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A Plant Viral "Reinitiation" Factor Interacts with the Host Translational Machinery

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Summary

The cauliflower mosaic virus transactivator, TAV, controls translation reinitiation of major open reading frames on polycistronic RNA. We show here that TAV function depends on its association with polysomes and eukaryotic initiation factor eIF3 in vitro and in vivo. TAV physically interacts with eIF3 and the 60S ribosomal subunit. Two proteins mediating these interactions were identified: eIF3g and 60S ribosomal protein L24. Transient expression of eIF3g and L24 in plant protoplasts strongly affects TAV-mediated reinitiation activity. We demonstrate that TAV/eIF3/40S and eIF3/TAV/60S ternary complexes form in vitro, and propose that TAV mediates efficient recruitment of eIF3 to polysomes, allowing translation of polycistronic mRNAs by reinitiation, overcoming the normal cell barriers to this process.

Introduction

Initiation of translation on eukaryotic mRNAs is a complex undertaking (Jackson, 2000). The main pathway of initiation on both cellular and viral mRNAs is cap-dependent ribosome scanning. According to the widely accepted model, initiation begins with assembly of the 43S ribosomal complex, comprising the 40S ribosomal subunit, eIF1, eIF1A, eIF3, and the eIF2/GTP/Met/tRNA$_i$ ternary complex (Hershey and Merrick, 2000). eIF5 has been implicated as participating in this complex by bridging eIF3 and eIF2 (Bandyopadhyay and Maitra, 1999; Asano et al., 2000). Attachment of the 43S complex to the capped 5′-end of the mRNA requires cap binding complex eIF4F (composed of eIF4E, eIF4G, and eIF4A), eIF4B, and ATP. Multiple interactions between eIF4E, eIF4G, eIF3, and the 40S ribosomal subunit play a critical role in recruitment of the latter to the 5′-end of the mRNA, resulting in formation of a 48S initiation complex. This latter complex scans the 5′-untranslated region, supported by eIF1 and eIF1A, searching for a suitable initiation codon (Pestova et al., 1998a).

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After codon-anticodon base pairing at the first AUG in a favorable initiation context, eIF5 stimulates GTP hydrolysis and, presumably, the release of initiation factors from 40S ribosomes. However, little is known about how, and when, interactions between initiation factors and the 40S ribosome within the 48S complex are disrupted. The 48S complex that assembles at the initiation codon is joined by the 60S ribosomal subunit, and recent data have clarified the role of eIF5B as the 60S subunit joining factor (Pestova et al., 2000), to form the functional 80S ribosome.

The pathway of initiation is significantly simplified in some specialized initiation mechanisms, such as cap-independent scanning, and internal initiation (reviewed by Jackson, 2000), thus providing examples of minimal factor requirements to start translation. These unusual translation initiation pathways are often exploited by viruses to regulate translation independently of host control and avoiding the need for numerous initiation factors. For example, in hepatitis C virus (HCV) and pestivirus, the 43S ribosomal preinitiation complex binds directly to an internal ribosome entry site (IRES) just upstream of the start codon; only eIF3 and the ternary complex are required for codon-anticodon base pairing (Pestova et al., 1998b).

Very little is known about factor requirements for reinitiation of translation, when ribosomes having terminated translation of an ORF give rise to 40S subunits capable of resuming scanning and reinitiating at a downstream AUG. Although RNAs with two or more long ORFs are rare in eukaryotic cells, reinitiation can occur in cases where the upstream ORF (uORF) is relatively short (2 to about 20 codons), with reinitiation frequency increasing with the distance between the uORF and the "main" ORF (Kozak, 1987; Hinnebusch, 1997; Fütterer and Hohn, 1992; Luukkonen et al., 1995). It has been speculated that loss of initiation factors may not be complete after translation of a short ORF (sORF) (Kozak, 1987). Remaining initiation factors might help 40S ribosomal subunits to resume scanning and/or allow them to stay reinitiation competent (Fütterer and Hohn, 1992). Factors influencing reinitation frequency are largely unknown, although a requirement for activated initiation factor eIF2 seems to be inherently obvious. The best known examples of reinitiation after uORF translation are the yeast GCN4 mRNA (Hinnebusch, 1997), where reinitiation at the main ORF depends on depletion of the active eIF2/GTP/Met-tRNA$_i$ ternary complex and requires timespan controls (Dever et al., 1992), and the polycistronic mRNAs of caulimoviruses, where reinitiation occurs also after translation of a long ORF and requires the viral protein TAV (transactivator; Bonneville et al., 1989; Gowda et al., 1989; Fütterer and Hohn, 1991). There are other reports demonstrating reinitiation after long ORF translation at AUG codons located a short distance from a termination codon in artificial RNA constructs in mammalian cells (Peabody and Berg, 1986a, 1986b).

CaMV is a plant pararetrovirus that uses reverse transcription for genome amplification (Rothnie et al., 1994).

Two promoters direct the production of the terminally redundant pregenomic 35S RNA and the 19S subgenomic RNA encompassing ORF VI. The 35S RNA, alternatively used as a replicative intermediate or as a polycistronic mRNA for expression of viral proteins, consists of a 600 nt long leader sequence containing several AUG codons, followed by seven to eight tightly arranged long ORFs encoding all of the viral proteins. Initiation of translation on the 35S RNA is 5′-cap-dependent (Fütterer and Hohn, 1991; Schmidt-Puchta et al., 1997), and leads first to recognition of the AUG of sORF A within the leader sequence (Dominguez et al., 1998; Ryabova and Hohn, 2000; Pooggin et al., 2000).

TAV—encoded by ORF VI—is very abundant and forms a dense matrix in the cytoplasm of infected cells. It has many functions in the life cycle of the virus (Rothnie et al., 1994). Most relevantly here, it stimulates reinitiation of translation of major ORFs on the 35S polycistronic RNA (Fütterer et al., 1990; Scholthof et al., 1992).

Two types of translation reinitiation have been demonstrated on the 35S RNA. First, reinitiation within, or downstream of, the CaMV leader (the latter accomplished by ribosomal shunting) after translation of sORF A (Ryabova and Hohn, 2000). This type of reinitiation is stimulated 2–3 fold by TAV (Pooggin et al., 2000). The second type is TAV-activated reinitiation after translation of the long viral ORFs (Bonneville et al., 1989; Fütterer et al., 1990; Scholthof et al., 1992); in this case reinitiation is greatly stimulated by TAV, whereas initiation at the first ORF is not significantly affected. Specific *cis* sequence signals are not required for transactivation, since TAV can activate reinitiation after translation of any first ORF in an artificial bicistronic RNA (Fütterer and Hohn, 1991, 1992). TAV-mediated reinitiation is not much affected by the distance between two ORFs. Notably, the presence of a sORF (optimally around 30 codons) upstream of both long ORFs strongly enhances the process. A long overlap of the major ORFs (130 nt) inhibits transactivation, whereas a short overlap (17 nt) is permissible.

We investigated whether interaction of TAV with host cell proteins might provide some clues as to its function in transactivation. We show that TAV interacts directly with several components of the host translational machinery, thereby changing its properties to allow polycistronic translation.

Results

TAV Interacts with eIF3g, a Subunit of Translation Initiation Factor eIF3, and with Ribosomal Protein L24

To investigate whether TAV exerts its effect on reinitiation of translation via an interaction with the translation machinery, purified recombinant TAV expressed in *E. coli* was incubated with polysomes isolated from turnip plants, yeast, and *E. coli*, and complexes were analyzed on sucrose gradients (Supplementary Figure S1, http://www.cell.com/cgi/content/full/106/6/723/DC1). When incubated with polysomes isolated from turnip plants, about 50% of the TAV protein was found specifically associated with turnip polysomes, and about 30% of TAV cosedimented with yeast polysomes in sucrose gradients. In contrast, there was no significant shift of TAV to polysome-containing fractions from *E. coli*. The interaction of TAV with a polysomal component thus appears to be specific for eukaryotes.

To identify host factors interacting with TAV, we used the yeast two-hybrid method to screen an *Arabidopsis thaliana* cDNA library (Fromont-Racine et al., 1997) with three versions of TAV: full-length, an amino terminal portion (NTAV, residues 26–242), and a carboxy terminal portion (CTAV, residues 242–520). An efficient mating strategy on filters, followed by direct plating on selective media, was employed. Six cDNA clones selected with CTAV and two selected with full-length TAV encoded a protein corresponding to a subunit g of initiation factor eIF3 (for nomenclature, see Burks et al., 2001). Assuming that translation starts at AUG^{109}, the cDNA for *A. thaliana* eIF3g encodes a protein of 294 amino acids with a mobility in SDS-PAGE of 35 kDa (accession number AJ 293728). This corresponds well to the p36 purified from the wheat germ eIF3 complex by Heufler et al. (1988). Indeed, monoclonal antibodies to purified p36 (Heufler et al., 1988) specifically recognized a version of our eIF3g fused to GST (Supplementary Figure S2).

Of the remaining cDNA clones isolated in the CTAV screen, one encoded a protein corresponding to one of two copies of the *A. thaliana* ribosomal protein L24 (accession number T47559), except for a minor difference in the C-terminal part (accession number AJ 293729). *A. thaliana* L24 (164 amino acids) is related to ribosomal proteins from other species, and is especially basic, even for a ribosomal protein.

TAV Interacts with eIF3g and L24 In Vitro

To validate our in vivo results, we overexpressed GST-fusions to eIF3g and L24 in *E. coli* and purified them using glutathione-Sepharose 4B beads. ^{35}S-labeled TAV and a globin control (see Experimental Procedures) were produced by in vitro translation in a wheat germ extract. The labeled proteins were either directly analyzed on SDS-PAGE (Figure 1A, lanes 1 and 2) or incubated with GST, GST-eIF3g, or GST-L24 bound to glutathione beads. After removal of supernatant with unbound proteins (unbound fraction, U) and extensive washing, proteins bound to glutathione beads were eluted (bound fraction, B), separated by SDS-PAGE (Figure 1A, lower panel), and visualized by autoradiography (Figure 1A, upper panel). The results show that TAV was present in the unbound fraction after incubation with GST alone, indicating no interaction (Figure 1A, lane 4). In contrast, after incubation with GST-eIF3g or GST-L24, TAV was found in the bound fraction (Figure 1A, lanes 9 and 13). The ^{35}S-labeled globin control was in the unbound fraction throughout. These observations strongly suggest specific binding of full-length TAV to eIF3g and L24. TAV contains two RNA binding domains in its C-terminal part, RBa and RBb (Figure 2A; De Tapia et al., 1993), and one in its N-terminal part, MAV (Figure 2A; Cerritelli et al., 1998). To show that interactions of TAV with eIF3g and L24 were not mediated by RNA, the mixture of interacting partners was digested by an RNase cocktail (Figure 1A, lanes 16–21). Again, TAV was found in bound fractions when incubated with GST-eIF3g and GST-L24 (lanes 18 and 20), but not with GST alone (lane 16). ^{35}S-labeled globin was not found in bound fractions.

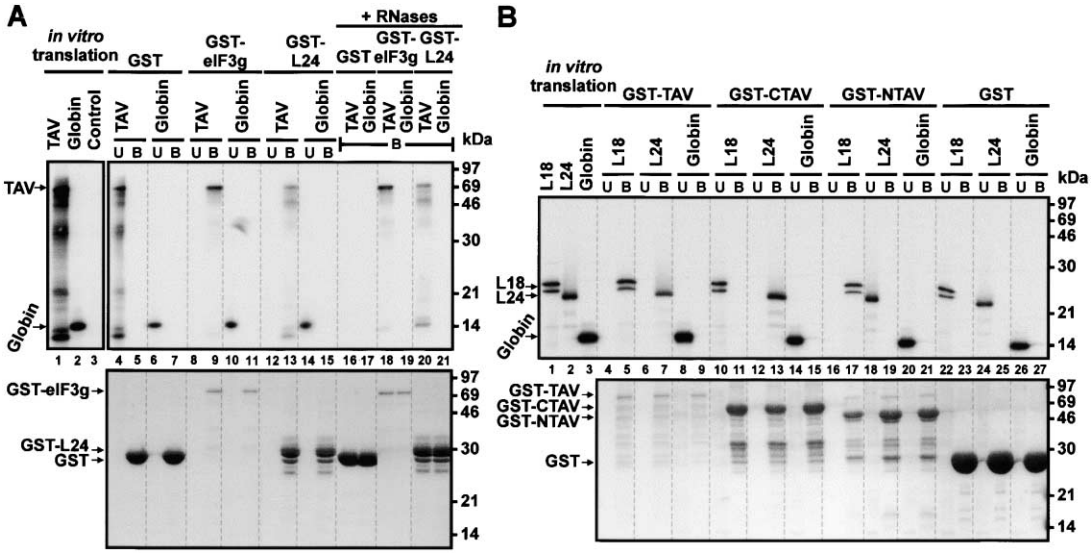

Figure 1. TAV Interacts with eIF3g, L24, and L18 In Vitro

(A) Autoradiography (upper panel) of SDS-PAGE gel of ^{35}S-labeled proteins produced by in vitro translation from TAV or globin mRNAs (lanes 1 and 2, respectively; lane 3, no-mRNA control). Lanes 4–21: interaction of GST, GST-eIF3g, or GST-L24 bound to glutathione Sepharose 4B beads with ^{35}S-labeled TAV or globin. U, unbound; B, bound fractions. Lanes 16–21 show the experiment performed in the presence of an RNase cocktail (see Experimental Procedures). The lower panel shows the same gel stained with Coomassie blue. The positions of size markers are indicated on the right.

(B) Autoradiography (upper panel) of SDS-PAGE of ^{35}S-labeled proteins produced by in vitro translation from L18, L24, and globin mRNAs (lanes 1–3, respectively). The upper band of L18 corresponds to 6His::L18 fusion (the first AUG of this fusion is in an unfavorable context for initiation of translation). Lanes 4–27: interaction of GST, GST-TAV, GST-CTAV, and GST-NTAV bound to glutathione Sepharose 4B beads with ^{35}S-labeled L24, L18, and globin. The lower panel shows the same gel stained with Coomassie blue.

This result suggests that RNA does not mediate these interactions.

Interaction of the N-terminal domain of TAV with *A. thaliana* 60S ribosomal protein L18 was recently demonstrated by Far Western assay (Leh et al., 2000). We used the GST pull-down assay to assess the specificity of binding of this ribosomal protein to TAV (Figure 1B). GST-TAV, GST-CTAV, GST-NTAV, or GST (bottom panel) bound to beads were incubated with ^{35}S-labeled L18 or L24 synthesized in wheat germ extract (Figure 1B, lanes 1 and 2, respectively). GST-TAV associated with ^{35}S-labeled L18 and L24, but not with ^{35}S-labeled globin (Figure 1B, upper panel, lanes 5, 7, and 8, respectively). A notable difference was observed: L18 interacted only with GST-NTAV (lane 17), and L24 only with GST-CTAV (lane 13). Thus, the two ribosomal proteins bind to different regions of TAV.

eIF3 and L24 Bind to the Same Central Region of TAV

To further delineate the regions of TAV involved in binding to eIF3g and L24, additional TAV truncated mutants were tested for binding in yeast two-hybrid assays. The results (Figure 2A) show that neither the N-terminal 242 amino acid (aa) residues (BD-NTAV) nor the C-terminal part encompassing aa 379–520 (BD-C5) could support significant binding to either eIF3g or L24 (AD-eIF3g and AD-L24), whereas a larger C-terminal fragment encompassing aa 242–520 (BD-CTAV) interacted strongly with both partners. Some of this activity was retained in the short central part (aa 242–310; BD-C1). The efficiency of both interactions was greatly increased by extending the central polypeptide C1 on both ends by including aa 216–241 and 311–320 (BD-C4), while neither one of these additions alone (BD-C2 or BD-C3) had any effect. This suggests that short sequences flanking the C1 region in concert have the potential to significantly improve the affinity of the TAV fusion protein for both eIF3g and L24, most probably by improving folding of the C1 region. The results also demonstrate that both eIF3g and L24 bind to the same central region of TAV.

The C1 segment contains the most conserved motif within TAV among different caulimoviruses (Figure 2D). According to secondary structure predictions (http://dodo.cpmc.columbia.edu/predictprotein), the unstructured part of this motif (aa 299–310) starting from Gly^{299} (Figure 2D) is located between two conserved α helices. Tyr^{305}, the central amino acid of this unstructured region, was mutated to Pro to test the involvement of this conserved TAV motif in binding. Indeed, this mutation (Y305P) abolished the interaction of TAV with both eIF3g and L24 (Figure 2A).

The regions of eIF3g and L24 involved in interaction with TAV were also mapped. The central part of eIF3g, spanning residues from 65–173 (segment F2), seems to be critical for the eIF3g-TAV interaction (Figure 2B). This segment contains a well-conserved Zn-finger motif. A single mutation in this motif (C139S) strongly interfered with eIF3g-TAV binding, while a double mutation (C139S, C142S) abolished the interaction (Figure 2B). The C-terminal part of eIF3g comprising the RNA recognition motif (AD-F3) was not necessary for TAV binding.

A short N-terminal segment of L24 (amino acids 1–65,

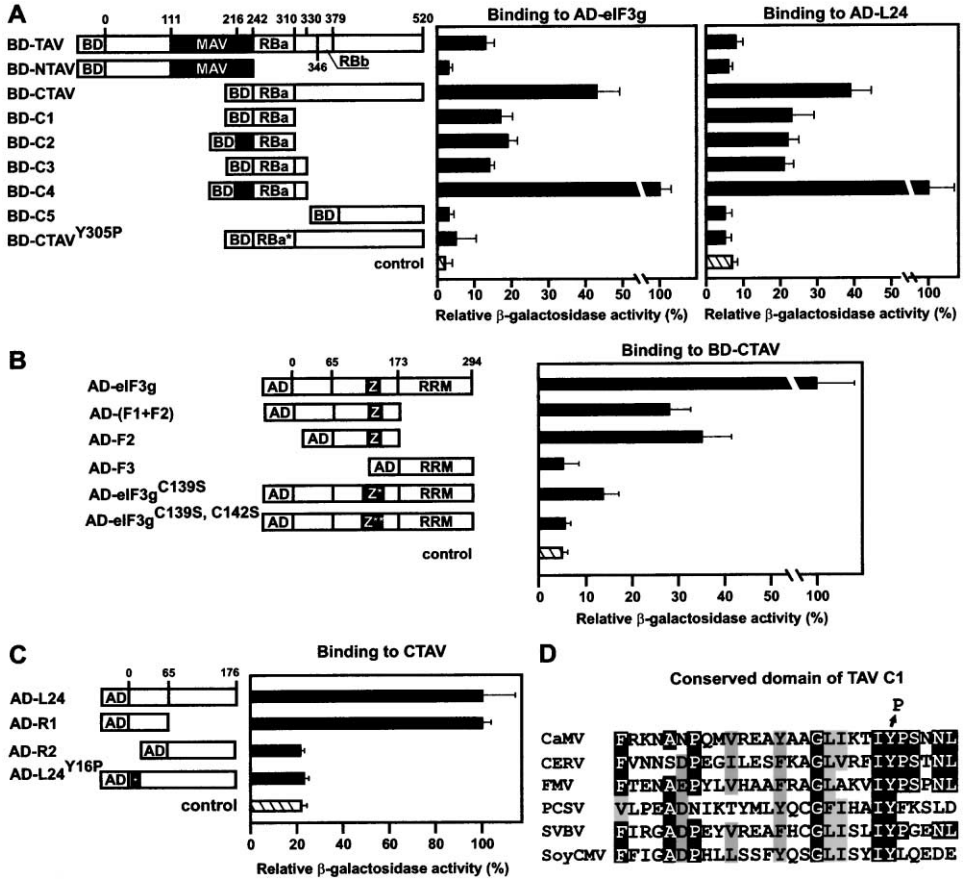

Figure 2. Mapping of Interacting Regions

(A) Quantitation of the interaction between TAV or TAV deletion mutants fused to the Gal4 binding domain (BD) and eIF3g or L24 fused to the Gal4 activation domain (AD) in the yeast two-hybrid system. Interactions were scored by measuring β-galactosidase activity in liquid assay. The highest value of β-galactosidase activity in the diploids transformed with the corresponding constructs is set to 100% (12 and 4 Miller units for BD-CTAV interactions with AD-eIF3g and AD-L24, respectively). MAV, minimal region required for transactivation; RBa and RBb, RNA binding domains.

(B) eIF3g and mutants and their binding activities to CTAV. Zinc-finger-like sequence (Z) and RNA recognition motif (RRM) are indicated.

(C) L24 and mutants and their binding activities to CTAV. Results in (A), (B), and (C) represent the mean values from triplicates ± standard deviation.

(D) Similarity between conserved motifs within RBa of TAV from different caulimoviruses. CaMV (283-310), CERV, carnation etched ring virus (274-301); FMV, figwort mosaic virus (274-301); PCSV, peanut chlorotic streak virus (219-246); SVBV, strawberry vein binding virus (292-319); SoyCMV, soybean chlorotic mottle virus (225-257). Identical residues are printed in reverse type and conserved residues are shaded in agreement with Blossom 62 and Jonson amino acid substitution matrixes. The position of the Y305P mutation is shown with an arrow. Profiles of conserved motifs between homologous caulimovirus TAVs were observed using the MEME motif-discovery tool (http://www.sdsc.edu/MEME/meme.2.2/website/meme-adv.html).

AD-R1) interacts with CTAV to the same extent as the full-length L24 (Figure 2C), while the C-terminal part, AD-R2, appears not to be involved in the interaction. The N terminus is the most conserved part of L24 in eukaryotes, and corresponds to the full-length protein in archaebacteria (Hatakeyama et al., 1989). According to secondary structure predictions, the N terminus of L24 (aa 12–20) harbors a Gly-rich unstructured portion followed by a short β sheet (aa 21–25). A single mutation in this region (Y16P) abolished L24-TAV binding (Figure 2C).

Expression levels of the different modified or truncated proteins were controlled by Western blotting, and no significant variation in the amount of protein expressed was observed compared to the wild-type fusion proteins, BD-TAV, AD-eIF3g, and AD-L24 (data not shown).

eIF3g and L24 Compete for TAV Binding

To investigate possible competition between TAV-interacting partners, we used a three-hybrid system (Tirode et al., 1997) involving simultaneous expression of three polypeptides to allow or prevent the formation of a transcriptional activator complex (see Figure 3A). Besides the two-hybrid fusion proteins, a third protein is expressed under the control of the Met25 promoter, which is positively regulated by lack of methionine.

We tested β-galactosidase expression in yeast cells transformed with a combination of the two plasmids, pAD-CTAV and pBrBD-L24/Met-eIF3g (see Figure 3A and its legend for details). LacZ expression depended on the presence of methionine (Figure 3B, cf. lanes 3 and 6), indicating that eIF3g outcompetes L24 for interaction with CTAV, leading to a LacZ$^-$ phenotype. In contrast,

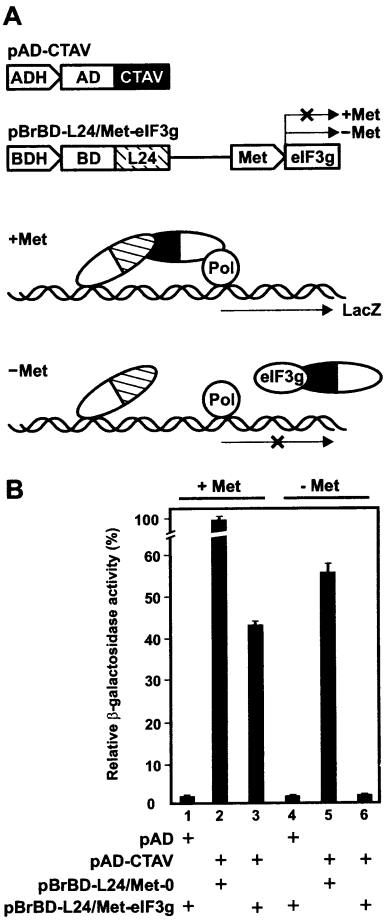

Figure 3. eIF3g Outcompetes L24 for TAV Binding

(A) The three-hybrid system vectors are depicted schematically with a model of transcription activation by reconstitution of Gal4 activity. (B) Quantitation of β-galactosidase activity for yeast cells cotransformed with the pairs of plasmids indicated. Note that under the conditions used, the presence or absence of methionine in the medium can itself influence results of three-hybrid tests (cf. lanes 2 and 5).

cells cotransformed with pAD-CTAV and pBrBD-L24/Met-0 show a LacZ$^+$ phenotype in both the presence and absence of methionine (Figure 3B, lanes 2 and 5).

These results suggest that eIF3g and L24 compete with each other for interaction with TAV in vivo.

60S Ribosomes and Intact eIF3 Interact with TAV

The results shown so far suggest a physical association of TAV with both the 60S subunit and eIF3. To test these interactions, wheat germ 60S or 40S subunits or complete rabbit eIF3 were incubated with GST-TAV or GST bound to glutathione beads. The results show significant association of 60S and eIF3 to GST-TAV (bound fractions, Figures 4A, lane 8, and 4B, lane 7). TAV does not significantly interact with 40S ribosomes in our assay (Figure 4A, lane 12). Neither 60S nor eIF3 interacted with GST alone (Figure 4A, lanes 5 and 9, and Figure 4B, lane 4). These data support the specific binding of TAV to these two components of the translational machinery.

To investigate whether TAV could also form a complex with 40S ribosomes via eIF3, rabbit eIF3 and wheat germ 40S subunits were incubated with GST-TAV or GST bound to glutathione beads. The results show formation of a TAV/eIF3/40S complex (bound fractions, Figure 4C, lane 8), while no complexes were formed when GST was used as a control (lane 6).

To determine whether TAV is able to mediate binding of eIF3 to 60S ribosomal subunits, recombinant His-TAV expressed in *E. coli* was incubated with GST-eIF3g bound to glutathione beads with and without 60S ribosomes. 60S ribosomes bound to eIF3g only in the presence of His-TAV, forming an eIF3g/TAV/60S complex (Figure 4D, cf. lanes 5, 7, and 9).

The TAV-eIF3 Complex Associates with Polysomes In Planta

To examine whether TAV and eIF3 are associated with polysomes, polysomes prepared from healthy and CaMV-infected turnip plants were fractionated on sucrose gradients. Remarkably, the polysomal fraction increased significantly in CaMV-infected plants (cf. A and B). The amount of TAV and eIF3 in fractions collected from these gradients was analyzed by Western blot analysis with polyclonal purified anti-TAV antiserum (Nakayashiki et al., 1993) and anti-human eIF3b (PRT1) antiserum (Lin et al., 2001) (Figures 5A and 5B).

A significant proportion of both TAV and eIF3b was found together in polysomes from CaMV-infected cells (Figure 5B, cf. fractions 6 and 7), while in healthy plants, eIF3b accumulates only in 40S fractions and monosomes (Figure 5A, fractions 1–3).

In a control experiment, a mixture of eIF3 and recombinant His-TAV was found on the top of the gradient in the absence of polysomes (data not shown).

Interactions between TAV and Host Factors Affect Reinitiation of Translation in Plant Protoplasts

We next tested the effect of TAV-interacting proteins on TAV transactivation capacity. TAV activity can be detected in transient expression experiments in plant protoplasts transfected with dicistronic constructs (Bonneville et al., 1989). MAV, a minimal segment of TAV (aa 111–242), which is still included in NTAV, supports a residual level of transactivation in *N. plumbaginifolia* protoplasts (De Tapia et al., 1993). To analyze the effect of transiently-expressed eIF3g on TAV- and MAV-mediated transactivation, a set of two expression units under the control of the 35S promoter was used (Figure 6A). The first construct, pmonoCAT, contains a single chloramphenicol acetyltransferase (CAT) ORF, and is used to measure the frequency of first translation initiation events. The second construct, pbiGUS, contains two ORFs: CaMV ORF VII and β-glucuronidase (GUS). GUS activity is used as a measure of the frequency of reinitiation events. In the absence of TAV, the CAT ORF expressed well, while GUS activity was barely detectable (Figure 6B, lane 1). Cotransfection of a plasmid expressing full-length TAV (p35S-P6; Kobayashi et al., 1998) resulted in the appearance of GUS activity (Figure 6B, lane 2), with MAV coexpression giving about 20% of the level of TAV-mediated transactivation activity (lane 11). TAV point mutant Y305P (TAV^{Y305P}) diminished TAV-mediated transactivation in *N. plumbaginifolia* (lane 9)

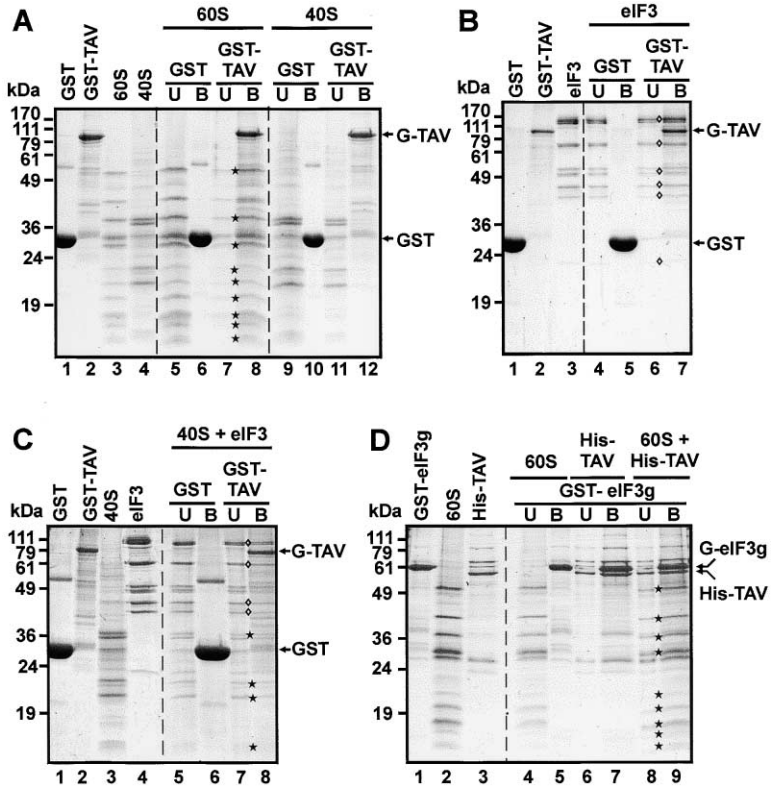

Figure 4. TAV Binds Directly to eIF3 and 60S

GST, GST-TAV, GST-eIF3g, and His-TAV were overexpressed in *E. coli* and purified by affinity chromatography; 60S, 40S, and eIF3 were purified from extracts. The left panel of each gel shows the purified components. For the pull-down experiments, GST and its derivatives were bound to glutathione beads and incubated with the components to be tested. The beads were then washed and the unbound (U) and bound (B) fractions assayed by SDS-PAGE and stained with Coomassie blue. The positions of size markers are indicated on the left.

(A) Interactions of 60S and 40S with GST and GST-TAV (lanes 5 to 12). Stars (lane 8) show 60S ribosomal proteins specifically coprecipitated with GST-TAV.

(B) GST and GST-TAV interactions with eIF3 (lanes 4–7). Diamonds (lane 7) show eIF3 subunits specifically coprecipitated with GST-TAV.

(C) GST and GST-TAV interactions with 40S and eIF3, both individually and together (lanes 5–8). Stars and diamonds (lane 8) indicate 40S and eIF3 subunits specifically coprecipitated with GST-TAV, respectively.

(D) GST-eIF3g interactions with 60S and His-TAV, both individually and together (lanes 4–9). Stars (lane 9) show 60S ribosomal proteins specifically coprecipitated with GST-eIF3g in the presence of TAV.

to a level comparable to that attributable to MAV, consistent with the observed disruption of TAV and eIF3g or L24 interactions (see Figure 2A) and suggesting direct involvement of TAV in sequestering of these proteins. TAV and TAV^{Y305P} were well expressed in *N. plumbaginifolia* protoplasts as controlled by Western blotting (data not shown).

Overexpression of increasing amounts of eIF3g led to significant inhibition of TAV-mediated GUS expression (Figure 6B, lanes 3–6). We explain this by competition of subunit g alone with the endogenous complete complex eIF3.

A single mutation within the Zn-finger motif of eIF3g (C139S) restored more than half of TAV-mediated activity, while simultaneous mutation of two cysteine residues (C139S and C142S) almost fully restored it (Figure 6B, lanes 7 and 8, respectively). This result correlates well with the finding that the eIF3g region containing the Zn-finger motif plays an important role in TAV-eIF3g interaction. No effect was observed when eIF3g was cotransfected together with TAV point mutant Y305P or MAV (cf. lanes 9 and 10, 11 and 12, respectively), in good agreement with our observation that NTAV (containing MAV) does not interact with eIF3g (see Figure 2A).

In contrast to eIF3g, overexpression of L24 stimulated TAV-mediated reinitiation (Figure 6C, lanes 3–5); again, TAV mutant Y305P- or MAV-mediated reinitiation was not affected (cf. lanes 8 and 9, 10 and 11, respectively). The L24 enhancing effect was abolished in L24^{Y16P} (lane 6), which correlates well with observed disruption of interactions between this mutant and TAV (see Figure 2C). Overexpression of both interacting partners, L24

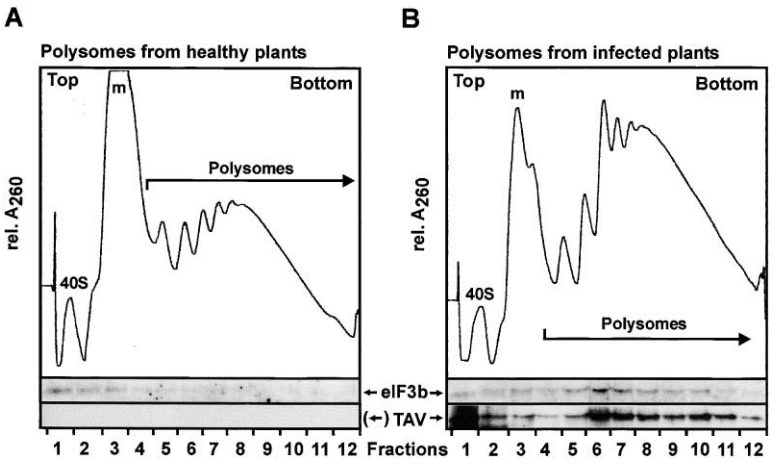

Figure 5. Cosedimentation of TAV-eIF3 Complexes with Polysomes

Polysomes from healthy (A) and CaMV-infected (B) plants were subjected to velocity sedimentation through sucrose density gradients. Gradients were fractionated while scanning at 254 nm, and the resulting absorbance profiles are shown. Positions of 40S, monosomes (m), and polysomes are indicated. Aliquots (300 μl) of each fraction were precipitated with 20% TCA and analyzed by SDS-PAGE and immunoblotting using polyclonal antibodies against TAV and eIF3b (lower panels).

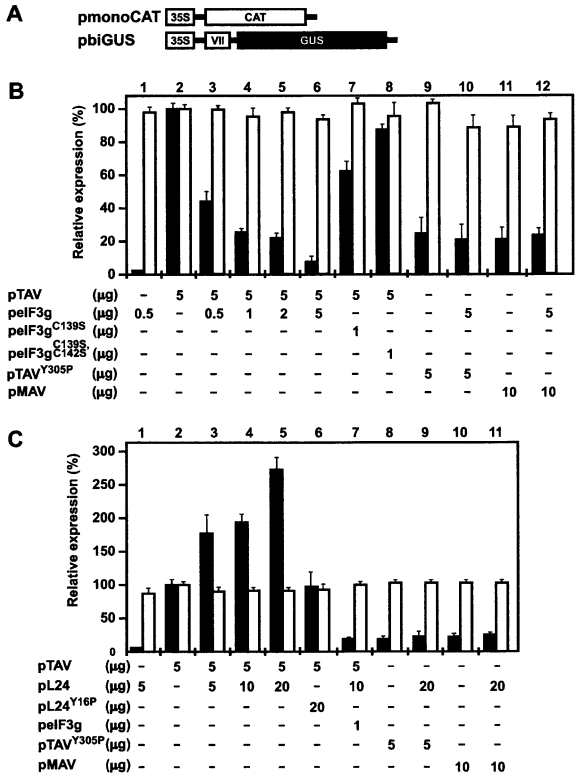

Figure 6. Interaction of TAV with the Host Translation Machinery Affects Polycistronic Expression

All transfection experiments in *N. plumbaginifolia* protoplasts included the two reporter plasmids which are shown in (A), as well as effector plasmids in the amounts indicated below the graphs in B and C. Results shown represent the means obtained in three independent experiments. CAT and GUS levels in the presence of the reporters and TAV only are set at 100%.

(B) Inhibition of TAV activity by eIF3g and the effect of its mutants.

(C) Stimulation of TAV by L24 and the effects of its mutants. CAT (white bars) and GUS (black bars) expression levels measured in protoplast extracts are indicated.

and eIF3g, inhibited transactivation by TAV (Figure 6C, lane 7), consistent with our finding that eIF3g outcompetes L24 for TAV binding (see Figure 3B).

TAV Point Mutants Abolish CaMV Infectivity

Wild-type TAV or mutant derivatives Y305P and Y305H were introduced into plasmids containing a TAV-deficient viral genome and used for infectivity studies in turnip plants. Two independent clones were used for each mutant. The introduction of wild-type TAV resulted in strong symptoms of infection two weeks after inoculation. Neither of the mutants showed any signs of infection, even at increased inoculum concentration, for as long as three months. This result shows that the eIF3g/L24-interacting domain of TAV involved with the TAV-mediated transactivation process is important for virus viability.

Discussion

CaMV TAV is an example of a viral protein involved in the process of reinitiation of translation in eukaryotes. TAV is associated with the host translational machinery, forming a complex with eukaryotic polyribosomes. It interacts with 60S ribosomal subunits and the key initiation factor eIF3. eIF3 is bound to TAV via subunit g, and interaction with 60S ribosomal subunits occurs via at least two ribosomal proteins, L24 and, as recently reported (Leh et al., 2000), L18.

TAV consists of two distinguishable domains having different functions contributing to high transactivation efficiency (De Tapia et al., 1993). The core functional domain of TAV (MAV; aa 111–241) can support residual transactivation activity when present in high surplus concentrations in protoplasts of *N. plumbaginifolia*, but not in *Orychophragmus violaceus* protoplasts (De Tapia et al., 1993). The C-terminal part of TAV can efficiently inhibit the transactivation activity of the entire protein, suggesting that CTAV is able to sequester host factors that are essential for transactivation activity.

eIF3 and Its Interactions

eIF3 is required for mRNA binding to the 40S ribosome, and stimulates binding of the ternary complex to the 40S ribosome (Hershey and Merrick, 2000). Subunit g, to which TAV binds directly, is one of the ten subunits shared by mammalian and plant eIF3s (Burks et al., 2001) and one of five subunits that form a functional "core" complex in yeast [TIF32 (eIF3a), PRT1 (eIF3b), NIP1 (eIF3c), TIF34 (eIF3i), and TIF35 (eIF3g)] (Phan et al., 1998). In yeast, a known binding partner of the N-terminal portion of eIF3g is eIF3i (Verlhac et al., 1997). Yeast eIF3g also interacts with eIF4B (Vornlocher et al., 1999). We observed similar interactions of *A. thaliana* eIF3g with *A. thaliana* eIF3i and eIF4B using GST pull-down assays (H.-S.P., T.H., and L.R., unpublished data). In yeast, the N-terminal part of eIF3g containing the Zn-finger motif is essential, whereas the C-terminal RNA binding domain is not required for assembly of a functional complex (Verlhac et al., 1997).

Our experiments show that the central region of eIF3g, including the Zn-finger motif, is required for TAV binding. This domain is probably available on the surface of eIF3, since the complete factor binds TAV (Figure 4B) and free subunit g significantly inhibits TAV-dependent reinitiation in plant protoplasts (Figure 6B), presumably due to competition with endogenous eIF3.

TAV and Its Interactions with the 60S Ribosome

L24 and L18 belong to the class of ribosomal proteins present only in eukaryotic and archaebacterial ribosomes (Ban et al., 2000), consistent with our results showing that TAV does not interact with *E. coli* polyribosomes. L24 from archaebacteria corresponds to the N-terminal portion of eukaryotic L24, and we have demonstrated that this portion is responsible for interactions with TAV. According to the recently published crystal structure of the archaebacterial 60S ribosome (Ban et al., 2000), the homolog of eukaryotic L18 (L18e) functions to stabilize the tertiary structure of 23S rRNA domain II on the external surface of the 60S subunit, somewhere near the neck region. L24e is located at the internal surface, close to the main factor binding site. A similar location, near the stalk of the 60S subunit, has been suggested for rat liver 60S ribosomal protein L24 (Marion and Marion, 1987). In rat liver 80S ribosomes, L24 could be crosslinked to the 40S subunit, consistent

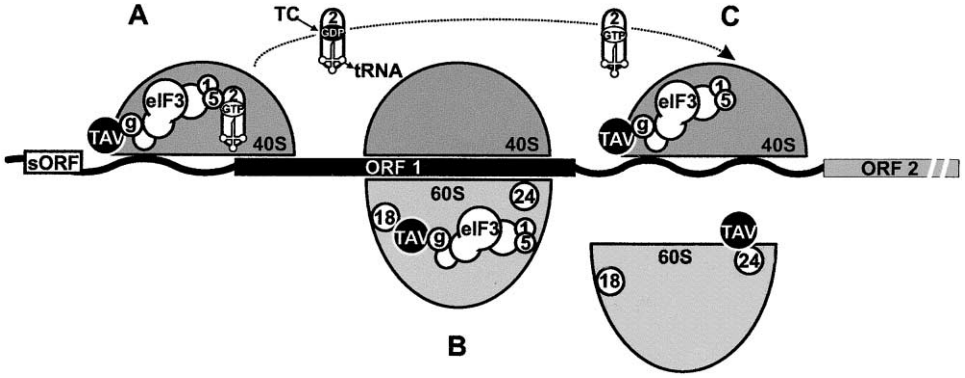

Figure 7. Model of TAV Function during the Reinitiation Process

(A) TAV interacts with eIF3 via subunit g during or after translation of an sORF. TAV/eIF3/40S complex reacquires a ternary complex (TC) and scans for ORF 1. eIF3, its subunit g (g), eIF1 (1), eIF2 (2), eIF5 (5), tRNA, L24 (24), L18 (18), and TAV are indicated. Recycling of TC is shown by a dotted arrow.

(B) After translation initiation of ORF 1, the TAV/eIF3 complex remains bound to polysomes, apparently via its translocation to 60S through interaction with L18. The TAV-eIF3 complex is relocated back to 40S during ORF 1 termination.

(C) TAV/eIF3/40S complex reacquires a ternary complex and scans for ORF 2.

with a 60S interface location (Uchiumi et al., 1986). Assuming the structure of plant 60S subunits indeed resembles that of archaebacterial 60S subunits, L24 and L18 would be located too far apart to interact with the same TAV molecule. Thus, we speculate that the 60S ribosome has at least two TAV binding sites and is capable of binding two TAV molecules simultaneously on its external and internal surface through interactions with L18 and L24, respectively.

Yeast L24, although dispensable for cell viability (Baronas-Lowell and Warner, 1990), is involved in the 60S subunit joining step during translation initiation, and its depletion causes the appearance of so called "halfmers," when polysomes are deficient in active 60S subunits (Baronas-Lowell and Warner, 1990; Dresios et al., 2000). The total level of 60S subunits is not affected in these mutants, suggesting that the defect lies in the ability to form functional 80S ribosomes. Overexpression of L24 in plant protoplasts led to significant enhancement of TAV-dependent reinitiation, while the first initiation event was not significantly affected (Figure 6C). This suggests that overexpressed L24 in a free form binds TAV and activates its proper function in reinitiation. Thus, L24 might also have an extraribosomal function in reinitiation of translation, like some other ribosomal proteins (Wool, 1996). Alternatively, the strong positive effect of free L24 might be due to relief from inhibition of transactivation mediated by ribosome-bound L24. L24 is one of the bridge points between the yeast 40S and 60S ribosomal subunits (R. Beckmann, personal communication); thus, interaction with TAV might affect 80S ribosome formation. Such an interaction might be favorable for virus replication late in infection, when TAV levels are high and the RNA should be used for packaging and/or reverse transcription, rather than translation.

No effect of L18 overexpression on transactivation in plant protoplasts was observed (our unpublished data). However, the location of L18 on the outside of the 60S ribosome might allow TAV to bind without directly affecting ribosome function in translation. Such binding might increase the local concentration of 60S ribosomes at the surface of the viral inclusion bodies that are composed of TAV. Accumulation of polysomes can in fact be observed around inclusion bodies (Shepherd, 1976). These "tethered" ribosomes would then direct their CaMV translation products into the inclusion bodies. Significantly, all proteins expressed from CaMV RNAs are found within these inclusion bodies (Givord et al., 1984; Martinez-Izquierdo et al., 1987), including heterologous nonviral proteins encoded by transgenic CaMV RNA (De Zoeten et al., 1989).

A recent study in mammals suggests a role for ribosomal protein L18 in the regulation of double-stranded RNA-activated protein kinase (PKR) competing with double-stranded RNA (dsRNA) for binding to PKR (Kumar et al., 1999). The binding of L18 inhibited both PKR autophosphorylation and PKR-mediated phosphorylation of eIF2α in vitro (Zhu et al., 1997). Thus, a possible function of the TAV-L18 interaction might also be modulation of a plant PKR-like activity. Such an effect might not be apparent in our plant protoplasts assay. Further exploration of this possibility is necessary, as the role of PKR in plants is not fully known.

Model of TAV Action

A current model of TAV action during the reinitiation process is shown in Figure 7. The intriguing observation that TAV-mediated polycistronic translation is highly enhanced by an upstream sORF (Fütterer and Hohn, 1991, 1992) is considered in this model.

We propose here that TAV interacts with 40S ribosome-bound eIF3, which is not removed during the short translation elongation event (Figure 7A). Such an acquisition of TAV could be induced by removal of eIF4B, since both eIF4B and TAV bind the same subunit of eIF3, namely eIF3g (Vornlocher et al., 1999). eIF3 is located distal to the 40S-60S joining surface, and thus would not necessarily block 80S ribosome formation (Srivastava et al., 1992). In our model, we assume that TAV acquisition by eIF3/40S occurs after initiation of sORF translation, most likely at the termination step. After termination of sORF translation, the TAV/eIF3/40S

complex can rerecruit the ternary complex via eIF3, resume scanning, and reinitiate at ORF 1 (Figure 7A).

If translation proceeds for long enough (e.g., at ORF 1, Figure 7B), TAV-bound eIF3 may disengage from 40S and translocate to 60S of the translating ribosome via L18. Indeed, we demonstrated in vitro that complexes between 40S ribosomes and TAV through bridging by eIF3, and between 60S ribosomes and eIF3 through bridging by TAV, can be formed (Figures 4C and 4D). Thus, TAV-bound eIF3 might shuttle between 60S (Figure 7B) and 40S ribosomal subunits (Figure 7C). Notably, recycling and phosphorylation of eIF2 on 60S subunits of polysomes have been observed (Ramaiah et al., 1992). The TAV/L24 interaction might enhance 60S subunit recycling during TAV-mediated polycistronic translation, affecting the 60S ribosome release as well as joining steps.

In conclusion, the interactions discovered between the CaMV viral protein TAV and the host cell translational machinery provide a basis for further progress in understanding translation reinitiation in eukaryotes.

Experimental Procedures

Polysome Isolation and Density Centrifugation

Healthy and CaMV-infected turnip plants (*Brassica rapa* cv. Just Right) were propagated in phytoboxes with illumination for 16 hr/day at 20°C. Turnip polysomes were isolated one week after symptoms appeared, as described in Jackson and Larkins (1976). Polysomes (14 A_{260} units) were loaded on 10%–50% sucrose gradients and centrifuged for 3.5 hr at 4°C in an SW41 rotor at 35,000 rpm.

Western Blots

Proteins were separated on 12.5% polyacrylamide gels and transferred to nitrocellulose membranes by electroblotting (1 hr, 1 mA/cm^2 gel). TAV and its mutants were detected using rabbit anti-TAV antiserum (De Tapia et al., 1993). TAV and eIF3b in plant polysomes were detected using a purified polyclonal rabbit anti-TAV antiserum (Nakayashiki et al., 1993) and anti-human eIF3b (PRT1) antiserum (Lin et al., 2001). BD-TAV and TAV truncated versions fused to Gal4 DNA binding domain (BD), AD-eIF3g, AD-L24, and eIF3g, and L24 truncated versions fused to Gal4 DNA activation domain (AD) were detected using rabbit antiserum against BD and AD, respectively (Clontech).

Two-Hybrid Strategy

The yeast strains Y187, CG1945, and diploid strain Y187xCG1945, and plasmids pAS2ΔΔ and pACTIIst (derived from pAS2 and pACTII), were kindly provided by M. Fromont-Racine and P. Legrain (Institute Pasteur, Paris). ORF VI fragments were fused in-frame to the Gal4 DNA binding domain (BD) in the yeast vector pAS2ΔΔ. Plasmid BD-NTAV was constructed by cloning the HindIII-BglII fragment from pHELP7 (Bonneville et al., 1989) into the filled-in BamH1 site of pAS2ΔΔ. pBD-TAV was produced by insertion of an oligonucleotide covering ORF VI from the ATG to the HindIII site, together with the HindIII-PstI fragment of ORFVI into BamH1 (filled in) and PstI sites of pAS2ΔΔ.

The mating strategy for two-hybrid screening was as described in Fromont-Racine et al. (1997) and in Clontech yeast protocols handbook PT 3024-1. The yeast strain Y187 was transformed according to standard procedures with an *A. thaliana* cDNA library (Clontech). Ten million transformed yeast colonies were collected and pooled. For each screen, a 1 ml vial was thawed and cells were mixed with CG1945 cells transformed with either AS2ΔΔ-TAV, AS2ΔΔ-NTAV, or AS2ΔΔ-CTAV. Plates contained 20 mM 3-aminotrizole (3-AT).

Two-hybrid analysis of interacting proteins was performed using a cotransformation procedure. Diploid strain GC1945xY187 was cotransformed with plasmids expressing BD fused with full-length or truncated TAV sequences and AD fused to full-sized or truncated eIF3g or L24 sequences. Plasmids AD-eIF3g and AD-L24 were rescued from yeast cells by transformation of *E. coli* DH5α as described by Clontech. CTAV deletion mutants (from C1 to C5, see Figure 2A) were produced by PCR and cloned between the SmaI and PstI sites of pAS2ΔΔ. eIF3g and L24 deletion mutants fused to AD were produced by PCR and inserted between the EcoRI and BamHI sites of pGAD424 (Clontech).

TAV, eIF3g, and L24 mutants for two-hybrid assays (pBD-$CTAV^{Y305P}$, pAD-$eIF3g^{C139S}$, pAD-$eIF3g^{C139S,\ C142S}$, and pAD-$L24^{Y16P}$) or transient expression experiments ($pTAV^{Y305P}$, $peIF3g^{C139S}$, $peIF3g^{C139S,\ C142S}$, and $pL24^{Y16P}$) were created by exchanging appropriate restriction fragments for mutagenised fragments. The sequences of oligonucleotides used are available on request.

The Yeast Three-Hybrid System

The three-hybrid system was performed according to standard Clontech protocols using the yeast strain HF7c. The CTAV ORF was fused to AD behind the ADH promoter of the pGAD424 vector carrying the LEU 2 selection gene (pAD-CTAV). A NotI-NotI eIF3g fragment obtained by PCR from pAD-eIF3g was introduced into the NotI site of the Bridge vector (pBr, Clontech). In addition, a BamHI-PstI fragment from pAD-L24 was fused to BD into the Bridge vector with or without eIF3g to produce pBrBD-L24/Met-eIF3g and pBrBD-L24/Met-0, respectively (see Figure 3A).

In Vitro Transcription and Translation

pT7-TAV, pT7-eIF3g, and pT7-L24, used for in vitro transcription with T7 polymerase, were obtained by ligating XhoI-Pst1 fragments prepared by PCR from pBD-TAV, pAD-eIF3g, and pAD-L24, respectively, into XhoI-Pst1-digested pLm (Ryabova and Hohn, 2000). pETKH-L18 (Leh et al., 2000) was kindly provided by M. Keller (Institute of Plant Molecular Biology, Strasbourg, France).

T7-directed transcripts were transcribed in the presence of the cap analog ^{7}mGpppG and translated in wheat germ extract (WGE) as described by Ryabova and Hohn (2000). Globin RNA and WGE were from Roche Molecular Biochemicals.

In Vitro GST Pull-Down Assay

Wheat germ 60S and 40S ribosomes were kindly provided by Dr. S. Zhanybekova (Institute of Molecular Biology and Biochemistry, Almaty, Kazakhstan). Rabbit eIF3 was kindly provided by Dr. T. Pestova (Dept. of Microbiology, SUNY HSC at Brooklyn, NY). pGST-TAV, pGST-NTAV, pGST-CTAV, pGST-eIF3g, and pGST-L24 were constructed by ligating BamH1-EcoRI fragments prepared by PCR from the corresponding plasmids (pBD-TAV, pBD-NTAV, pBD-CTAV, pAD-eIF3g, and pAD-L24) into pGEX-2TK (Pharmacia Biotech) as in-frame fusions with the GST-domain, and expressed in *E. coli* BL21. pHis-TAV was constructed by subcloning 6 His residues fused to the N terminus of the HindIII-PstI fragment from pHELP7 into pQE11 (Qiagen).

The in vitro GST pull-down assay was performed as described previously (Herzog et al., 2000). In RNase treatment experiments, the binding reaction was treated with a cocktail of 10 μg RNase A, 10 U RNase T1, and 8 U RNase V1 for 1h at 30°C. Binding of GST or GST-TAV to ribosomal subunits and eIF3 (or GST-eIF3g to 60S and His-TAV) was carried out in a 300 μl reaction containing 10 mM Tris-HCl, [pH 7.5], 50 mM NaCl, 8 mM $MgCl_2$, 6 mM β-mercaptoethanol with either 5 μg of rabbit reticulocyte eIF3 or 5 μg His-TAV, and/or 5 A_{260} of 40S subunits or 60S subunits. Aliquots of the bound (5 μl), as well as 25 μl of unbound fraction, were separated by 12.5% SDS-PAGE, and the ^{35}S-labeled proteins were visualized by autoradiography.

Transient Expression

The eIF3g and L24 coding sequences from pAD-eIF3g and pAD-L24 were subcloned under the control of the CaMV 35S RNA promoter of pTAV (p35S-P6; Kobayashi et al., 1998). pmonoCAT and pbiGUS were described by Bonneville et al. (1989), and pminiTAV (pMAV) by De Tapia et al. (1993).

Leaf protoplasts derived from *N. plumbaginifolia* were prepared, and samples of 6×10^5 protoplasts were used for polyethylene glycol-mediated transfection as described previously (De Tapia et al., 1993). All transfections were performed with 5 μg pbiGUS and 2 μg pmonoCAT. For transactivation, 5 μg pTAV (or derivatives) or

10 μg pMAV was added. Increasing concentrations of peIF3g or pL24 were also cotransformed. CAT and GUS activities were determined in protein extracts prepared after overnight incubation as described previously (Pooggin et al., 2000). The values given are the means from more than three independent experiments.

Infectivity Studies

Four turnip plants were mechanically inoculated for each mutant. DNA preparation from plants and PCR of viral progeny were performed as described previously (Pooggin et al., 1998).

TAV and mutants TAV^{Y305P} and TAV^{Y305H} were cloned into the recombinant viral vector pECad6 lacking ORF VI (K. Kobayashi and T.H., unpublished data). Its genome consists of a synthetic transcriptional enhancer, a portion of the strain CM1841 (which includes the 35S RNA promoter and ORF VII), and the complementing portion of the genome of the strain CM4-184 without ORF VI bearing a natural deletion of 420 nt within the aphid transmission factor gene.

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