VIROLOGY 234, 84–92 (1997)
ARTICLE NO. VY978634

Interaction of the Viral Protein Genome Linked of Turnip Mosaic Potyvirus

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Received March 3, 1997; returned to author for revision April 3, 1997; accepted May 16, 1997

The yeast LexA interaction trap was used to screen a cDNA library from *Arabidopsis thaliana* in order to identify proteins that interact with the viral protein genome linked (VPg)–proteinase of turnip mosaic potyvirus. The screen allowed the isolation of four candidate cDNA clones. Clones pHC4, pHC21, and pHC40 were partially sequenced but no homologies to known proteins were found. However, the amino acid sequence deduced from the complete nucleotide sequence of pSW56 revealed that it was the eukaryotic initiation factor (iso) 4E [eIF(iso)4E]. Deletion analysis indicated that the VPg domain was involved in the interaction with the plant protein. Interaction between the viral protein and the cellular protein was confirmed by ELISA-based binding experiments. eIF(iso)4E plays an essential role in the initiation of the translation of capped mRNAs and its association with VPg would point to a role of the viral protein in the translation of the virus.

INTRODUCTION

Turnip mosaic virus (TuMV) belongs to the potyvirus group, a member of the supergroup of "picorna-like" viruses (Riechmann *et al.,* 1992). The TuMV genome has been sequenced and is a single RNA molecule of positive polarity featuring a 5′ nontranslated region (NTR) of 129 nucleotides (nt), an open reading frame of 9489 nt, and a 3′ NTR of 212 nt, followed by a poly(A) tract (Nicolas and Laliberté, 1992). The polyprotein encoded by the open reading frame has an expected molecular weight of 358 kDa and is processed by three viral proteinases into at least 10 mature polypeptides (Fig. 1). The viral protein genome linked–proteinase (VPgPro), which is analogous to the nuclear inclusion a protein of tobacco etch potyvirus (Dougherty and Parks, 1991), is responsible for most of the proteolytic events on the polyprotein, one of them being its autocatalytic cleavage into VPg and Pro (Laliberté *et al.,* 1992). The proteolytic activity resides in the C-terminal domain (Dougherty and Parks, 1991) while the N-terminal domain represents the VPg, a structural protein that is covalently linked to the 5′ end of the viral RNA (Riechmann *et al.,* 1992). In picornaviruses, the covalent attachment of the VPg to viral RNA is required for genome replication (Flanegan *et al.,* 1990; Kuhn and Wimmer, 1987). Consequently, by virtue of its association with the viral RNA and by analogy with the role played by its picornaviral counterpart, it is believed that the TuMV VPg, and possibly VPgPro, is involved in viral RNA replication.

Viral replication is the result of a complex interplay between functions encoded by the viral and host genomes. For instance, the RNA-dependent RNA polymerase (RdRp) of bacteriophage Qβ consists of one viral and three host-encoded subunits, and an additional host protein is needed for negative-strand synthesis on the positive-strand template (Blumenthal and Carmichael, 1979; Kajitani and Ishihama, 1991). Likewise, replication complexes have been described for several plant viruses and it was shown that host proteins cofractionated with viral proteins during purification. The best example for interaction between plant and viral proteins is perhaps the purification and solubilization of the cucumber mosaic virus (CMV) RdRp which supported the replication of the viral RNA: the replication complex contained the 1a and 2a proteins of CMV along with one host polypeptide (Hayes and Buck, 1990). The exact nature of these interacting plant proteins is not known, except for brome mosaic virus (BMV) where it has been shown that the viral 2a protein associated with the translational eukaryotic initiation factor (eIF) 3 (Quadt *et al.,* 1993).

While purification of replication complexes has allowed some insight as to the participation of host proteins in this process, genetic approaches are required for the identification and role of these host proteins. One potential genetic model system for the study of plant–virus interaction is *Arabidopsis thaliana* (Ishikawa *et al.,* 1991; Takahashi *et al.,* 1994), while another is yeast that has been shown to support replication of BMV (Janda

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0042-6822/97 $25.00

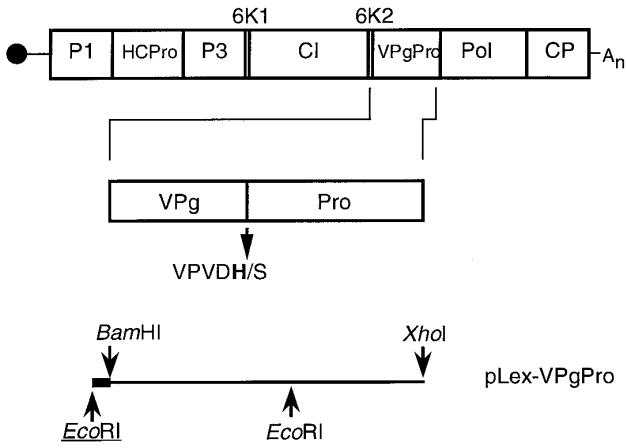

FIG. 1. Schematic representation of the genetic map of TuMV and the VPgPro cDNA recombinant molecule used in this study. Boxes represent the coding region of the viral RNA, and VPg is represented as black circle. Vertical solid lines indicate defined cleavage sites of the polyprotein. Restriction sites indicated above were used for the construction of the pLex-VPgPro, pLex-VPg, and pLex-Pro. The underlined *Eco*RI site comes from the vector. Single-letter amino acids represent the cleavage site recognized by Pro and the hydrolyzed peptide bond is marked (/). The replacement of a glutamic (E) for a histidine (H) residue preventing the autocatalytic cleavage of VPgPro is denoted by the boldface amino acid.

and Ahlquist, 1993). Equally, the yeast two-hybrid system is a powerful procedure for investigating protein–protein interactions. This procedure has permitted the characterization of protein associations *in vivo* where biochemical experiments, such as copurification, failed to show interaction (Brondyk *et al.,* 1995; Huet *et al.,* 1994; Rossi *et al.,* 1996), likely due to kinetic parameter constraints (Brent, 1996).

To further elucidate the replication strategy of TuMV, we employed the yeast LexA interaction trap (Gyuris *et al.,* 1993) to identify proteins from *A. thaliana,* a TuMV host, that interact with VPgPro. Here, we report the identification of several candidate interactors, among which is the cap-binding protein eIF(iso)4E, a key component in the initiation of translation.

MATERIALS AND METHODS

Microorganisms and media

Manipulations of bacterial as well as yeast strains and of nucleic acids and proteins were by standard methods (Golemis *et al.,* 1996; Sambrook *et al.,* 1989). *Escherichia coli* strain XL1-blue was used for subcloning and BL21(DE3) for protein expression. *Saccharomyces cerivisiae* strain EGY48 (*MATa, trp1, his3, ura3,* 6op-*Leu2*) was used for the interaction trap. Yeast cells were transformed by the lithium acetate method, omitting the addition of dimethyl sulfoxide as indicated (Golemis *et al.,* 1996). Yeast complete minimal (CM) medium lacking specific amino acids and/or uracil (dropout), supplemented with 2% glucose or 2% galactose/1% raffinose (w/v), was used.

Plasmids and cDNA library

The following plasmids were employed for the interaction trap study and are described in detail (Golemis *et al.,* 1996). pEG202 was used for the fusion of the bait protein to the DNA-binding domain of LexA. The pJG4-5 plasmid was used to express the *A. thaliana* cDNA library as translational fusions to a cassette consisting of the SV40 nuclear localization sequence, the acid blob B42, and the hemagglutinin epitope tag; expression is under the control of the *GAL1* inducible promoter. The *lacZ* reporter plasmids were pJK101 (for repression assay), pSH18-34 containing eight *lexA* operators (for screening and affinity assays), pJK103 containing two *lexA* operators, and pRB1840 containing one *lexA* operator. These last two plasmids were used for testing the affinity of the bait protein for the prey protein. Control plasmids were pSH17-4, which carries a transcriptionally active LexA–Gal4 fusion (positive control), and pRFHM1, which carries a transcriptionally inert LexA–bicoid fusion. The above plasmids contain growth selectable marker, the 2-μm origin of replication to allow propagation in yeast, the ampicillin resistance gene, and the pBR322 origin of replication to allow propagation in *E. coli.* The yeast strain and all the yeast plasmids used in this work were a generous gift of Roger Brent (Massachusetts General Hospital, Boston).

The bait plasmid pLex-VPgPro was constructed as follows: the region coding for VPgPro in plasmid pET-Pro/24 (Laliberté *et al.,* 1992) was amplified by the polymerase chain reaction (PCR) using the forward and reverse polynucleotide primers 5′-AAAGGCAGGATCCAAAGACAG-3′ and 5′-TTTGTGCCTCGAGTGCCGTGCTAT-3′. The amplified fragment was digested with the enzymes *Bam*HI and *Xho*I, ligated with similarly restricted pEG202, and introduced into *E. coli.*

The *A. thaliana* cDNA library was cloned in pJG4-5 and was provided by H. M. Goodman (Massachusetts General Hospital, Boston). The library was made from mRNAs of 6- to 8-day-old plants and insert sizes ranged from 0.5 to 2.4 kb.

Interaction hunt

The interaction hunt and the β-galactosidase filter assay followed exactly the procedure as previously described (Golemis *et al.,* 1996). The degree of interaction was quantified using the β-galactosidase liquid assay. The precultures were grown in selective medium supplemented with 2% glucose. A 1:20 dilution of the overnight cultures was transferred into the induction medium (2% galactose/1% raffinose) and cells were grown to midlog phase ($OD_{600} \sim 0.5$ to 1.0). Aliquots of between 0.05 and 0.20 ml were taken and the reaction volume was adjusted

to 1.0 ml with Z buffer (0.06 *M* Na_2HPO_4, 0.04 *M* NaH_2PO_4, 0.001 *M* $MgSO_4$, and 0.05 *M* β-mercaptoethanol, pH 7.0). The cells were permeabilized with sodium dodecyl sulfate (SDS) and chloroform. The *o*-nitrophenyl-β-D-galactopyranoside was used as substrate. The optical density of the reaction was measured by spectrophotometry at 420 and 550 nm. The β-galactosidase activity was calculated using the following equation, units $= 1000 \times (OD_{420} - 1.75 \times OD_{550})/(T \times V \times OD_{660})$, where T is time in minutes and V is volume in milliliters of culture used. Values reported are the averages of duplicate assays with at least three independently isolated yeast colonies, along with the standard deviation.

Recombinant protein expression and purification

Plasmid pSW56 was digested with *Eco*RI and *Xho*I and the 0.7-kb insert was ligated with similarly restricted pET21a (Novagen). The resulting plasmid pETtag(iso)4E codes for eIF(iso)4E fused at its N-terminal end to the 11-amino-acid N-terminal peptide of T7 gene 10 protein which is recognized by the anti-T7tag monoclonal antibody (Novagen). A second plasmid, pET(iso)4E, was generated by omitting the T7tag sequence; plasmid pET14-(iso)4E (kindly provided by C. Robaglia, INRA, France; this clone was independently obtained but is identical to the gene encoded by pSW56, except for a few nucleotides at the 5′ end) was digested with *Nde*I and *Bam*HI and the insert was ligated with similarly restricted pET21a. Those plasmids were introduced into *E. coli* BL21(DE3). An overnight culture was diluted 1:100 in fresh medium and after 3 hr of growth at room temperature, protein expression was induced with 0.4 m*M* isopropylthio-β-galactoside (IPTG).

The recombinant proteins, eIF(iso)4E and tag-eIF-(iso)4E, were purified as described (Van Heerden and Browning, 1994) with the following modifications: The cell pellets from 1 liter of induced *E. coli* harboring pET(iso)4E or pETtag-(iso)4E were resuspended in 5 ml of buffer B-500 (20 m*M* HEPES [pH 7.6], 1 m*M* DTT, 0.1 m*M* EDTA, 10% glycerol, 500 m*M* KCl) containing 0.5 m*M* PMSF. The cells were passed through a french press. The supernatant was diluted 1:5 in buffer B-0 (B-500 buffer without KCl) and was applied on a 1-ml m^7GTP–Sepharose resin (Pharmacia) equilibrated in buffer B-100 (B-500 buffer with 100 m*M* KCl). The column was washed with 40 ml of buffer B-100. Recombinant proteins were eluted with 0.1 m*M* m^7GTP. All purification steps were done at 4°. VPgPro was purified as previously described (Laliberté *et al.,* 1992). Purified eIF(iso)4E, tag-eIF(iso)4E, and VPgPro were dialyzed extensively against a buffer containing 20 m*M* HEPES [pH 7.6], 100 m*M* KCl, and 1 m*M* DTT.

ELISA-based binding assay

Purified VPgPro was adsorbed to the wells of an ELISA plate (1 μg/well) by overnight incubation at 4°. The remaining binding capacity of the wells was blocked with Blotto 5%/PBS 1×. The purified tag-eIF(iso)4E was diluted in Blotto 1%/PBS 1× with Tween 0.2% and was incubated for 1 hr at 4° with the previously coated wells. In the case of the competition assay, the VPgPro-coated wells were co-incubated with tag-eIF(iso)4E (1 μg) and increasing concentrations of eIF(iso)4E. The detection of the specific interaction was achieved as in ELISA with the anti-T7tag antibody and peroxidase-labeled goat anti-mouse immunoglobulin G (KPL). After each incubation the wells were washed three times with H_2O/Tween 0.05%.

Sequencing and sequence analysis

Nucleotide sequences were determined using the Sanger dideoxy-termination method on recombinant pJG4-5 with the sequencing primer BCO1, 5′-CCAGCCT-CTTGCTGAGTGGAGATG-3′, or on pTZ18U following subcloning. The reactions were run on the ALF DNA sequencer (Pharmacia). Sequence comparisons were performed using the default settings of the BLAST program (Altschul *et al.,* 1990) on the National Center for Biotechnology Information World Wide Web server.

The GenBank Accession No. for the *A. thaliana* eIF(iso)4E is U62044.

RESULTS

Nucleotides 5899 to 7203 of the TuMV genome encoding VPgPro were amplified by PCR and introduced into pEG202 by directional cloning to create pLex-VPgPro (Fig. 1). To prevent autocatalytic cleavage of the protein, a mutant form was chosen in which the glutamic acid residue preceding the hydrolyzed peptide bond was replaced by a histidine residue; the proteinase retains its full activity but is not able to cleave the VPg/Pro site (Laliberté *et al.,* 1992). pLex-VPgPro was introduced into EGY48 yeast cells along with the *lacZ* reporter plasmid pSH18-34. The stable expression of the fusion protein was confirmed by immunoblotting of protein extracts using an anti-TuMV VPgPro rabbit serum (data not shown). Activation and repression assays (Golemis *et al.,* 1996) confirmed that the the fusion protein by itself did not activate the reporter genes and that it was localized in the nucleus (data not shown).

EGY48 cells, harboring pLex-VPgPro and pSH18-34, were transformed with the *A. thaliana* cDNA library inserted into pJG4-5 and plated on glucose CM medium lacking his, trp, and ura. The primary transformant cells (10^5 CFU) were pooled and were plated for selection. Approximately 200 colonies grew on galactose/raffinose CM medium lacking his, trp, ura, and leu. These colonies were picked and were filter-assayed for β-galactosidase activity following growth on glucose or galactose/raffinose plates: 10% of the colonies turned blue on galactose/raffinose plates and not on glucose plates. The recombinant pJG4-5 plasmids were recov-

TABLE 1

β-Galactosidase Activities Displayed between Various DNA-Binding Fusion and Activating Fusion Plasmids

Binding domain	Activating domain	β-Galactosidase activity (U)
pLex-VPgPro	pHC4	25 ± 8
pLex-VPgPro	pHC21	444 ± 143
pLex-VPgPro	pHC40	50 ± 26
pLex-VPgPro	pSW56	588 ± 69
pLex-VPgPro	pJG4-5	⩽0.05
pLex-gPV	pHC4	⩽0.05
pLex-gPV	pHC21	⩽0.05
pLex-gPV	pHC40	⩽0.05
pLex-gPV	pSW56	⩽0.05
pRFHM1	pHC4	⩽0.05
pRFHM1	pHC21	⩽0.05
pRFHM1	pHC40	⩽0.05
pRFHM1	pSW56	⩽0.05
pSH17-4 (positive control)		813 ± 164

ered from these positive clones. To reduce the number of clones to be tested for specificity, redundant clones were eliminated by restriction enzyme mapping and Southern blot analyses.

EGY48 yeast cells were retransformed with individual cDNA plasmids purified from yeast colonies which were positive on filter assay along with pLex-VPgPro, or with plasmids directing the expression of other proteins fused to LexA, in order to evaluate the specificity of the interaction. Independent colonies from each transformation were tested for Leu2 activation. All yeast transformants grew well on glucose-based medium containing leucine, while none grew on glucose-based medium lacking leucine. Equally, transformants grew well on galactose/raffinose-based medium containing leucine while only the candidate cDNA plasmids paired with pLex-VPgPro grew on galactose/raffinose-based medium lacking leucine. No colonies were observed with the combination pLex-gPV/candidate cDNA plasmids, pRFHM1/candidate cDNA plasmids, or pLex-VPgPro/pJG4-5. pLex-gPV contains the reverse complement of the gene coding for the VPg domain and pRFHM1 codes for the homeodomain of bicoid protein from *Drosophila melanogaster* fused to lexA. The specificity assay led to the isolation of at least four distinct clones: pHC4, pHC21, pHC40, and pSW56. In addition, pSW56 was picked up on three independent screenings and is an indication, at least for this clone, that its selection was not an episodic event.

β-Galactosidase activity was measured to evaluate the degree of interaction of the protein encoded by four different plasmid clones with VPgPro. In this test, pLex-gPV, pRFHM1, and pJG4-5 were used as negative controls. Table 1 lists the activity measured for each particular plasmid combination expressed in units. Candidate interactors showed significant activity only with pLex-VPgPro. In particular, pSW56 had β-galactosidase levels comparable to those generated by the Gal4 transcriptional activation domain directly fused to LexA (pSH17-4).

Partial sequences for pHC4, pHC21, and pHC40 were obtained (data not shown) and homology searches in databases were undertaken using the BLAST program. pHC4, pHC21, and pHC40 corresponded to the *A. thaliana* dBEST clones 118B1T7 (Accession No. T42858), 127B24T7 (Accession No. T44588), and VBVWD03 (Accession No. F14051), respectively. However, the encoded proteins did not have any significant homology to known proteins.

On the other hand, the complete nucleotide sequence of pSW56 was determined and is shown in Fig. 2a, along with the deduced amino acid sequence. Searches in databases indicated that pSW56 and its encoded protein have a strong homology with the cap-binding protein eIF4E from several organisms and would thus be the *A. thaliana* representative of this initiation factor. However, knowledge about the actual N-terminus of the protein encoded by pSW56 is lacking. Amino acid sequences of the *A. thaliana* protein, eIF(iso)4E from wheat, and eIF4E from wheat are shown in Fig. 2b. The comparison indicates that the *A. thaliana* protein is more similar to wheat eIF(iso)4E (54% identity) than to wheat eIF4E (40% identity). This would suggest that pSW56 codes for the eIF(iso)4E isomer. Allen *et al.* (1992) reported the existence of two isoforms for eIF4E in wheat, eIF(iso)4E (p28 subunit) and eIF4E (p26 subunit). These cap-binding proteins associate with p82 and p200 subunits to form eIF (iso)4F and eIF4F, respectively. The 28- and 82-kDa polypeptides of eIF(iso)4F are antigenically distinct from the 26- and 220-kDa polypeptides of eIF4F (Browning *et al.,* 1987). Both have functional properties that are very similar; in fact the eIF4F can be substituted with the isoform in an *in vitro* system (Lax *et al.,* 1985). Additionally, eIF4E is characterized by a high proportion of tryptophan residues whose positions are well conserved. There are 10 tryptophan residues in the *A. thaliana* protein, 1 more than in the wheat proteins and 2 more than in mammalian cap-binding factor; these residues were shown to be important for binding the cap structure of mRNAs (Altmann *et al.,* 1988).

To determine which domain of the VPgPro interacts with eIF(iso)4E, pEG-VPg was constructed by deleting most of the region coding for Pro. The deletion was obtained by partial digestion of pLex-VPgPro with *Eco*RI and complete digestion with *Xho*I (Fig. 1). The interaction was evaluated by measuring β-galactosidase activity. Levels of activity similar to those found with VPgPro were obtained (Table 2), indicating that the interaction is mediated by VPg and that the Pro domain does not have any effect on the strength of the interaction. pLex-Pro was also constructed by excising the *Eco*RI fragment from pLex-VPgPro and was assayed for interaction with pSW56; no activity was measured.

Estojak *et al.* (1995) found that the strength of protein–

a

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GAA TTC GGC ACG AGA CCA AAA GCC GTC TCA GAA GAA AAC ACA ACT GCG AAG AAT ATG GCG   60
 E   F   G   T   R   P   K   A   V   S   E   E   N   T   T   A   K   N   M   A   20

ACC GAT GAT GTG AAC GAG CCT CTC CCG GCG GCG GCG GAA TTA CCG GCG ACA GAG GCG GAG  120
 T   D   D   V   N   E   P   L   P   A   A   A   E   L   P   A   T   E   A   E   40

AAA CAA CCA CAC AAG CTC GAA AGA AAG TGG AGT TTC TGG TTC GAT AAC CAA TCA AAG AAA  180
 K   Q   P   H   K   L   E   R   K   W   S   F   W   F   D   N   Q   S   K   K   60
                                     1           2

GGC GCC GCC TGG GGA GCT TCT CTT CGT AAA GCC TAT ACT TTC GAC ACC GTC GAA GAT TTT  240
 G   A   A   W   G   A   S   L   R   K   A   Y   T   F   D   T   V   E   D   F   80
             3

TGG GGA TTG CAC GAG ACT ATA TTT CAG ACT AGC AAA TTG ACA GCG AAT GCT GAA ATT CAC  300
 W   G   L   H   E   T   I   F   Q   T   S   K   L   T   A   N   A   E   I   H  100
 4

TTG TTC AAA GCT GGT GTT GAG CCA AAG TGG GAA GAT CCA GAG TGT GCT AAT GGA GGA AAG  360
 L   F   K   A   G   V   E   P   K   W   E   D   P   E   C   A   N   G   G   K  120
                                     5

TGG ACT TGG GTT GTT ACT GCT AAT AGG AAG GAA GCT TTA GAC AAA GGC TGG CTT GAA ACT  420
 W   T   W   V   V   T   A   N   R   K   E   A   L   D   K   G   W   L   E   T  140
 6       7                                                   8

TTG ATG GCT TTG ATT GGA GAG CAA TTT GAT GAA GCA GAT GAG ATT TGT GGT GTG GTT GCT  480
 L   M   A   L   I   G   E   Q   F   D   E   A   D   E   I   C   G   V   V   A  160

AGT GTC CGT CCA CAG TCG AAG CAA GAC AAG CTC TCC TTG TGG ACT AGG ACG AAG TCT AAT  540
 S   V   R   P   Q   S   K   Q   D   K   L   S   L   W   T   R   T   K   S   N  180
                                             9

GAA GCT GTT CTG ATG GGT ATT GGG AAG AAG TGG AAG GAG ATA CTT GAT GTC ACC GAC AAG  600
 E   A   V   L   M   G   I   G   K   K   W   K   E   I   L   D   V   T   D   K  200
                                     10

ATA ACT TTC AAT AAC CAT GAT GAT TCG AGA AGA AGC CGA TTC ACT GTT TGA AGAAACGAGTG  662
 I   T   F   N   N   H   D   D   S   R   R   S   R   F   T   V   *              216

TGAAACCAGAGGAAGATGCAATATGGCTTTTTTTTTGTGATACTGTTTGGTCGGTTTACTCAAATAGCTGAGACATTTA  741
GGGACATTCACTTTGAGGTGTAAACTCTACAAAGTTTTCTGTTTCATTAAAATGATTTTTTGTTATAAAAA         812
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b

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                                                                    1  2            3
A. thaliana clone 56   EFGTRPKAVSEENTTAKNMATDDVNEPLPAAAELPATEAEKQPHKLERKWSFWFDNQ··SKKGAAWGASLRKAYT
wheat eIF(iso)4E       ........MA-VEAA···LPVAATET-EV--EGDAGAAEA-G----Q-Q-T--Y-IQTKP-P-----T--K-G--
wheat eIF4E            ·····················EGEIADDGDGSSA-AAGRITA-P--NA-T-----PQGKSRQV---STIHPIH-

                              4                                  5          6 7              8
A. thaliana clone 56   FDTVEDFWGLHETIFQTSKLTANAEIHLFKAGVEPKWEDPECANGGKWTWVVTANRKEALDKGWLETLMALIGEQ
wheat eIF(iso)4E       -----E--C-YDQ--RP---VGS-DF-------------------------··-ISS--TN--TM----C-------
wheat eIF4E            -S--------YNN-HNP---NVG-DF-C--NKI-------I----------··ISCG-GKS·-TF--H--L-M----

                                                      9                10
A. thaliana clone 56   FDEADEICGVVASVRPQSKQDKLSLWTRTKSNEAVLMGIGKKWKEILDVTDKITFNNHDDSRR·····SRFTV
wheat eIF(iso)4E       ---SQ----------Q··R---------K-A-----QVD-------VI-YN--MVYSF-----SQKPSRGG-Y--
wheat eIF4E            --FG-----A-V---Q····ERVAI--KNAA---AQIS---Q---F--YK-S-G-IV-E-AK-SDKGPKN-Y--
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FIG. 2. (a) Nucleotide and deduced amino acid sequences of pSW56. Nucleotide residues are numbered on the top; amino acid residues are numbered on the bottom. Tryptophan residues are indicated in boldface letters and are numbered. The nucleotides indicated in italic represent the *Eco*RI site used for cloning in pJG4-5 and provide the correct reading frame. (b) Comparison of the amino acid sequence for cap-binding protein of *A. thaliana* clone pSW56 and wheat. Amino acid sequences were aligned using MSA Version 2.0 provided on the World Wide Web server. Dashes indicate amino acid identical to that of the *A. thaliana* clone pSW56. Dots indicate a gap inserted by the alignment program.

protein interactions can be estimated using the interaction trap by measuring the level of β-galactosidase activity with *lacZ* reporter plasmids showing thresholds of activation, such that weak interactions ($K_d > 1\ \mu M$) were not detected. In order to discriminate whether VPgPro–eIF(iso)4E and VPg–eIF(iso)4E interactions are of high, intermediate, or low affinity, β-galactosidase activity was measured using *lacZ* reporter vectors which differ in their sensitivity to transcriptional activation (one, two, and eight *lexA* operators) (Table 2). Both pLex-VPgPro and pLex-VPg paired with pSW56 provided substantial activation with all three reporters and the activity measured with the least sensitive reporter plasmid was still relatively high. As a preliminary estimate, the strength of the interaction between the viral protein and the initiation factor maybe considered high and likely to be physiologically probable.

The cDNA insert from clone pSW56 was subcloned into an IPTG-inducible *E. coli* expression vector which allowed the synthesis of tag-eIF(iso)4E. In this case, the

TABLE 2

Interaction-Dependent β-Galactosidase Activity of the *lacZ* Reporter Containing Different Numbers of *lexA* Operators

Binding domain	Activating domain	β-Galactosidase activity (U) *lacZ* reporter pRB1840 1op-*lacz*	pJK103 2op-*lacz*	pSH18-34 8op-*lacz*
pLex-VPgPro	pSW56	125 ± 17	490 ± 39	588 ± 69
	pJG4-5	⩽0.05	⩽0.05	⩽0.05
pLex-VPg	pSW56	381 ± 86	565 ± 118	498 ± 79
	pJG4-5	⩽0.05	⩽0.05	⩽0.05
pRFHM1	pSW56	⩽0.05	⩽0.05	⩽0.05
	pJG4-5	⩽0.05	⩽0.05	⩽0.05

initiation factor is fused at its N-terminal to the 11-amino-acid N-terminal peptide of T7 gene 10 protein. The recombinant protein was purified by affinity chromatography on m^7GTP–Sepharose (Fig. 3) and reacted with anti-T7tag monoclonal antibody (data not shown). It also reacted with antibodies raised against wheat eIF(iso)4E (data not shown), which further indicated that the isolated cDNA encodes a cap-binding protein. The apparent molecular weight of the recombinant protein is about 30 kDa, which is slightly higher than the expected size from the amino acid data. eIF(iso)4E [same as tag-eIF(iso)4E but omitting the T7tag sequence] was similarly purified (data not shown). To demonstrate direct association between VPgPro and eIF(iso)4E, these proteins were subjected to an ELISA-based interaction assay (Heuer *et al.,* 1996). The wells of an ELISA tray were coated with VPgPro and were incubated with different concentrations of purified tag-eIF(iso)4E. The adsorbance measured fitted a saturation curve with increasing concentrations of the translational factor (Fig. 4a). A background level signal was measured when the wells had not been precoated with VPgPro, and no significant interaction was detected when an *E. coli* lysate not expressing VPgPro was used. When a competition assay between a tag-eIF(iso)4E and eIF(iso)4E for binding VPgPro was performed, the absorbance reading was reduced with increasing concentrations of eIF(iso)4E (Fig. 4b). These results indicate that the interaction was specific for the eIF(iso)4E domain and that interaction observed was not conferred by the presence of the T7tag moiety.

DISCUSSION

VPgPro plays an important role in the maturation of the potyviral polyprotein and possibly in viral replication (Riechmann *et al.,* 1992). However, replication requires the contribution of host factors which have yet to be identified. In this report, the yeast interaction trap, based on the LexA activator protein, was used to identify host proteins that form a complex with VPgPro. Following the screening of a cDNA library from *A. thaliana,* four candidate interactor proteins were isolated and were shown by genetic tests to be specific for the potyviral protein. These clones provided galactose-dependent (condition under which the protein encoded by the cDNA is expressed) growth of yeasts on plates lacking leucine only in combination with pLex-VPgPro. Growth on solid medium lacking leucine was further corroborated using *lacZ* expression, and the measured β-galactosidase activity was of the same order of magnitude as observed for known interacting proteins (Campbell *et al.,* 1995; Estojak *et al.,* 1995; McBride *et al.,* 1996). The direct association between the protein encoded by pSW56 and VPgPro that was uncovered with the interaction trap was further confirmed by *in vitro* binding experiment. The affinity of this last interacting pair is likely to be significant since either pLex-VPgPro/pSW56 or pLex-VPg/pSW56 provided high β-galactosidase activity, whether the *lacZ* reporter plasmids contained one, two, or eight *lexA* operators.

Database searches did not provide any information as to the nature of the protein encoded by pHC4, pHC21, and pHC40. On the other hand, sequence comparison indicated that pSW56 codes for eIF(iso)4E, an isomeric form of the cap-binding protein eIF4E, although the N-terminal amino acids need to be determined. The existence of the isomeric form is observed so far only in higher plants: wheat, maize, and cauliflower (Browning *et al.,* 1992). In mammalian cells, eIF4E is a component of the eIF4F complex; the other subunits are the eIF4A (helicase) and eIF4G proteins (Merrick, 1992). Most eukaryotic mRNAs contain a cap structure [m^7G(5′)ppp(5′)N; where N is any nucleotide] at their 5′-terminus. This structure is required for efficient attachment of the mRNA to the 40S ribosomal subunit and the association is mediated by eIF4F (through binding to eIF4E) and eIF3 (Merrick, 1992). eIF4E plays a key role in the regulation of translation (Hershey, 1991): it is present in limiting amounts in the cell, its phosphorylation state correlates

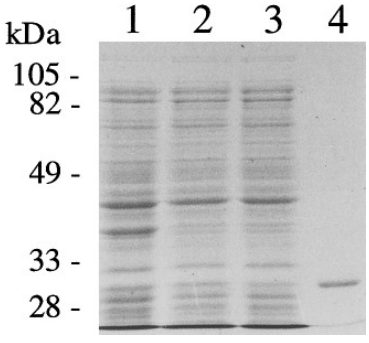

FIG. 3. Purification of tag-eIF(iso)4E. Expression and purification were as described under Materials and Methods. Samples were loaded on a SDS–polyacrylamide gel and were stained with Coomassie blue. Lane 1, induced pETtag(iso)4E; lane 2, supernatant from the cell lysate; lane 3, effluent; and lane 4, m^7GTP–Sepharose eluate.

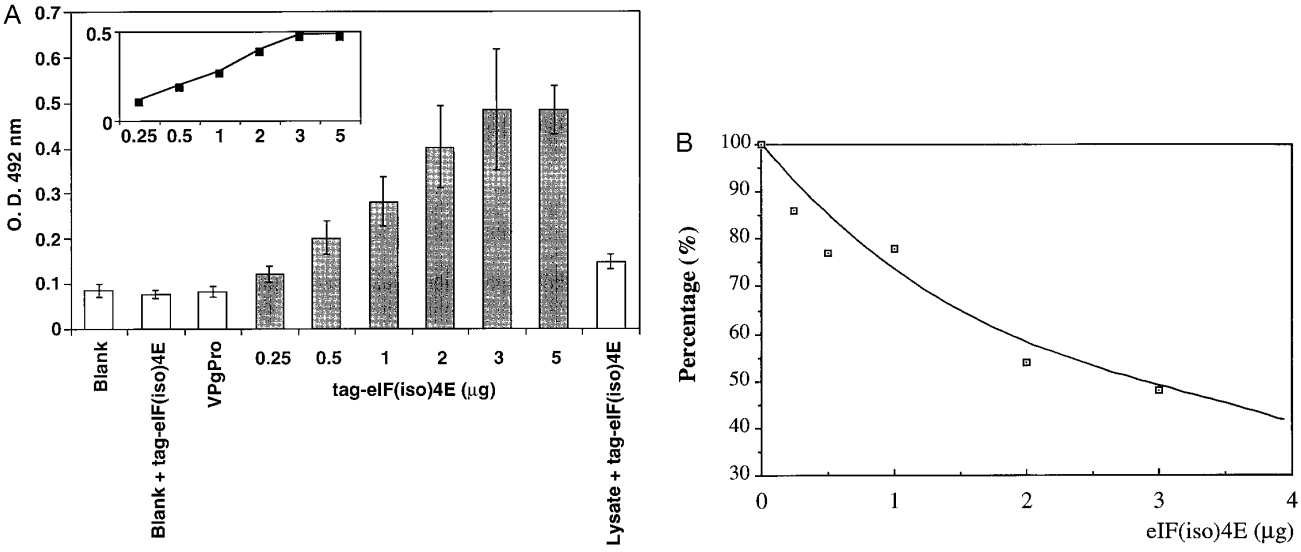

FIG. 4. (a) ELISA-based binding assay. Wells precoated with VPgPro were incubated with increasing amount (μg) of tag-eIF(iso)4E protein (represented by gray columns). Empty columns are control experiments in which wells were coated with Blotto only and no tag-eIF(iso)4E was added (blank), coated with Blotto only and incubated with 2 μg of tag-eIF(iso)4E [blank + tag-eIF(iso)4E], coated with VPgPro and no tag-eIF(iso)4E was added (VPgPro), and coated with an *E.coli* lysate not expressing VPgPro and 2 μg of tag-eIF(iso)4E was added [lysate + tag-eIF(iso)4E]. Error bars represent standard deviations for triplicate wells. (Inset) Data are represented as a saturation binding curve. (b) Competition assay. Wells precoated with VPgPro were incubated with 1 μg of tag-eIF(iso)4E protein and with increasing amounts of eIF(iso)4E. The detection of tag-eIF(iso)4E interacting with the VPgPro is expressed as percentage of binding with respect to no eIF(iso)4E added.

with the rate of protein synthesis, and it is able to transform rodent cells when overexpressed (De Benedetti *et al.,* 1994; Lazaris-Karatzas *et al.,* 1990). The activity of eIF4E is modulated by two specific binding proteins (4E-BP1 and 2) (Haghighat *et al.,* 1995; Lin *et al.,* 1994; Mader *et al.,* 1995). The association of 4E-BP1 with eIF4E decreases the translation of capped mRNAs but not of uncapped mRNAs. This is achieved by direct competion of 4E-BP1 with eIF4G for binding to eIF4E (Haghighat *et al.,* 1995). eIF4E is also found in the nucleus (Lejbkowicz *et al.,* 1992; Ptushkina *et al.,* 1996) and it has been suggested that it plays a role in nucleocytoplasmic transport (Rousseau *et al.,* 1996). eIF4E is thus an important regulatory element for the initiation of translation.

The interaction between VPg and eIF(iso)4E may therefore be a key event for the establishment of TuMV infection. However, the interaction between the translational initiation factor and the potyviral protein uncovered using the yeast two-hybrid system and confirmed by *in vitro* binding experiments must above all make biological sense in an infected plant cell. For true *in planta* contact to be considered, the interacting pair must be found in the same cell compartment. This would be the case for VPgPro and eIF(iso)4E since VPgPro-containing potyviral replication complexes are associated with the rough endoplasmic reticulum (Martin *et al.,* 1995), which also contains eIF4E. Similarly, presence of translational initiation factors in replication complexes has also been reported for BMV; eIF3 was shown to interact with the viral protein 2a (RdRP) (Quadt *et al.,* 1993).

The functional significance of the interaction between VPg and eIF(iso)4E remains to be determined. Animal viruses often alter the host cell translational machinery to allow more efficient expression of virally encoded proteins. In many cases, eIF4E is directly or indirectly involved (Feigenblum and Schneider, 1993; Gingras *et al.,* 1996; Huang and Schneider, 1991; Kirchweger *et al.,* 1994, Lamphear *et al.,* 1995; Liebig *et al.,* 1993; Zhang *et al.,* 1994). Translation of the viral RNA proceeds by a cap-independent mechanism whereby ribosomes bind internally at specific sites on the viral RNAs (Ohlmann *et al.,* 1995; Pelletier and Sonenberg, 1988) or because of a reduced requirement for the initiation factor (Huang and Schneider, 1991). Consequently, one possibility is that the interaction of VPg with eIF(iso)4E leads to inactivation of the translational factor, resulting in host protein synthesis shutdown. Evidence for this latter event is a report that showed pea-seed-borne mosaic potyviral infection causing inhibition of host gene expression and protein accumulation along the infection front (Wang and Maule, 1995). Inactivation of eIF(iso)4E should then be accompanied by a lower dependence of the viral RNA for this factor in order to be efficiently translated. Cap-independent translation has been demonstrated for a certain number of plant viral RNAs. Of particular interest for this discussion, the 5′ leader sequence of tobacco mosaic virus RNA renders translation of reporter mRNAs eIF4E-independent in *S. cerevisiae* extracts (Altmann *et al.,* 1990). Furthermore, potyviral RNA 5′ NTR provides cap-independent translation (Carrington and Freed, 1990; Riechmann *et al.,* 1991), and evidence for internal ribosome entry for TuMV RNA has been reported (Basso

et al., 1994). Alternatively, interaction of VPg with eIF(iso)4E may not lead to inactivation of the host factor but would rather secure the limiting amount of eIF(iso)4E for viral RNA translation at the expense of cellular mRNA translation. Finally, it is also possible that this interaction is important for replication.

This report showed that VPg interacts with eIF(iso)4E of *A. thaliana.* Future *in vitro* studies will be required to determine the kinetic parameters in order to evaluate the strength of the association of the two proteins and if indeed interaction leads to inactivation of eIF(iso)4E.

ACKNOWLEDGMENT

We deeply appreciate the provision of plasmids for the two-hybrid system by R. Brent and his colleagues, and we thank as well H. Zhang and H. M. Goodman for providing the *A. thaliana* cDNA library. We are grateful to C. Robaglia for the plasmid pET14-(iso)4E. We thank K. S. Browning from the University of Texas for a generous gift of the antiserum against wheat anti-eIF(iso)4E. We thank F. Shareck and L. Trempe for the oligonucleotide synthesis as well as J. Roger and L. Forget for nucleic acid sequencing. This work was supported by the Natural Sciences and Engineering Research Council of Canada.

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