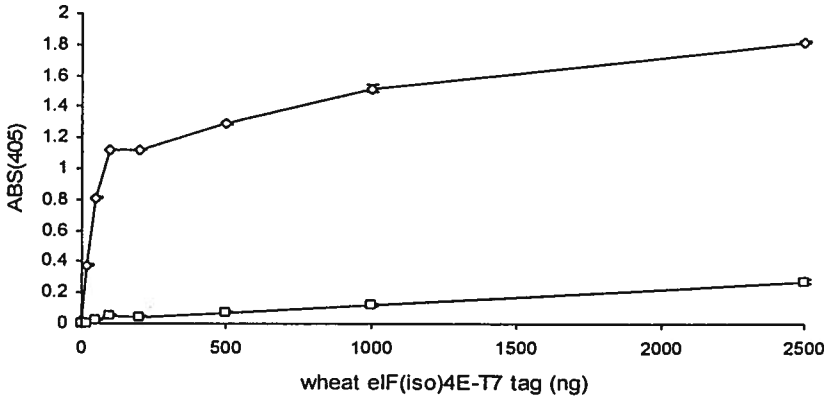


Figure 3. *In vitro* interaction between TuMV VPgPro, eIF(iso)4G and wheat eIF(iso)4E. (3A) A gradient of purified wheat eIF(iso)4E was applied to ELISA plate wells previously coated with 1 µg of VPgPro (◊). The same gradient of purified wheat eIF(iso)4E was applied to wells without pre-adsorption of VPgPro (□). The amount of eIF(iso)4E captured in the wells was measured with mouse monoclonal anti-T7 tag followed by colorimetric detection. (3B) *In vitro* interaction between wheat eIF(iso)4E and wheat eIF(iso)4G. A gradient of purified wheat eIF(iso)4G was applied to ELISA plate wells previously coated with 1 µg of wheat eIF(iso)4E (◊). The same gradient was also applied to wells without pre-adsorption of eIF(iso)4E (□). The amount of eIF(iso)4G captured in the wells was measured with rabbit polyclonal anti-eIF(iso)4G antibodies followed by colorimetric detection. Standard deviations (from two replicates) are indicated by error bars.


ABS(405)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
500
1000
1500
2000
2500
wheat eIF(iso)4E-T7 tag (ng)

B

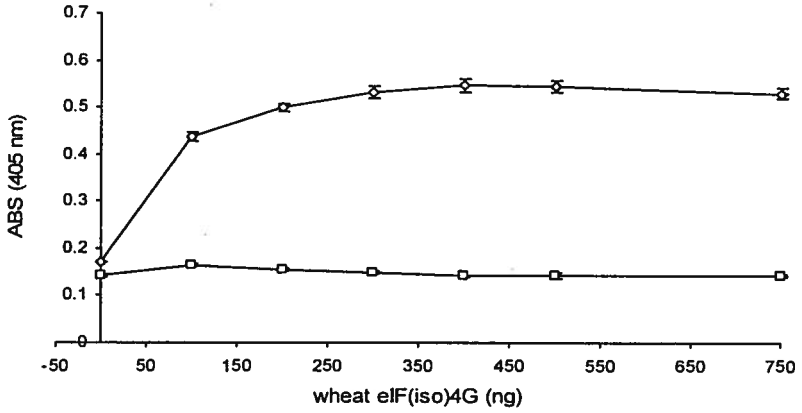

ABS (405 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-50
50
150
250
350
450
550
650
750
wheat eIF(iso)4G (ng)

Figure 4. Formation of a tripartite complex *in vitro* between VPgPro, eIF(iso)4E and eIF(iso)4G. ELISA plate wells were pre-adsorbed with VPgPro (2 μg). A constant amount of eIF(iso)4E was added along with a gradient of eIF(iso)4G. The amount of captured eIF(iso)4G was monitored (◊). The amount of retained eIF(iso)4G was measured (□) in wells that were pre-adsorbed with VPgPro and exposed to a gradient of eIF(iso)4G. The fate of theVPgPro-eIF(iso)4E complex in the presence of eIF(iso)4G was measured: VPgPro-coated wells were exposed to a constant amount of eIF(iso)4E and a gradient of eIF(iso)4G. The capture of eIF(iso)4E was measured with monoclonal anti-T7 tag antibodies (dotted line). The level of background generated by the antibodies was determined by applying eIF(iso)4E to wells previously coated with VPgPro and using a rabbit polyclonal anti-eIF(iso)4G serum for detection (Δ).

Figure 4

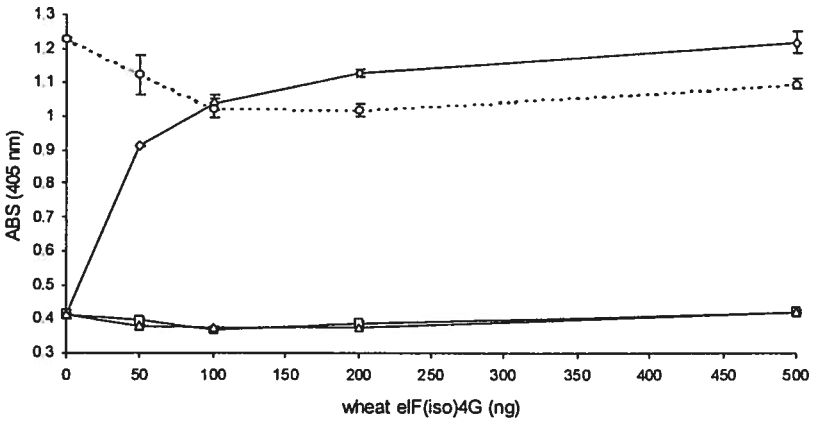

ABS (405 nm)
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0
50
100
150
200
250
300
350
400
450
500
wheat eIF(iso)4G (ng)

Figure 5. Inhibition by m^7GTP of VPg-eIF(iso)4E complex formation using an ELISA-based binding assay. Wells were coated with 1.0 μg of VPgPro and incubated with 2.0 μg of eIF(iso)4E with different concentrations of m^7GTP or GTP, in the absence (black column) or in the presence of 100 ng of eIF(iso)4G (grey column). Values represent the absorbance at 492 nm for the ELISA detection of eIF(iso)4E and are averages of three replicates from a typical experiment.

Figure 5

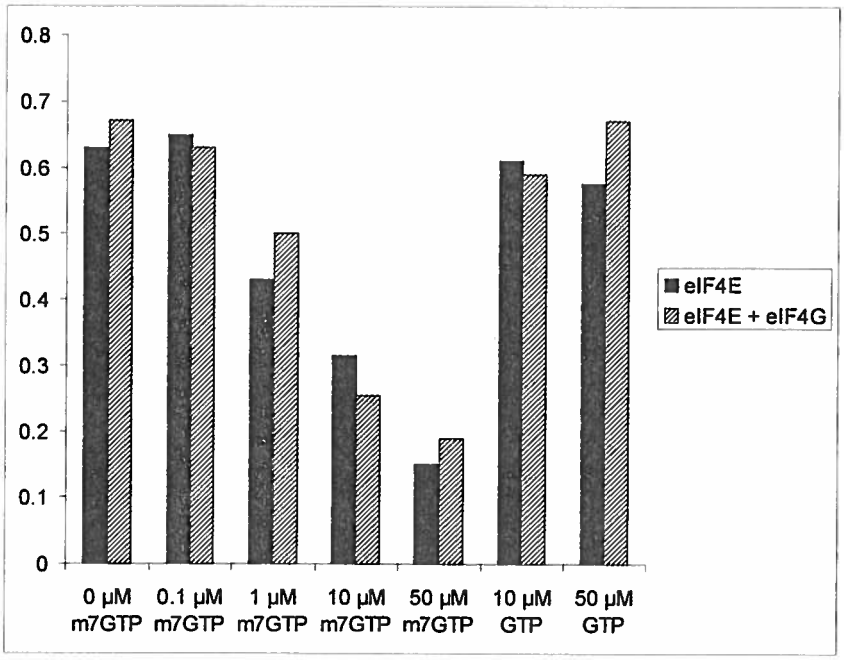
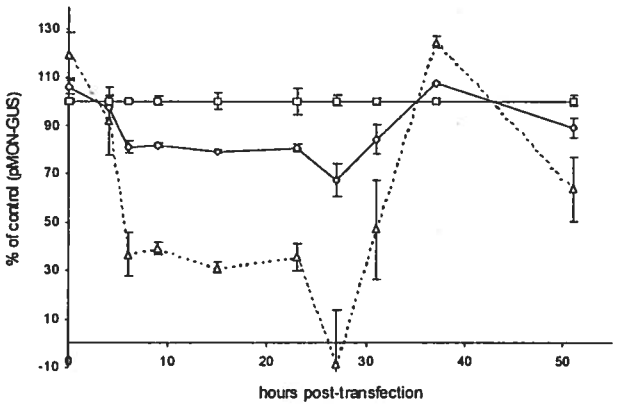

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 µM m7GTP
0.1 µM m7GTP
1 µM m7GTP
10 µM m7GTP
50 µM m7GTP
10 µM GTP
50 µM GTP
eIF4E
eIF4E + eIF4G

Figure 6. [35S]-methionine incorporation into proteins in protoplasts transfected with p35TuNOS (A) or with p35TuD77N (B). Tobacco protoplasts were transfected with either plasmid or pMON-GUS and kept in culture. At regular time intervals, protoplasts were incubated with [^{35}S]-methionine. Incorporated radioactivity into proteins was determined by TCA-precipitation and liquid scintillation. For each time point, the activity measured for p35TuNOS-transfected protoplasts (◊) is expressed as a percentage of the activity measured for pMON-GUS-transformed protoplasts (□). The dotted line represents the amount of [^{35}S]-methionine incorporated when only transfected protoplasts are considered. The proportion of transfected protoplasts was estimated with a parallel protoplast transformation using pMON-GUS. Each point represents the average of three replicates; standard deviations are indicated by vertical bars.

Figure 6

A


% of control (pMON-GUS)
130
110
90
70
50
30
10
-10
0
10
20
30
40
50
hours post-transfection

B

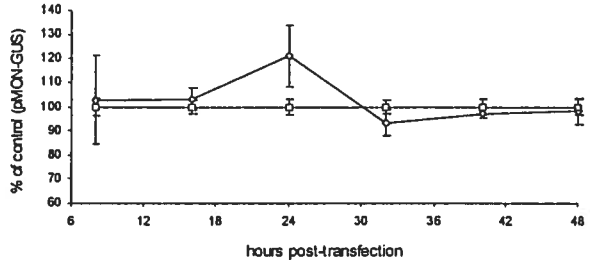

% of control (pMON-GUS)
140
130
120
110
100
90
80
70
60
6
12
18
24
30
36
42
48
hours post-transfection

Figure 7. Expression of CP in transfected protoplasts. Tobacco protoplasts were transfected with either p35TuNOS, p35D77N or pMON-GUS. Aliquots were removed from cultured after 48 hrs and subjected to Western blot analysis with anti-CP rabbit polyclonal antibodies. Lane 1: purified recombinant CP; lane 2: p35Tunos-tranfected cells; lane 3: p35D77N-tranformed cells; lane 4: pMON-GUS-transformed cells.

Figure 7

Annexe III

Liste des publications et des communications

Publications

Complex formation between potyvirus VPg and translation eukaryotic initiation factor 4E correlates with virus infectivity. **Léonard S**, Plante D, Wittmann S, Daigneault N, Fortin MG, Laliberte JF. **J Virol. 2000 Sep**;74(17):7730-7.

Interaction between the *Tomato ringspot virus* proteinase and the translation eukaryotic initiation factor (iso)4E from *Arabidopsis thaliana* in vitro. **Simon Léonard**, Joan Chisholm, Jean-François Laliberté and Hélène Sanfaçon. **Journal General of virology (2002) Aug**;83(Pt 8):2085-9.

Interaction of *Turnip Mosaic Virus* VPgPro with the Translation Initiation Factor 4E and the Poly(A) Binding Protein *in Planta.* **Simon Léonard**[1], Catherine Viel[1], Chantal Beauchemin[1], Nicole Daigneault[1], Marc G. Fortin[2] and Jean-François Laliberté[1*]
(Article accepté dans **Journal General of virology Nov. 2003)**

Multi-protein interaction with VpgPro of turnip mosaic virus in infected cells. Simon Léonard[1*], Catherine Viel[1], Nicole Daigneault[1], Marc G. Fortin[2] and Jean-François Laliberté[1*]
(Soumis à **Journal of Virology** novembre 2002)

Interaction of *Turnip mosaic virus* VPg protein with translation initiation factor eIF4F and inhibition of host protein synthesis during infection. Daniel Plante[1], Simon Léonard[2], Hiroyuki Tampo[1], Jean-François Laliberté[2], Marc G. Fortin[1*]
(Soumis à **Journal General of virology Dec 2002)**

Communications

Sixth International Symposium on Positive Strand RNA Viruses. May 28-June 2, 2001 INSTITUT PASTEUR, Paris, France
Sélectionné pour une présentation orale

Fifth symposium of INTERNATIONAL SOCIETY FOR PLANT MOLECULAR BIOLOGY (ISPMB) June 18-24, 2000, Centre des congrès, Québec, Canada
Sélectionné pour une présentation orale

1[cr] Congrès interne de L' Institut Armand-Frappier. Magog, Québec, Canada Octobre 1999
Sélectionné pour une présentation orale

Fifth International Symposium on Positive Strand RNA Viruses
June 1998, St-Petersburg, Floride, USA
Présentation d'une affiche

Annexe IV

Copies des articles publiés

Journal of General Virology (2002), 83, 2085–2089. Printed in Great Britain SHORT COMMUNICATION

Interaction *in vitro* between the proteinase of *Tomato ringspot virus* (genus *Nepovirus*) and the eukaryotic translation initiation factor iso4E from *Arabidopsis thaliana*

Simon Léonard,[1] Joan Chisholm,[2] Jean-François Laliberté[1] and Hélène Sanfaçon[2]

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Eukaryotic initiation factor eIF(iso)4E binds to the cap structure of mRNAs leading to assembly of the translation complex. This factor also interacts with the potyvirus VPg and this interaction has been correlated with virus infectivity. In this study, we show an interaction between eIF(iso)4E and the proteinase (Pro) of a nepovirus (*Tomato ringspot virus*; ToRSV) *in vitro*. The ToRSV VPg did not interact with eIF(iso)4E although its presence on the VPg-Pro precursor increased the binding affinity of Pro for the initiation factor. A major determinant of the interaction was mapped to the first 93 residues of Pro. Formation of the complex was inhibited by addition of m^{7}GTP (a cap analogue), suggesting that Pro-containing molecules compete with cellular mRNAs for eIF(iso)4E binding. The possible implications of this interaction for translation and/or replication of the virus genome are discussed.

A key step in the replication cycle of viruses is translation of the viral genome. Optimal translation is achieved by recruiting, and in some cases selectively altering, host translation factors (Thompson & Sarnow, 2000; Gale *et al.*, 2000). This in turn often results in the inhibition of host mRNA translation. Most viral RNAs differ in structure from cellular mRNAs. This provides opportunities for viruses to redirect the host translation machinery in favour of viral protein synthesis. For example, the 5′ end of the genomic RNA from picorna-like viruses (including animal and insect picornaviruses, and plant poty-, como- and nepoviruses) does not have a cap structure (m^{7}GpppN, where N is any nucleotide) as found in cellular mRNAs. Instead, the RNA is covalently linked to a virus-encoded protein termed VPg. Translation proceeds in a cap-independent manner through the use of an internal ribosome-entry site (IRES) (Martínez-Salas *et al.*, 2001; Gallie, 2001).

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Viral proteins are likely to participate in the regulation of viral genome translation (Thompson & Sarnow, 2000; Gale *et al.*, 2000). A case in point is the VPg of *Turnip mosaic virus* (TuMV; genus *Potyvirus*), which interacts with the eukaryotic initiation factor eIF(iso)4E of *Arabidopsis thaliana* (Wittmann *et al.*, 1997; Léonard *et al.*, 2000). eIF(iso)4E is a plant isomer of eIF4E (Rodriguez *et al.*, 1998) which binds the cap structure of cellular mRNAs and plays an important role in the regulation of translation initiation (Sonenberg & Gingras, 1998). The cap analogue m^{7}GTP, but not GTP, inhibits VPg–eIF(iso)4E complex formation, suggesting that VPg and cellular mRNAs compete for eIF(iso)4E binding. Plants inoculated with TuMV infectious cDNA containing a mutation in the eIF(iso)4E binding domain of VPg remain symptomless and do not show accumulation of virus coat protein, indicating that there is a correlation between VPg–eIF4E binding *in vitro* and virus viability *in planta* (Léonard *et al.*, 2000). The NIa protein (also called VPg-Pro) of *Tobacco etch virus* also interacts with eIF4E from tomato and tobacco, and the interaction was shown to enhance genome amplification (Schaad *et al.*, 2000). Although the precise biological function of the VPg (or VPg-Pro)–eIF4E interaction remains to be elucidated, it may either play a role in recruiting host factors for the translation and/or replication of the viral RNA or be involved in host translational shut-down, possibly through disruption of the interaction between cellular mRNAs and cap-binding translation initiation factors.

Nepoviruses are closely related to potyviruses in term of their genomic structure and genome expression strategy but differ from potyviruses in at least two significant aspects. First, the nepovirus genome is bipartite, with RNA1 encoding most of the proteins involved in virus replication (including VPg and Pro). Second, nepovirus VPgs are much smaller than potyvirus VPgs, which range from 22 to 24 kDa (Riechmann *et al.*, 1992). For example, the VPg of *Tomato ringspot virus* (ToRSV) is composed of 27 residues (Wang *et al.*, 1999). There is no amino acid sequence homology between potyvirus and nepovirus VPgs (Mayo & Fritsch, 1994). It was therefore of interest to determine if the nepovirus VPg, or larger precursor forms,

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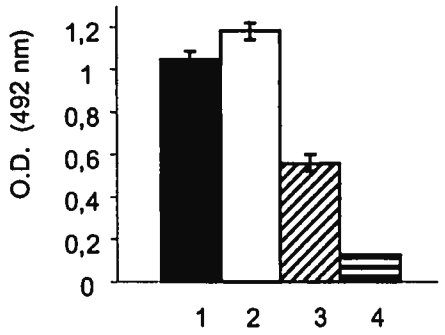

Fig. 1. Demonstration of VPg-Pro interaction with eIF(iso)4E using an ELISA-based binding assay. Wells were coated with 1 μg of VPg-Pro from TuMV (lane 1), VPg-Pro (lane 2) or Pro (lane 3) from ToRSV and then incubated with 2 μg of eIF(iso)4E of *A. thaliana*. In lane 4, wells were coated with Blotto only and incubated with 2 μg of eIF(iso)4E. Retention of eIF(iso)4E was detected using anti-T7 tag antibodies. Values are averages of three replicates from a typical experiment. Error bars represent the standard deviation.

interacts with eIF(iso)4E. One possible precursor of VPg is VPg-Pro. VPg-Pro (the functional equivalent of the potyvirus NIa) was found to accumulate during *in vitro* translation of larger precursors as a result of inefficient processing of the VPg-Pro cleavage site (Wang *et al.*, 1999; Wang & Sanfaçon, 2000), although accumulation of VPg-Pro in infected plants has not yet been demonstrated.

The interaction between VPg-Pro as well as Pro of ToRSV and eIF(iso)4E of *A. thaliana* was tested using an ELISA-based binding assay (Wittmann *et al.*, 1997; Léonard *et al.*, 2000). ToRSV has the ability to replicate in *A. thaliana* (R. I. Hamilton, personal communication). eIF(iso)4E was produced in *E. coli* and purified by m^7GTP–Sepharose chromatography (Wittmann *et al.*, 1997). The factor was fused to the N-terminal peptide of the T7 gene-10 protein (T7 tag), which allows its recognition by an anti-T7 tag monoclonal antibody (Novagen). ToRSV proteins were produced in *E. coli*, purified (Chisholm *et al.*, 2001) and adsorbed to wells of ELISA plates (1·0 μg per well) by overnight incubation at 4 °C. Purified eIF(iso)4E (2 μg) diluted in 1% Blotto in PBS containing 0·2% Tween was incubated for 1 h at 4 °C in the coated wells. Detection of bound initiation factor was achieved in an ELISA with the anti-T7 tag antibody and peroxidase-labelled goat anti-mouse immunoglobulin G (KPL). VPg-Pro from TuMV was purified as described previously (Wittmann *et al.*, 1997) and used as positive control. As shown previously (Wittmann *et al.*, 1997), the VPg-Pro of TuMV interacted with eIF(iso)4E (Fig. 1). VPg-Pro of ToRSV also interacted with eIF(iso)4E. The interaction was specific for the viral protein since the factor was not retained when wells were coated with an *E. coli* lysate not containing any VPg-Pro (Wittmann *et al.*, 1997). An interaction was also detected between the mature Pro and eIF(iso)4E, although retention of the factor was less than for VPg-Pro (0·55 OD units for Pro vs 1·2 OD units for VPg-Pro). This suggests that the interacting domain resides within Pro, but that the presence of VPg increases the affinity of the viral protein for eIF(iso)4E. In contrast, the interacting domain of the TuMV VPg-Pro resides within VPg, and the TuMV VPg-Pro and VPg have the same binding affinity for eIF(iso)4E (Wittmann *et al.*, 1997).

The domain(s) of the ToRSV VPg-Pro involved in the interaction with eIF(iso)4E were mapped by Far-Western experiments. Mutants (Fig. 2a) were generated by amplifying cDNA fragments from plasmid pET21d-VPg-Pro (Chisholm *et al.*, 2001) using specific oligonucleotides. The PCR products were inserted into plasmids pET21d (Novagen) or pTrxFus (Invitrogen). Exceptions to this are plasmid pET15bVPg-Pro-del*Bam*HI, which was constructed by deleting a small *Bam*HI fragment from plasmid pET15bVPg-Pro-N-Pol (Wang *et al.*, 1999), and plasmid pET21dVPg-Pro-del*Cla*I, which was constructed by inserting a *Nco*I–*Cla*I fragment from plasmid pET21d-VPg-Pro into plasmid pET21d. Protease mutants were expressed in *E. coli* as described by the supplier (Novagen). *E. coli* proteins were separated by SDS–PAGE and electroblotted to PVDF membranes (Bio-Rad). The membranes were probed with approximately 60 μg of the purified eIF(iso)4E essentially as described (Kao *et al.*, 1992). Binding of eIF(iso)4E was revealed by immunodetection using the anti-T7 tag monoclonal antibody and a goat anti-mouse secondary antibody linked to alkaline phosphatase (Sigma). The different proteinase forms were seen as a predominant band in *E. coli* extracts, as shown by Coomassie blue staining (Fig. 2b). The identity of these proteins was confirmed by immunodetection with polyclonal antibodies raised against the recombinant VPg-Pro (data not shown). Interaction of VPg-Pro with eIF(iso)4E was observed (Fig. 2b, c, lane 1) and no additional interactions were detected with *E. coli* proteins from the extract, indicating that the interaction was specific. An interaction was also detected with Pro (lane 7) but not with a protein containing the VPg domain fused to thioredoxin (lane 12).

Point mutations in the putative catalytic triad (H^{1283}D) and in the putative substrate-binding pocket (H^{1451}L) that abolished the proteolytic activity of the proteinase (Hans & Sanfaçon, 1995) did not affect the binding affinity for the factor (Fig. 2c, lanes 2 and 3), indicating that the determinants for the interaction were distinct from those for proteolytic activity. All proteins containing the first 93 amino acids of the Pro domain were shown to interact with eIF(iso)4E (lanes 1–7 and 11). Truncated proteinases containing a deletion of the N-terminal 93 residues could interact with the factor (lanes 8 and 10), but a truncated protein harbouring the last 81 amino acids of Pro did not (lane 9). Taken together these results indicate that a major determinant of the interaction resides within the first 93 amino acids of the Pro domain, although an additional domain within the C-terminal two-thirds of Pro may contribute to the binding.

To test whether ToRSV proteins and the cap structure of cellular mRNAs compete for eIF(iso)4E binding, the influence of the cap analogue m^7GTP on the formation of the VPg-

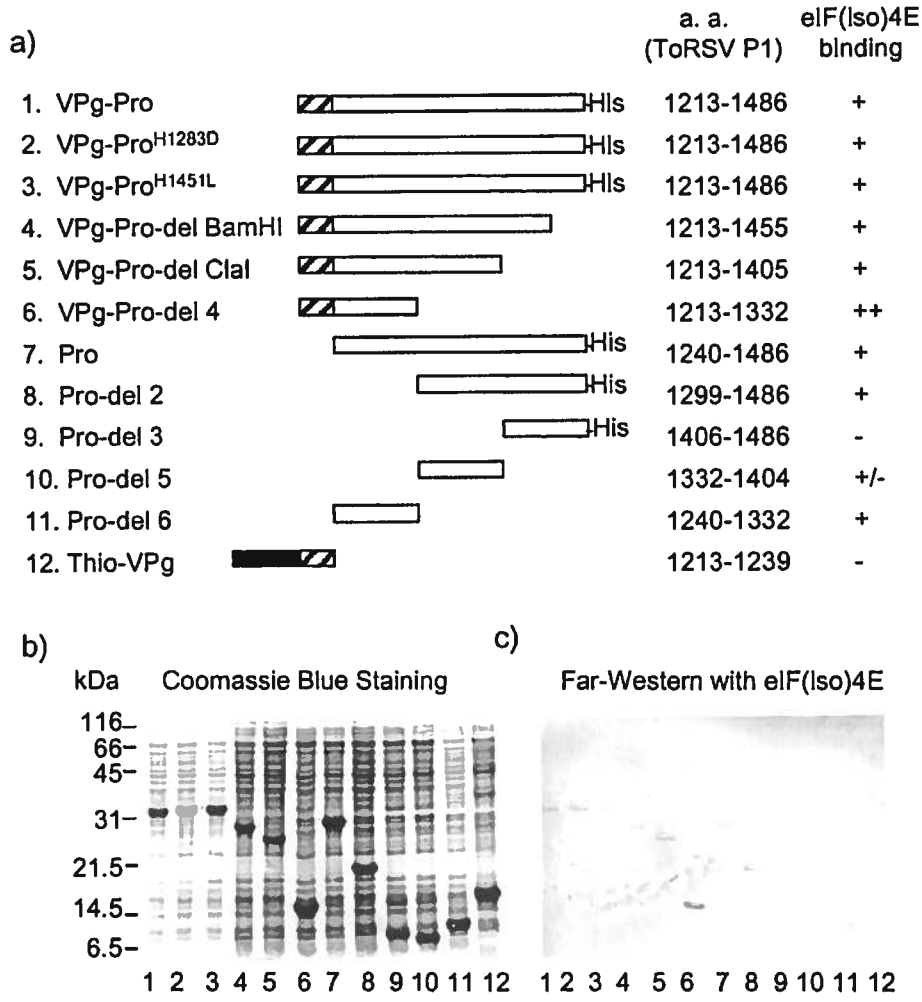

Fig. 2. Definition of the domains in ToRSV VPg-Pro involved in the interaction with eIF(iso)4E using a Far-Western assay. (a) Schematic representation of the ToRSV protease mutants. The white boxes represent regions of the Pro domain, the hatched boxes represent the VPg domain and the black box represents the thioredoxin protein (from vector pTrxFus; Invitrogen) fused in-frame with the VPg coding region. A histidine tail (His) at the C termini of the proteins is shown when present. The amino acids of the ToRSV RNA1-encoded polyprotein (P1) present in each recombinant protein are indicated [numbering from the first amino acid of the polyprotein according to Rott *et al.* (1995)]. (b and c) Analysis of the interaction of the ToRSV protease mutants with eIF(iso)4E using the Far-Western assay. After induction of the expression of viral proteins, *E. coli* cells were resuspended in 50 mM NaH_2PO_4, pH 8·0, 0·3 M NaCl, 0·1 % Triton X-100. After sonication the extracts were separated by SDS–PAGE and either stained by Coomassie blue staining (b) or electroblotted to PVDF membranes (Bio-Rad). Interaction with eIF(iso)4E was tested using the Far-Western method as described in the text (c). The migration of molecular mass standards is indicated on the left of the gels. The following proteins were tested in each lane. Lane1, VPg-Pro; lane 2, VPg-Pro^{H1283D}; lane 3, VPg-Pro^{H1451L}; lane 4, VPg-Pro-del *Bam*HI; lane 5, VPg-Pro-del *Cla*I; lane 6, VPg-Pro-del 4; lane 7, Pro; lane 8, Pro-del 2; lane 9, Pro-del 3; lane 10, Pro-del 5; lane 11, Pro-del 6; lane 12, Thio-VPg.

Pro–eIF(iso)4E and Pro–eIF(iso)4E complexes was tested. ELISA plate wells were coated with 1·0 μg of viral proteins and incubated with 2·0 μg of eIF(iso)4E and either 10 or 20 mM m^7GTP. The cap analogue inhibited the formation of the complexes by approximately 30 % at a concentration of 20 μM (Fig. 3). No inhibition was observed with 20 μM of GTP, indicating that the inhibition was cap-related. As previously shown, 20 μM of m^7GTP inhibited the interaction of eIF(iso)4E with the VPg-Pro of TuMV by 60 % (Léonard *et al.*, 2000).

One possible role for the interaction between the nepovirus Pro and eIF(iso)4E is that it could promote assembly of the translation complex on the viral RNA. In the circular translation model, efficient translation of cellular mRNAs requires interactions between initiation factors bound to the 5′ cap structure and the poly(A)-binding protein (PABP) bound to the 3′ poly(A) tail (Sachs, 2000). Similarly, translation of several viruses has been shown to be mediated by protein bridges between the 5′ and 3′ termini of the RNA which involve viral

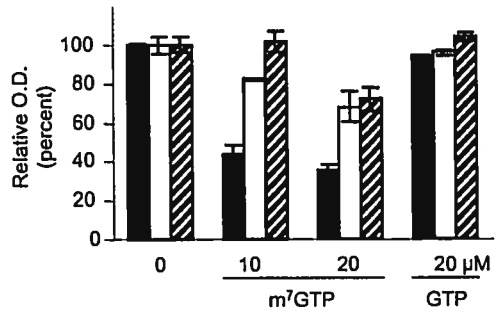

Fig. 3. Inhibition by m^7GTP of VPg-Pro-eIF(iso)4E complex formation as determined by ELISA-based binding assay. Wells were coated with 1 μg of VPg-Pro from TuMV (black column), VPg-Pro (white column) and Pro (hatched column) from ToRSV and were incubated with 2 μg of eIF(iso)4E of *A. thaliana*, with no cap analogue, 10 and 20 μM m^7GTP or 20 μM GTP. Retention of eIF(iso)4E was detected using anti-T7 tag antibodies. Values are averages of three replicates from a typical experiment and error bars represent the standard deviation. Values in the absence of m^7GTP were arbitrarily adjusted to 100% for each protein.

proteins and cellular translation initiation factors. For instance, the rotavirus NSP3 protein promotes genome circularization and efficient translation of the viral RNA by simultaneously binding to the 3′ untranslated region of the non-polyadenylated viral RNA and to eIF4G, which in turn binds to eIF4E (Piron *et al.*, 1998, 1999; Vende *et al.*, 2000). In the case of picornaviruses, the IRES interacts directly with eIF4E, eIF4G and eIF3 (Ali *et al.*, 2001; Borman *et al.*, 2001; Lopez de Quinto *et al.*, 2001). Given that eIF4G interacts with PABP and picornavirus RNA is polyadenylated, circularization was expected. Indeed, eIF4G–PABP interaction was required for poly(A) tail-mediated stimulation of IRES translation (Michel *et al.*, 2001).

The formation of protein bridges leading to genome circularization may also be an important feature of the translation of plant virus genomes. Recently, the coat protein of *Alfalfa mosaic virus* was shown to stimulate translation of viral RNAs, presumably by acting as a functional analogue of PABP (Neeleman *et al.*, 2001). The nepovirus Pro could similarly participate in genome circularization by acting as a bridging element between host initiation factors and the viral RNAs. Pro is a member of the 3C-like proteinase family and is likely to have RNA-binding properties (Blair *et al.*, 1998). It could thus interact with the 5′ end of the viral genome, as shown for the picornavirus 3C (Gamarnik & Andino, 2000; Kusov *et al.*, 1997; Kusov & Gauss-Muller, 1997; Harris *et al.*, 1994; Walker *et al.*, 1995) and the potyvirus NIa proteinases (Daros & Carrington, 1997). Alternatively, Pro as a precursor protein with VPg may be covalently linked to viral RNAs. In addition to possibly improving translation of the viral RNAs, genome circularization could provide several advantages for virus replication, including the coordination of translation and RNA synthesis, the localization of the viral polymerase at the appropriate start site and a control mechanism for the integrity of the viral genome (Herold & Andino, 2001). Finally, the interaction of the ToRSV Pro with eIF(iso)4E may also have other biological functions, one of which may be a direct or indirect role in a possible shut-down of host translation.

The presence of the VPg domain on a precursor of the ToRSV Pro regulates the different activity of the proteinase as it enhances its ability to interact with eIF(iso)4E (this study) and decreases its ability to release the movement protein and coat protein from RNA2-derived substrates (Chisholm *et al.*, 2001). The results presented here provide additional support for our previous suggestion that the inefficient cleavage at the VPg-Pro site may play an important role in the biology of ToRSV (Chisholm *et al.*, 2001).

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Complex Formation between Potyvirus VPg and Translation Eukaryotic Initiation Factor 4E Correlates with Virus Infectivity

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The interaction between the viral protein linked to the genome (VPg) of turnip mosaic potyvirus (TuMV) and the translation eukaryotic initiation factor eIF(iso)4E of *Arabidopsis thaliana* has previously been reported. eIF(iso)4E binds the cap structure (m^7GpppN, where N is any nucleotide) of mRNAs and has an important role in the regulation in the initiation of translation. In the present study, it was shown that not only did VPg bind eIF(iso)4E but it also interacted with the eIF4E isomer of *A. thaliana* as well as with eIF(iso)4E of *Triticum aestivum* (wheat). The interaction domain on VPg was mapped to a stretch of 35 amino acids, and substitution of an aspartic acid residue found within this region completely abolished the interaction. The cap analogue m^7GTP, but not GTP, inhibited VPg-eIF(iso)4E complex formation, suggesting that VPg and cellular mRNAs compete for eIF(iso)4E binding. The biological significance of this interaction was investigated. *Brassica perviridis* plants were infected with a TuMV infectious cDNA (p35Tunos) and p35TuD77N, a mutant which contained the aspartic acid substitution in the VPg domain that abolished the interaction with eIF(iso)4E. After 20 days, plants bombarded with p35Tunos showed viral symptoms, while plants bombarded with p35TuD77N remained symptomless. These results suggest that VPg-eIF(iso)4E interaction is a critical element for virus production.

Potyviruses belong to the supergroup of "picorna-like" viruses. The viral genome is a single RNA molecule of positive polarity of close to 10,000 nucleotides with a poly(A) tract at its 3′ end. It codes for one large polyprotein which is processed into at least 10 mature proteins by three viral proteinases (Pro) (47). The 5′ end of the viral RNA does not have a cap structure (m^7GpppN, where N is any nucleotide) but is covalently linked to a virus-encoded protein termed VPg via a tyrosine residue (39, 40). VPg has several suggested roles in the virus life cycle. Interactions of VPg with the viral RNA polymerase in yeast (25, 33) and in vitro (15) support a role in viral RNA synthesis. Additionally, VPg has been implicated in overcoming resistance in plants (27, 35, 41, 42, 55). VPg also performs a yet-to-be-defined function in the nucleus. Indeed, NIa protein of tobacco etch potyvirus, a precursor form of VPg, has been found in the nucleus (10, 23, 46), and mutations in the VPg domain resulting in the inhibition of nuclear transport debilitated viral genome amplification (54). Recently, an interaction was shown to take place between the VPg of turnip mosaic potyvirus (TuMV) and the translation eukaryotic initiation factor (eIF) iso 4E of *Arabidopsis thaliana* (65). eIF4E is a component of the eIF4F complex and binds the cap structure of cellular mRNAs (6, 36, 38, 43). The cap mediates attachment of mRNAs to small ribosomal subunits, and the association is mediated by eIF4F (through binding to eIF4E) and eIF3 (38, 43). The interaction between VPg and eIF(iso)4E suggests the participation of the viral protein in the initiation of translation of the viral RNA.

Initiation is the rate-limiting step of translation in eukaryotes, and eIF4E has a regulatory role in this cellular event (38, 43, 60). In mammals, eIF4E is the least abundant of the initiation factors (13), although this assertion has been challenged (45). Its cap-binding activity is modulated by phosphorylation (62, 64). It is also regulated by eIF4E-binding proteins (4E-BPs) (31) which, by binding eIF4E, prevent the formation of the eIF4F complex (21, 34, 44). As a consequence, eIF4E plays an important role in the control of cell growth (58). In *Saccharomyces cerevisiae*, disruption of the gene coding for eIF4E is lethal, and mutants with altered mRNA cap-binding affinity reprogram mRNA selection by ribosomes (2). In mammals, overexpression of eIF4E has been shown to transform cells in tissue culture (11, 32). Elevated eIF4E expression results in the selective increase of a few proteins whose mRNAs are normally translationally repressed, such as ornithine decarboxylase and cyclin (49, 50). Just as elevated levels of eIF4E contribute to the development of a transformed cellular state, the reduction of eIF4E levels, using antisense RNA, has been shown to lengthen cell division times (12). The results of these in vitro studies, which emphasize the importance of eIF4E in the regulation of the cell division cycle, have been extended to clinical observations: eIF4E amounts have been found to be elevated in some human carcinomas (16, 27).

eIF4F is targeted by several animal viruses in their attempt to control host translation for preferential viral mRNA translation. For instance, adenoviruses and influenza viruses affect the phosphorylation state of eIF4E (14, 66). Encephalomyocarditis virus inactivates the initiation factor by enhancing 4E-BP1 binding (18). Finally, picornaviruses induce the cleavage eIF4G, with the consequence that cellular mRNAs linked to eIF4E cannot interact with 40S ribosome complexes (22, 59).

Although most of these observations relating to the role of eIF4E have been made in mammalian cells, the similarities in translation initiation in mammals, plants, and yeasts and the sequence homologies of different translation initiation factors (6, 17) suggest that the plant eIF4E plays as important a role as its mammalian homologue in the regulation of cellular pro-

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TABLE 1. List of oligonucleotides used in this study for plasmid construction and site-directed mutagenesis

Plasmid construct	5′ Oligonucleotide sequence (5′→3′)	3′ Oligonucleotide sequence (5′→3′)
pEGVPg$_{7-191}$	AAAGGCAGGATCCAAAGACAG	AGTTACTCTCGAGGTCCACT
pEGVPg$_{94-191}$	CCATTCACGGAATTCACCCTTGTA	AGTTACTCTCGAGGTCCACT
pEGVPg$_{62-191}$	GAACAGGAGGATCCTTAACA	AGTTACTCTCGAGGTCCACT
pEGVPg$_{7-63}$	AAAGGCAGGATCCAAAGACAG	GATCAAACTCGAGCATGTTA
pEGVPgΔ_{59-93}	AAAGGCAGGATCCAAAGACAG[a]	CATGTTAATGAATTCCCTGTTCTT
	CCATTCACGGAATTCACCCTTGTA	AGTTACTCTCGAGGTCCACT
pETeIF4E*At*	TAATTTAGGGAATTCGGAGAAACA	GCAAAGATTCTCGAGGTTTCAAGC
pEGVPg$_{F59A}$	CATGAAGCGGAATTCAAGAGGCAA	CTGACTGTTCTCGAGTGGCATTAT
	AACAGGGCAATCAACATGTAT	ATACATGTTGATTGCCTTCCTGTT
pEGVPg$_{Y63A}$	CATGAAGCGGAATTCAAGAGGCAA	CTGACTGTTCTCGAGTGGCATTAT
	TTCATCAACATGGCCGGCTTTGAT	ATCAAAGCGGGCCATGTTGATGAA
pEGVPg$_{D77A}$	CATGAAGCGGAATTCAAGAGGCAA	CTGACTGTTCTCGAGTGGCATTAT
	CGTTTCGTGGCGCCACTCACAGGA	TGTGAGTGGCGCCACGAAACG
pEGVPg$_{D77E}$	CATGAAGCGGAATTCAAGAGGCAA	CTGACTGTTCTCGAGTGGCATTAT
	CGTTTCGTGGAGCCACTCACAGGA	TCCTGTGAGTGGCTCCACGAAACG
pEGVPg$_{D77N}$	CATGAAGCGGAATTCAAGAGGCAA	CTGACTGTTCTCGAGTGGCATTAT
	CGTTTCGTGAACCCACTCACAGGA	TCCTGTGAGTGGGTTCACGAAACG

[a] Oligonucleotides on the same line were used in pairs, and amplified fragments were assembled as described in Materials and Methods.

cesses. In this study, we investigated the interaction between the VPg of TuMV and eIF(iso)4E and its consequences for viral infection. We found that the cap analogue m^{7}GTP competed with VPg for eIF(iso)4E binding. Furthermore, TuMV whose VPg was mutated at a single residue which abolished in vitro interaction with eIF(iso)4E was debilitated for viral infection in whole plants.

MATERIALS AND METHODS

Microorganisms and media. Manipulations of bacterial as well as yeast strains and of nucleic acids and proteins were done by standard methods (19, 52). *Escherichia coli* XL1-Blue was used for subcloning, and *E. coli* BL21(DE3) (Novagen) was used for protein expression. *S. cerevisiae* EGY48 (MATa *trp1 his3 ura3 8op-Leu2*) (19) was used for the interaction study.

Yeast two-hybrid system. Plasmids employed for the interaction study were as described by Golemis et al. (19). pEG202 was used for the fusion of VPg and its derivatives to the DNA-binding domain of LexA. pJG4-5 was used to express eIF(iso)4E of *A. thaliana* (pSW56) (65) as a translation fusion to a cassette consisting of the simian virus 40 nuclear localization sequence, the acid blob B42, and the hemagglutinin epitope tag; expression was under the control of the GAL1 inducible promoter. The *lacZ* reporter plasmid was pSH18-34 containing eight *lexA* operators. Strength of the interaction was quantified using the β-galactosidase liquid assay (19). β-Galactosidase units were calculated using the following equation: units = 1,000 × (optical density at 420 nm [OD_{420}] − 1.75 × OD_{550})/($T \times V \times OD_{660}$), where T is time in minutes and V is the volume of culture used in milliliters.

The pLex-VPg plasmids were constructed as follows. The region coding for VPg in plasmid pETPro/24 (30) was amplified by PCR using the 5′ and 3′ primer pairs listed in Table 1. The amplified fragment was digested with *Bam*HI and *Xho*I, ligated with similarly restricted pEG202, and introduced into *E. coli* XL1 and ultimately into *S. cerevisiae* EGY48. pEGVPgΔ_{59-93} was produced by amplification of pETPro/24 with a first set of primers (Table 1); the amplified fragment was digested with *Eco*RI and *Xho*I and ligated into similarly digested pKS pBluescript I (Stratagene) to produce pKS-VPg3′. Plasmid pETPro/24 was also amplified with a second set of primers, and the amplified fragment was digested with *Bam*HI and *Eco*RI and ligated in similarly digested pKS-VPg3′. This plasmid was digested with *Bam*HI and *Xho*I, and the VPg-containing fragment was ligated into *Bam*HI- and *Xho*I-digested pEG202.

Recombinant protein expression in *E. coli* and purification. Plasmid pETtag(iso)4E*At* codes for eIF(iso)4E of *A. thaliana* and was produced by digestion of plasmid pSW56 with *Eco*RI and *Xho*I and ligation of the 0.7-kb insert with similarly restricted pET21a (Novagen). The resulting eIF(iso)4E is fused at its N-terminal end to the 11-amino-acid N-terminal peptide of the T7 gene 10 protein (T7 tag), which is recognized by the anti-T7 tag monoclonal antibody (Novagen). Plasmid pETtag(iso)4E*Ta* codes for eIF(iso)4E of *Triticum aestivum* (wheat) and was produced by digestion of plasmid pGAG424/eIF(iso)4E (a generous gift from K. S. Browning, University of Texas) with *Eco*RI and *Sal*I and ligation with *Eco*RI- and *Xho*I-restricted pET21a. The resulting protein is fused at its N-terminal end with the T7 tag. Plasmid pETtag4E*At* codes for eIF4E of *A. thaliana* and was produced by amplification of plasmid pET14b/eIF4E (kindly provided by C. Robaglia, Centre d'Énergie Atomique) with the primers listed in Table 1; the amplified fragment was digested with *Eco*RI and *Xho*I and ligated with *Eco*RI- and *Xho*I-restricted pET21a. The resulting recombinant protein is fused at its N-terminal end with the T7 tag. Plasmids were introduced into *E. coli* BL21(DE3). Recombinant proteins were purified as described earlier (65).

VPgPro was purified as previously described (37). VPgΔPro was produced as follows. pETPro/24 and pEGVPgΔ_{59-93} were digested with *Nco*I and *Stu*I. The 5.5- and 0.4-kb fragments from pETPro/24 and pEGVPgΔ_{59-93}, respectively, were purified and ligated. The ligation product was introduced into *E. coli* XL1-Blue and ultimately into BL21(DE3). The recombinant protein was expressed and purified as described above for VPgPro.

ELISA-based binding assay. Purified VPgPro was adsorbed to the wells of an enzyme-linked immunosorbent assay (ELISA) plate (1.0 μg/well) by overnight incubation at 4°C, and the wells were blocked with 5% Blotto in phosphate-buffered saline (PBS). Purified initiation factor was diluted in 1% Blotto in PBS with 0.2% Tween and was incubated for 1 h at 4°C with the previously coated wells. Detection of bound initiation factor was achieved as in the ELISA assays with the anti-T7 tag antibody and peroxidase-labeled goat anti-mouse immunoglobulin G (KPL). Wells were washed three times with 0.05% Tween between incubations.

Site-directed mutagenesis. PCR site-directed mutagenesis by the overlap extension method was done as described previously (24). Primers used for mutagenesis are listed in Table 1, and plasmid p35Tunos (53) was used as a template. Amplification was performed with the *Pwo* DNA polymerase (Roche).

Particle bombardment. Plasmid p35TuD77N was constructed by digesting p35Tunos (53) with *Cla*I and ligating the 3.8-kb fragment with similarly digested pKS pBluescript I (Stratagene), resulting in the recombinant plasmid pKS-Tunos/Cla. Plasmid pEGVPgD77N was digested with *Pml*I and *Spe*I, and the corresponding fragment was inserted into pKS-Tunos/Cla linearized with *Spe*I and partially digested with *Pml*I. This last construction was digested with *Cla*I, and the fragment was ligated back into p35Tunos. Proper assembly was verified by nucleic acid sequencing. Particle bombardment was done in the Biolistic PDS-1000/He instrument (Bio-Rad). Then, 7 μg of DNA was mixed with 3 mg of gold particles in 2.5 M $CaCl_2$ and 0.1 M spermidine. This mixture was diluted 1:5 in ethanol, and 5 μl was placed in the center of a 900-lb/in^2 rupture disk. *Brassica perviridis* plants at the two-leaf stage were used.

RESULTS

Interaction of VPg with eIF4E of *A. thaliana* and eIF(iso)4E of *T. aestivum*. Plants have two isomers of the cap-binding initiation factor, namely, eIF(iso)4E and eIF4E (7, 8). A third factor was recently identified (51), but its involvement in translation initiation relative to the eIF4E isomers is unclear. Since both isomers of eIF4E participate in translation initiation, it was speculated that VPg would bind to both forms and to eIF(iso)4E from a monocotyledenous species such as *T. aestivum* (wheat). *A. thaliana* is a dicotyledenous plant and is infected by TuMV, whereas wheat is not a host of the virus. Interactions between the viral protein and these initiation factors were investigated using an ELISA-based binding assay. The initiation factors were produced in *E. coli* as recombinant

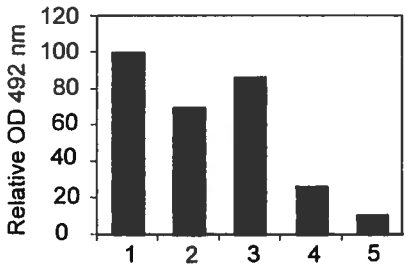

FIG. 1. VPg interaction with eIF4E isomers as demonstrated by ELISA-based binding assay. Wells precoated with 1.0 μg of VPgPro were incubated with 2.0 μg of eIF(iso)4E (lane 1) and eIF4E (lane 2) from *A. thaliana*, eIF(iso)4E (from *T. aestivum* (lane 3), or no added initiation factor (lane 4). In lane 5, wells were coated with Blotto only and incubated with 2.0 μg of eIF(iso)4E from *A. thaliana*. Complexes were detected using anti-T7 tag antibodies. Values are averages of two replicates from a typical experiment.

proteins fused at their N-terminal end to the 11-amino-acid N-terminal peptide of the T7 gene 10 protein (T7 tag), which is recognized by an anti-T7 tag monoclonal antibody. The proteins were purified by m^7GTP-Sepharose chromatography. ELISA plate wells were coated with 1.0 μg of recombinant VPgPro (see protein purity in Fig. 2A, lane 1) and incubated with 2.0 μg of the different initiation factors. VPgPro, a precursor form of VPg, was used because it is purified more easily than VPg in *E. coli* and because it had been shown that the Pro domain does not participate in eIF(iso)4E binding (65). Complex formation was detected using anti-T7 tag antibodies. Figure 1 shows that VPgPro interacted most effectively with eIF(iso)4E of *A. thaliana*, and the level of that interaction was given as a relative value of 100 (lane 1). The interaction was specific for the viral protein since the initiation factor was not retained when wells were not coated with VPgPro (lane 5), nor was it retained with an *E. coli* lysate not containing VPgPro (65). Figure 1 also shows that eIF4E from *A. thaliana* (lane 2) and eIF(iso)4E from wheat (lane 3) interacted with VPgPro. Once the OD values were corrected for background noise (i.e., the OD value obtained in the absence of initiation factors [lane 4]), the binding values of VPgPro to eIF4E from *A. thaliana* and eIF(iso)4E from wheat were 60 and 80%, respectively, of the binding to eIF(iso)4E from *A. thaliana*. This experiment indicated that the VPg of TuMV interacted with several initiation factor species, with similar binding affinities.

Mapping of the VPg interaction domain. Since VPg interacted with the different isomers of the initiation factor and since the interaction is likely to be important for all potyviruses, it was hypothesized that the VPg domain responsible for the eIF(iso)4E interaction would be conserved among different potyviral VPgs. The VPg domain involved in the interaction with eIF(iso)4E was mapped using the yeast two-hybrid system. Deletions in the VPg gene were made by PCR and were fused to the gene coding for the DNA-binding domain of LexA in pEG202. These recombinant plasmids were introduced into the yeast EGY48 strain, which contained either pJG4-5 (carrying the activation domain without insert) or pSW56 which codes for eIF(iso)4E of *A. thaliana* fused to the activation domain of pJG4-5. The *lacZ* reporter plasmid pSH18-34 was also present in the yeast cells. Interaction between the different deleted VPg domains and eIF(iso)4E was measured by β-galactosidase assay. The near-full-length VPg comprising amino acids 7 to 191 (VPg_{7-191}) strongly interacted with eIF(iso)4E, providing on average 659 U of β-galactosidase activity (Table 2). No activity was measured when the initiation factor was omitted. VPg fragments comprising amino acids 7 to 63 (VPg_{7-63}) or amino acids 94 to 191 (VPg_{94-191}) failed to interact with the initiation factor. However, the VPg fragment comprising amino acids 62 to 191 (VPg_{62-191}) strongly interacted with eIF(iso)4E. This suggests that the region comprising amino acids 62 to 93 was involved in the interaction. This was confirmed by the deletion of amino acids 59 to 93 from VPg; this deletion mutant ($VPg\Delta_{59-93}$) exhibited extremely low levels of interaction (17 U of β-galactosidase).

The lack of interaction with eIF(iso)4E by $VPg\Delta_{59-93}$ could, however, have been the result of degradation of the fusion protein or lack of nuclear transport in the yeast. To test that this was not the case, in vitro binding assays with purified proteins were performed. The deletion mutant gene was subcloned in the plasmid pET21a and expressed as a Pro fusion (VPgΔPro) in *E. coli*. The protein was purified using the same procedure as for VPgPro. While VPgPro was purified as a 49-kDa species (Fig. 2A, lane 1), multiple forms of VPgΔPro,

TABLE 2. β-Galactosidase activity displayed by various VPg deletions in yeast expressing eIF(iso)4E from *A. thaliana* fused to the B42 activation domain

Schematic representation of VPg deletion fused to DNA binding domain of LexA	VPg residues (range)[a]	Interactor	β-Galactosidase units
	7–191	None[b]	0[c]
		eIF(iso)4E	659
	7–63	None	6
		eIF(iso)4E	6
	94–191	None	5
		eIF(iso)4E	6
	62–191	None	9
		eIF(iso)4E	1,081
	Δ59–93[d]	None	5
		eIF(iso)4E	17

[a] Numbers represent first and last residues of VPg fused to DNA binding domain of LexA.
[b] Yeast containing pJG4-5.
[c] Average value of two replicates from a typical experiment.
[d] Symbol and numbers represent deleted residues on VPg_{7-191}.

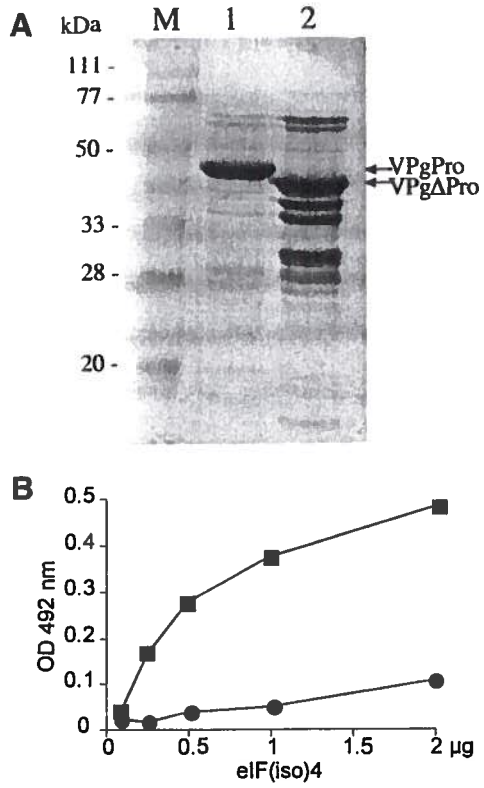

FIG. 2. VPgPro and VPgΔPro interaction with eIF(iso)4E of *A. thaliana* as demonstrated by ELISA-based binding assay. (A) Purification of VPgPro and VPgΔPro. Expression and purification were as described in Materials and Methods. Samples were loaded on a sodium dodecyl sulfate-polyacrylamide gel and stained with Coomassie blue. Lane 1, VPgPro (5 μg); lane 2, VPgΔPro (20 μg); lane M, molecular mass standards. (B) ELISA-based binding assay. Wells were coated with 1.0 μg of VPgPro (■) or 4.0 μg of VPgΔPro (●) and then incubated with increasing concentrations of eIF(iso)4E from *A. thaliana*. Complexes were detected using anti-T7 tag antibodies. Values are averages of two replicates from a typical experiment.

TuMV	59	FINMYGFDPEDFSAVRFVDPLTGATLDDNPFTDIT	93
PPV	1915	-V----Y--T-YNF---------H---E--LM--N	1949
LMV	2080	-V----YN---Y-FI--L-----K-M-EQV----S	2114
TVMV	1856	-V----VS-DEY-Y--YL--V------ES-M--LN	1890
PVY	1923	---------TEY-FIQ--------QIEE-VYA--R	1957
TEV	1907	---------T---YI---------H-I-ESSTNA-D	1941
BCMV	1920	------VE--NY-TL---------H-M-ES-RV--R	1954
PRSV	2152	-VAT---K---Y-Y--YL-----E---ES-Q---S	2186
ZYMV	1911	-VHL--VE--NY-FI---------H---ESTH---S	1945

FIG. 3. Amino acid sequence of the eIF(iso)4E-binding domain of VPg and comparison with corresponding region from other potyviruses. Amino acid sequences were aligned using BLAST software with the BLOSUM62 matrix provided on the NCBI World Wide Web server. The numbers for TuMV represent the first and last residue positions of VPg; for the other viruses, the numbers represent the first and last residue positions of the polyprotein. Dashes indicate amino acids identical to that of the TuMV VPg. PPV, plum pox potyvirus (accession number S47508); LMV, lettuce mosaic potyvirus (P89876); TVMV, tobacco vein mottling potyvirus (P09814); PVY, potato mosaic potyvirus (1906388); TEV, tobacco etch potyvirus (P04517); BCMV, bean common mosaic potyvirus (Q65399); PRSV, papaya ringspot potyvirus (Q01901); ZYMV, zucchini yellow mosaic potyvirus (Q89330).

with a main band at 46 kDa, were observed (lane 2). This degradation of VPgΔPro suggests that deletion of the amino acids caused the protein to be more susceptible to degradation than the complete VPgPro in *E. coli*. Once purified, VPgΔPro was not susceptible to further degradation. Conditions for the binding assay were adjusted so that similar concentrations of VPgPro and nondegraded VPgΔPro were used. ELISA plate wells were coated with either 1.0 μg of VPgPro or 4.0 μg of VPgΔPro and then incubated with increasing concentrations of eIF(iso)4E. Compared with wild-type VPgPro, VPgΔPro bound approximately fivefold less initiation factor (Fig. 2B). This experiment suggests that amino acids 59 to 93 of VPg are largely responsible for the binding of eIF(iso)4E.

The 35 amino acids identified above are listed in Fig. 3 and were compared with the corresponding region of eight potyviruses. The comparison indicates that the region is highly conserved among the different potyviruses: of the 35 amino acids, 8 residues are identical for all listed viruses, 13 are identical for most of the listed viruses, and 7 residues belong to the same class.

Site-directed mutagenesis of the phenylalanine at position 59, the tyrosine at position 63, and the aspartic acid at position 77 of the VPg was undertaken to determine their importance for eIF(iso)4E binding. Phe59 and Asp77 are conserved in all listed potyviruses and are adjacent to other highly conserved residues; Tyr63 is the residue which is covalently linked to the viral RNA (39, 40, 47). The VPg from an infectious TuMV cDNA clone (p35Tunos) derived from the UK1 strain (53) was used for these mutagenesis experiments since introduced mutations could be transferred back into infectious cDNA plasmids without introducing changes elsewhere in the viral genome. The VPg sequence of the Quebec and UK1 strains differ at several nucleic acid positions (mainly at position 3 of the codons) but differ by only four amino acid residues clustered in the middle of the protein. However, these residues are outside of the eIF(iso)4E binding region mapped above. The affinity of VPg from both strains for eIF(iso)4E of *A. thaliana* was similar, as determined with the yeast two-hybrid system (data not shown).

PCR site-directed mutagenesis by overlap extension was used to introduce substitutions, and the interaction of the VPg mutants with eIF(iso)4E was measured using the yeast two-hybrid system. Here, a portion of Pro was introduced along with VPg in pEG202 for subsequent subcloning into p35Tunos. Mutants VPg_{F59A} and VPg_{Y63A}, which introduced alanine residues at positions 59 and 63, respectively, produced β-galactosidase activity levels similar to that of the wild-type VPg, indicating that their modification did not affect VPg interaction with the initiation factor (Table 3). Mutants VPg_{D77A}, VPg_{D77E}, and VPg_{D77N}, which introduced either an alanine, a glutamic acid, or an asparagine, respectively, at position 77, failed, however, to interact with the translation factor. The importance of the aspartic acid in the interaction is stressed by the fact that replacement with related amino acids such as glutamic acid and asparagine abolished binding.

Effect of m^7GTP on the formation of VPg-eIF(iso)4E complex. eIF(iso)4E's role in the cell is to initiate assembly of the translation apparatus by binding to the 5′ m^7G residue of the mRNAs. In order to test whether the VPg and mRNAs would compete for eIF(iso)4E interaction, the influence of the cap analogue m^7GTP on the formation of the VPg-eIF(iso)4E complex was tested. ELISA plate wells were coated with 1.0 μg of recombinant VPgPro and incubated with 2.0 μg of eIF(iso)4E and various concentrations of m^7GTP. Complex formation was detected with anti-T7 tag antibodies. Figure 4A shows that increasing concentrations of the analogue progressively prevented the formation of the VPg-eIF(iso)4E complex. These concentrations appear to be physiologically relevant since m^7GTP at 4 μM greatly inhibited in vitro translation of

TABLE 3. β-Galactosidase activity displayed by mutants of VPg in yeast expressing eIF(iso)4E from *A. thaliana* fused to the activation domain B42

VPg	Interactor	β-Galactosidase units
Wild type	None[a]	0[b]
	eIF(iso)4E	178
F59A[c]	None	0
	eIF(iso)4E	125
Y63A	None	0
	eIF(iso)4E	198
D77A	None	0
	eIF(iso)4E	0
D77E	None	0
	eIF(iso)4E	0
D77N	None	0
	eIF(iso)4E	0

[a] Yeast containing pJG4-5.
[b] Average value of three replicates from a typical experiment.
[c] First and second letters represent original and modified residues, respectively; the number is the residue position on VPg.

RNAs in rabbit reticulocyte lysates (9). The cap analogue m^7GTP used at a concentration of 10 μM inhibited complex formation by 60%, while GTP used at the same concentration had no effect on the formation of the complex. To determine what type of ligand relationship (i.e., competitive or noncompetitive) existed between VPg and m^7GTP, ELISA plate wells were coated with 1.0 μg of recombinant VPgPro and incubated with increasing concentrations of eIF(iso)4E in the absence or in the presence of 0.5 and 1.0 μM m^7GTP. Binding data were treated as enzyme kinetic data and were represented as a Lineweaver-Burk plot [i.e., $1/OD_{492}$ versus 1/eIF(iso)4E] (Fig. 4B). The experimental points were not expected to fall on a straight line since VPg and eIF(iso)4E are in the same concentration range, while in enzyme kinetics the substrate concentrations are much higher than the enzyme concentrations. Curves were fitted across the experimental points using least-square analysis, assuming a binomial equation of the following type: $y = ax - bx^2 + c$. The three lines crossed at a single point left of the y axis. Such a pattern is indicative of mixed-type noncompetitive ligand binding, meaning that VPg and m^7GTP can simultaneously bind eIF(iso)4E, but the binding of one ligand decreases the binding affinity for the second ligand (56). This binding relationship is depicted in Fig. 5, where K_1 and K_2 are the dissociation constants for the respective complexes, and "a" is the factor by which the constants increase when the other ligand is already bound. Data of the type shown in Fig. 4B may be used to extract the dissociation constants (K_d) for the VPg-eIF(iso)4E and the m^7GTP-eIF(iso)4E complexes (56). When 1/[eIF(iso)4E] approaches zero (i.e., [eIF(iso)4E] > [VPgPro]), the bx^2 term becomes negligible and the equation is now $y = ax + c$ and has the same form as the Lineweaver-Burk equation, $1/v = K_{app}/(V_{max}[S]) + 1/V_{max}$. Using the values estimated for the constants a and c for each curve, the calculated K_d for the VPg-eIF(iso)4E complex is 0.9 μM, and the K_d for m^7GTP is 0.4 μM. The dissociation constant for m^7GTP measured here is slightly lower when compared with the K_d of 2 to 9 μM previously obtained for the dissociation of m^7GTP with wheat eIF(iso)4E (57, 63) and can be explained by the different experimental procedures used to determine the constant value. Furthermore, the factor by which the K_d of one ligand increases when the other ligand occupies its binding site is estimated to be 4.3.

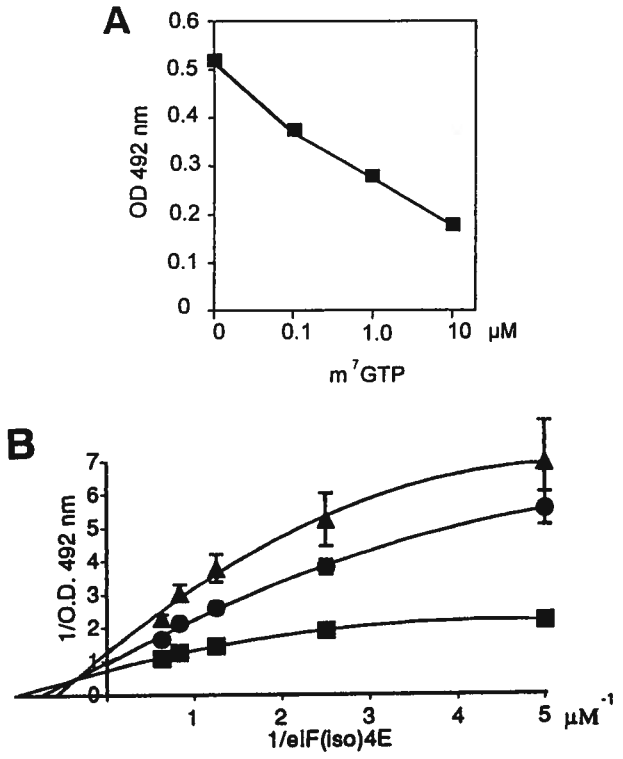

FIG. 4. Inhibition by m^7GTP of VPg-eIF(iso)4E complex formation as determined by ELISA-based binding assay. (A) Wells were coated with 1.0 μg of VPgPro and incubated with 2.0 μg of eIF(iso)4E from *A. thaliana* with increasing concentration of m^7GTP. Values are averages of two replicates from a typical experiment. (B) Lineweaver-Burk reciprocal representation of binding data. Wells were coated with 1 μg of VPgPro and incubated with increasing concentrations of eIF(iso)4E from *A. thaliana* in the absence (■) or presence, at 0.5 μM (●) or 1.0 μM (▲), of m^7GTP. Values are averages of two replicates from a typical experiment. Solid lines present the best fit of the data to equation $y = ax - bx^2 + c$.

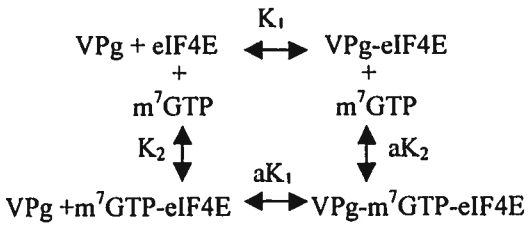

FIG. 5. Binding of VPg and m^7GTP to eIF(iso)4E.

Infection of whole plants. To determine if there is a correlation between the lack of in vitro interaction between VPg and eIF(iso)4E and debilitation of viral production, *B. perviridis* plants were infected with p35Tunos and p35TuD77N by particle bombardment. p35Tunos is an infectious cDNA clone of TuMV (53), and p35TuD77N is a p35Tunos derivative which contained the D77N mutation in the VPg domain that abolished the interaction with eIF(iso)4E. After bombardment, the plants were kept under an 18-h light regime at 22°C. After 8 days, plants bombarded with the wild-type infectious plasmid showed initial vein clearing followed by systemic mosaic symptoms. After 20 days, 14 of the 15 plants thus bombarded showed full symptoms of TuMV infection. On the other hand, plants bombarded with p35TuD77N remained symptomless. The presence or absence of viral proteins was confirmed by immunoblot analysis using a rabbit anti-TuMV capsid serum.

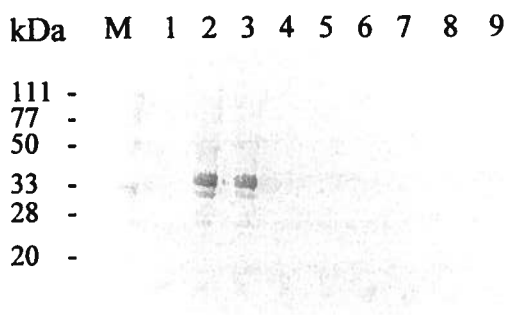

FIG. 6. Immunoblot analysis of *B. perviridis* plants bombarded with TuMV plasmid cDNA. After bombardment, plants were placed in a growth chamber for 10 days. Proteins were extracted from the new leaf emerging above the one bombarded, separated on a sodium dodecyl sulfate-polyacrylamide gel, transferred on a nitrocellulose membrane, and incubated with a rabbit anti-TuMV capsid serum. Lane 1, plant bombarded with gold particles not coated with DNA; lanes 2 and 3, plants bombarded with p35Tunos; lanes 4 to 9, plants bombarded with p35TuD77N; lane M, molecular mass standards.

No immunoreactive signal was found in mock-bombarded plants (Fig. 6, lane 1), while a strong signal of the expected molecular weight for the capsid protein was observed in plants bombarded with p35Tunos (lanes 2 and 3). No immunoreactive species were found in those plants bombarded with p35TuD77N (lanes 4 to 9).

DISCUSSION

Viruses use the cellular machinery for their replication, and this implies that viral proteins interact with proteins from the host. In this study, experiments were undertaken to investigate the biological importance of the interaction between the VPg of TuMV and eIF(iso)4E of *A. thaliana*. In *A. thaliana*, eIF4E and eIF(iso)4E share 70% identity in their amino acid sequence, and the identity between eIF(iso)4E from *A. thaliana* and from wheat is equally high at 70% (48). This high sequence homology is found in other plant species as well (6). The two factors are mechanistically equivalent for the translation process but exhibit differences in their ability to bind m^7GTP and other cap analogues (8), as well as in their expression in different organs (48). Because of this homology in sequence and function, VPg binding to eIF4E and eIF(iso)4E from *A. thaliana* as well as to eIF(iso)4E from wheat was expected and indicated that it can take place in many cell types and plant species, both monocotyledenous and dicotyledenous. In addition, the identification of the VPg domain interacting with eIF(iso)4E in a conserved region among potyviruses suggests that this interaction exists with other potyviruses as well. Preliminary experiments with the VPg of tobacco vein mottling potyvirus and plum pox potyvirus showed that indeed they can interact with eIF(iso)4E of *A. thaliana* (M. G. Fortin et al., unpublished results). Interaction with various initiation factor isomers and the identification of the binding domain in a highly conserved region of the VPg are indications that the interaction plays an important role in the viral life cycle. This presumed important role is supported by the fact that a mutation in VPg which abolished the interaction with the translation factor in vitro debilitated viral infection in whole plants.

The ELISA-based binding experiments indicated that the initiation factor can simultaneously make a complex with VPg and m^7GTP. Ligand binding showed negative cooperativity (i.e., one ligand decreases the affinity of the initiation factor for the other ligand). Lower ligand affinity can result from the binding of the first ligand physically hindering the binding of the second ligand. It can also be the consequence of eIF(iso)4E undergoing a conformational change, which is known to take place when eIF(iso)4E binds m^7GTP (57). This binding cooperativity seems to be a feature of eIF4E to regulate its activity. For instance, binding of mammalian eIF4G to eIF4E increased the affinity of the latter for the cap analogue (22), and the wheat germ poly(A) binding protein enhanced the binding affinity of eIF4E isomers for the cap analogue (63). A consequence of negative binding cooperativity would be that the interaction of VPg with eIF(iso)4E can lower the affinity of the initiation factor for the cap structure of mRNAs in planta, which may lead to a decrease in host protein synthesis.

Interaction of plant viruses with the host translation machinery and its consequence on protein synthesis has not been intensively investigated (4). Recently, inhibition of host gene expression has been associated with potyvirus replication. By examining the front of virus invasion in immature pea embryos infected with pea seed-borne mosaic potyvirus, decreased levels of host transcripts were observed (61), but not for all transcripts (5). Although no experimental explanation was provided, reduced transcript levels can result from an inhibition of transcription and/or from hydrolysis of mRNAs. However, transcript hydrolysis may be the consequence of inhibition of translation since there is a relationship between translatability and mRNA stability (1); it has been proposed that factors that stimulate translation initiation minimize the rate of entry of mRNA into decay pathways (26). For instance, a *cis*-acting mRNA stability determinant is the m^7Gppp cap. If less eIF(iso)4E is available for cap binding, the cap structure of mRNAs may become more susceptible to hydrolysis by decapping enzyme(s) (29), which then leads to degradation by 5′→3′ exonuclease(s) (1, 26). It remains to be seen if the interaction between VPg and eIF4E has any part to play in the observed inhibition of host gene expression during potyvirus infection.

This study showed that the ability of VPg to make a complex with eIF(iso)4E in vitro correlated with viral infection in planta. We are now attempting to elucidate the precise role of the VPg-eIF(iso)4E interaction in virus replication, i.e., whether VPg, when linked to viral RNA, can still bind the initiation factor and provide for the viral RNA a competitive edge over cellular mRNAs in translation initiation.

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