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Loss-of-Susceptibility Mutants of *Arabidopsis thaliana* Reveal an Essential Role for eIF(iso)4E during Potyvirus Infection

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Summary

The *Arabidopsis thaliana*-potyvirus system was developed to identify compatibility and incompatibility factors involved during infection and disease caused by positive-strand RNA viruses [1, 2]. Several *Arabidopsis* mutants with increased susceptibility to *Tobacco etch potyvirus* (TEV) were isolated previously [1], revealing a virus-specific resistance system in the phloem [3–5]. In this study, *Arabidopsis* mutants with decreased susceptibility to *Turnip mosaic potyvirus* (TuMV) were isolated. Three independent mutants that conferred immunity to TuMV were isolated and assigned to the same complementation group. These mutants were also immune or near-immune to TEV but were susceptible to an unrelated virus. The locus associated with decreased susceptibility was named *loss-of-susceptibility to potyviruses 1* (*lsp1*). The *LSP1* locus was isolated by map-based cloning and was identified as the gene encoding translation factor eIF(iso)4E, one of several known *Arabidopsis* isoforms that has cap binding activity. eIF4E and eIF(iso)4E from different plant species were shown previously to interact with the genome-linked protein (VPg) of TEV and TuMV, respectively. Models to explain the roles of eIF(iso)4E during virus infection are presented.

Results and Discussion

Infection of young *Arabidopsis* (ecotype C24) plants by TuMV resulted in severe symptoms and developmental defects within 14 days postinoculation (d.p.i.). Vegetative tissue of TuMV-infected plants exhibited stunting, reduced apical dominance, curled bolts, and mosaic patterns on leaves (Figure 1A). Reproductive organs displayed several developmental defects, including narrow sepals, split carpels, and other organ malformations (data not shown). Infected plants were sterile or had reduced fertility. The developmental defects caused by TuMV infection formed the basis for a genetic screen to identify mutants with altered susceptibility or response. Approximately 159,600 plants from the M2 generation of 14,000 ethyl methanesulfonate (EMS)-mutagenized plants were inoculated three times with TuMV, resulting in a 99.7% systemic infection rate (Figure 1B). Fertile plants that lacked symptoms after TuMV inoculation were easily identified among plants with the parental phenotype (Figure 1C). A total of 445 mutant candidates that lacked both virus-induced symptoms and detectable levels of TuMV capsid protein (CP) were identified. The M3 plants resulting from self-fertilization of mutant candidates were rescreened. A total of 11 candidates yielded M3 plants that each lacked virus-induced symptoms and contained no, or decreased levels of, TuMV CP in inflorescence tissue at 18 d.p.i. They were then crossed with parental C24 plants for dominance testing in the F1 generation and with plants from each mutant for complementation analysis.

Three independent mutants (C12-21, C15-8, and C18-78) each contained a single recessive locus and were placed in the same complementation group. TuMV-inoculated M3 plants from each mutant were indistinguishable from noninoculated parental C24 plants (Figure 2A). Each mutant lacked detectable levels of TuMV CP in inflorescence tissue at 14 and 21 d.p.i. (Figure 2B and data not shown). In leaves inoculated with TuMV-GFP, a TuMV strain that was engineered to express a green fluorescent protein, parental C24 contained infection foci that expanded over time (Figure 2C). In contrast, the three mutants had no visible infection foci in leaves inoculated with the same TuMV-GFP preparation ($n = 80$ leaves for each mutant, Figure 2C). The entire surface area from each inoculated mutant leaf was surveyed using epifluorescence microscopy to detect infection foci consisting of one or a few cells. No single-cell or other microscopic TuMV infection foci were detected. It should be noted that single-cell infections by potyviruses are easily detected using GFP-expressing viruses [6]. Based on the number of infection foci detected in parental C24 plants, and the fact that no single- or multicell infection foci were detected in any of the 240 inoculated leaves from the three mutants, it was calculated that parental plants were at least 10^3-fold more susceptible at the single-cell level than were the mutant plants. These findings strongly suggest that the mutants were defective in supporting TuMV genome expression and/or replication.

The C12-21 and C15-8 mutants were also tested for susceptibility to the related potyvirus, TEV-GUS, which contains the coding sequence for β-glucuronidase [7]. TEV-GUS systemically infects wild-type *Arabidopsis* C24 plants (Figure 2B) but does not induce symptoms [2]. None of the mutants were systemically infected by TEV-GUS (Figure 2B). Additionally, infection foci of any size were either never detected (C12-21) or rarely detected (C15-8) in inoculated leaves (Figure 2D). The rare infection foci in C15-8-inoculated leaves were relatively small, and the GUS histochemical substrate stained them less intensely (Figure 2D).

The susceptibility of C12-21 and C15-8 mutants to an unrelated virus, *Turnip crinkle carmovirus* (TCV), was also tested. Parental C24 plants that were inoculated with TCV were severely stunted and died prematurely

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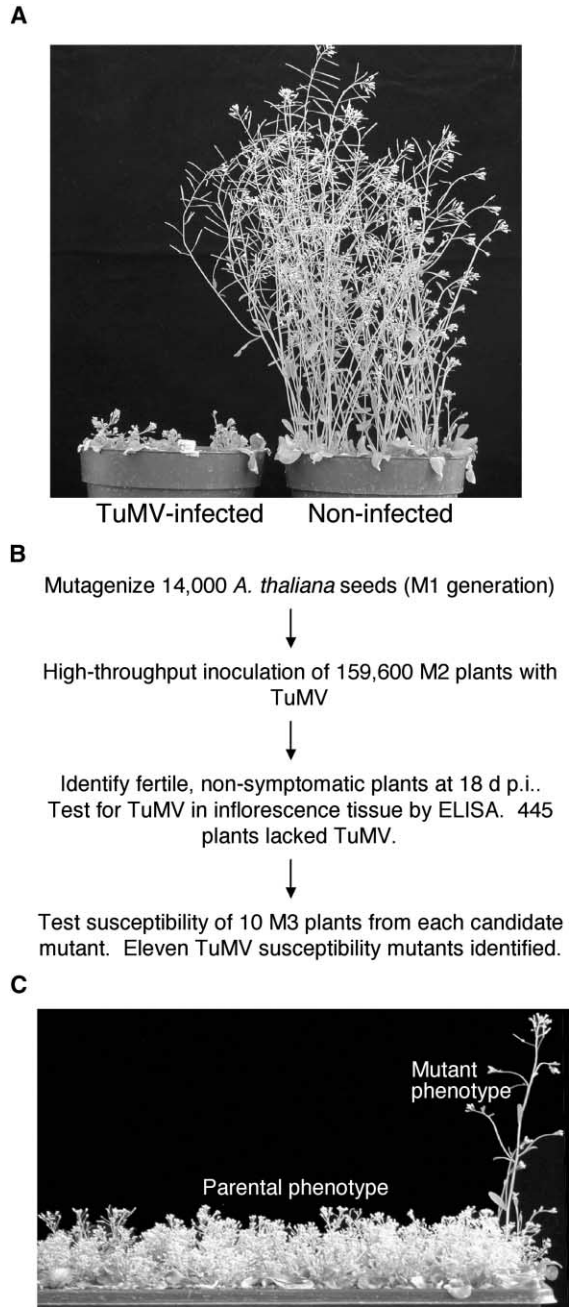

Figure 1. Screen for Loss-of-Susceptibility Mutants of *Arabidopsis*

The TuMV strain was obtained from an infectious clone, p35TuNOS (a gift of Dr. Fernando Ponz, Madrid). Large-scale inoculum was prepared using TuMV-infected *Brassica rapa* or *B. perviridis*.

(A) Stunting phenotype caused by TuMV in *Arabidopsis* ecotype C24 at 18 d.p.i. Each pot contained 12 plants.

(B) A flow chart for the recovery of loss-of-susceptibility mutants. Approximately 35,000 *Arabidopsis* ecotype C24 seeds were subjected to treatment with ethyl methanesulfonate (EMS, 0.002%) for 12 hr at room temperature. Germination of the mutagenized seed yielded 14,000 viable plants, which were divided into 15 batches. Approximately 159,600 plants from the M2 generation (self-fertilized progeny) were inoculated with TuMV using an artist airbrush [1] and were reinoculated after 3 and 10 days. Seed from plants that failed to show characteristic stunting and TuMV symptoms were saved, and M3 plants were inoculated with TuMV or TuMV-GFP. Lines that lacked TuMV susceptibility in the M3 generation were tested using ecotype-specific markers [22] to verify that the plants were C24 ecotype.

(C) Identification of mutant plants after repeated inoculation with TuMV.

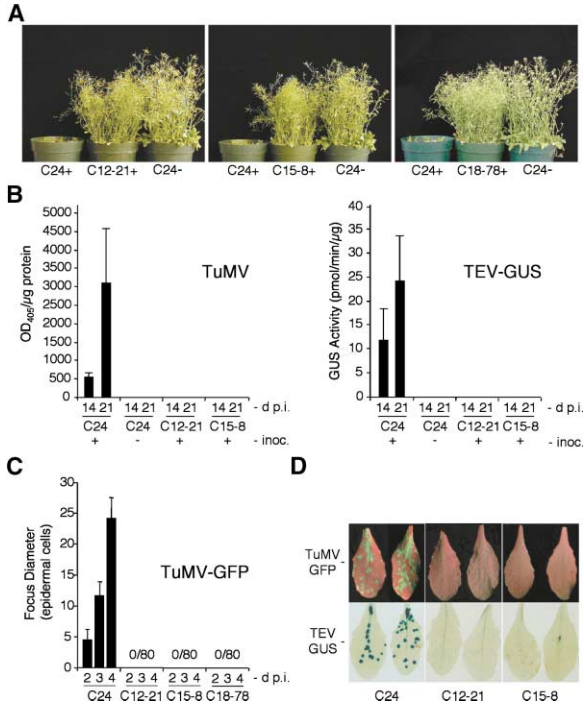

Figure 2. Characterization of Potyvirus Susceptibility of Three *Arabidopsis* Mutants

(A) Visible phenotypes of TuMV-infected parental *Arabidopsis* plants (C24+), TuMV-inoculated mutant plants (C12-21+, C15-8+, and C18-78+), and noninoculated parental plants (C24−). Each pot contained 12 plants.

(B) Analysis of TuMV and TEV-GUS in inflorescence (systemic) tissue of parental *Arabidopsis* C24 and C12-21 and C15-8 mutant plants. Normalized protein extracts from TuMV-inoculated plants were prepared at 14 and 21 d.p.i. and were tested for TuMV capsid protein (CP) by indirect ELISA using anti-TuMV CP serum PVAS 134 (ATCC) and anti-rabbit-Ig horseradish peroxidase conjugate (Amersham Pharmacia) using standard methods. Normalized extracts from TEV-GUS-inoculated plants were tested for GUS activity using approximately 100 mg inflorescence tissue as described [2].

(C) Analysis of cell-to-cell movement of TuMV-GFP in inoculated leaves. The diameter (number of epidermal cells in transect) of random infection foci was measured at 2, 3, and 4 d.p.i. in wild-type C24 plants (n = 30 foci/time point) as described [7]. No infection foci were found in any of the mutant plants (n = 80 inoculated leaves/mutant).

(D) Visualization of TuMV-GFP and TEV-GUS in inoculated leaves at 4 d.p.i. TuMV-GFP infection foci appear as green spots under UV illumination (upper panels). Details of the construction of p35STuMV-GFP, the infectious clone that yields TuMV-GFP, are available upon request. TEV-GUS infection foci appear as blue spots after infiltration with a GUS histochemical substrate (lower panels).

(Figure 3). Each of the mutant plants exhibited a response that was indistinguishable from parental C24 plants after inoculation with TCV and contained comparable levels of TCV in total plant extracts (Figure 3), indicating that the mutations had little or no effect on susceptibility to the heterologous virus. Because the

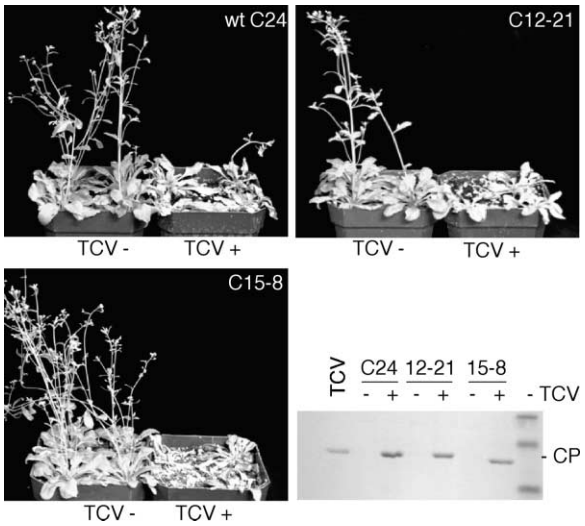

Figure 3. Characterization of Susceptibility of C12-21 and C15-8 Mutants to TCV

Inoculum was prepared as described [23] from infected *N. benthamiana* tissue. Visible phenotypes of noninoculated (TCV−) and TCV-inoculated (TCV+) parental C24 and C12-21 and C15-8 mutant plants. Each pot contained four plants. Turnip crinkle virus infection was assayed by a whole virion extraction procedure from total aerial tissue of inoculated plants using a low-pH enrichment method [23], which yields near-homogeneous virus preparations. Normalized extracts were analyzed by SDS-PAGE and were visualized by staining with Coomassie Brilliant blue, revealing TCV capsid protein (CP). Purified TCV was analyzed in parallel as a control.

mutants had potyvirus-specific susceptibility defects specific among the viruses tested, they were designated *loss-of-susceptibility to potyvirus* (*lsp*). The affected loci in C12-21, C15-8, and C18-78 were designated *lsp1-1*, *lsp1-2*, and *lsp1-3*, respectively.

The genome position of *LSP1* was determined using a mapping population (F2) of 342 TuMV-nonsusceptible progeny from a cross between mutant C12-21 (*lsp1-1*) and the polymorphic ecotype, La-*er* (dominant *LSP1* allele). The *lsp1* locus was mapped to an interval on chromosome V of approximately 200 kb between markers MOK9-39s (5 recombinants/684 chromosomes) and MXH1-36116 Taq (3 recombinants/684 chromosomes) (Figure 4A).

Examination of candidate genes in the defined interval revealed the gene encoding eIF(iso)4E, one of two cap binding protein isoforms that are known to interact with the potyviral genome-linked protein, VPg [8, 9]. The TuMV VPg was shown to interact with the isoform encoded by the gene located in the interval mapped here, whereas the TEV VPg was shown to interact with a different isoform (eIF4E) from tomato. The TEV VPg was not tested previously using the *Arabidopsis* isoforms. The complete eIF(iso)4E coding sequence from the C12-21 (*lsp1-1*), C15-8 (*lsp1-2*), and C18-78 (*lsp1-3*) mutants, and from the parental C24 ecotype, was sequenced. The eIF(iso)4E coding sequence from each of the mutants contained a single point mutation resulting in a premature stop codon (Figure 4A). The *lsp1-1* allele contained a TGG to TGA change at codon 63, while the *lsp1-2* and *lsp1-3* alleles contained the identical TGG to TGA change at codon 120. The *lsp1-2* and *lsp1-3* alleles were distinguished, however, by the presence of an additional point mutation in intron 1 of the *lsp1-3* allele. A dCAPS marker [10, 11] that detected the eIF(iso)4E mutation cosegregated with the mutant phenotype in all TuMV-nonsusceptible individuals in the mapping population (Figure 4A). Immunoblot analysis using a heterologous eIF(iso)4E antiserum revealed that eIF(iso)4E was absent or below detection levels in each mutant but was detected in parental C24 plants (Figure 4B).

To confirm that mutations affecting eIF(iso)4E were the cause of the loss-of-susceptibility phenotype, the C12-21 (*lsp1-1* allele) and C15-8 (*lsp1-2* allele) mutants were transformed with a wild-type genomic fragment containing the gene encoding eIF(iso)4E or an empty vector construct as a control. Susceptibility to both local (inoculated leaves) and systemic (inflorescence tissue) infection by TuMV-GFP was restored in mutant plants containing the wild-type gene encoding eIF(iso)4E but not the empty vector (Figure 4C). These data confirm that the nonsusceptible phenotype was due to loss of eIF(iso)4E.

The *Arabidopsis* genome encodes at least three cap binding proteins, eIF4E, eIF(iso)4E, and novel cap binding protein (nCBP) [12]. The finding that mutants lacking eIF(iso)4E are unable to support TuMV and TEV infection suggests that the other cap binding proteins cannot compensate for the loss of eIF(iso)4E during virus infection. It is possible that potyviruses interact with different eIF4E or eIF(iso)4E isoforms in different host plants, or that interactions with multiple isoforms are required. Both eIF(iso)4E and eIF4E are abundant, functional translation initiation factors [12–14], although the spectrum of mRNAs that use one or the other factor has yet to be defined. Because the *lsp1* mutants exhibited normal growth and development under standard propagation conditions, it seems likely that eIF(iso)4E is involved in the translation of either a narrow spectrum of mRNAs or mRNAs that are active under unusual or specific growth conditions. Alternatively, other cap binding factors might compensate adequately for the loss of eIF(iso)4E during translation of cellular mRNAs.

The identification of potyvirus-nonsusceptible mutants with eIF(iso)4E defects provides unambiguous evidence for a role of eIF(iso)4E during potyvirus infection of *Arabidopsis* at the single-cell level. Previous protein-protein interaction data support the idea that the essential function of eIF(iso)4E involves an interaction with the VPg protein [8, 9]. The previous studies, however, were insufficient to distinguish between an eIF(iso)4E function required for potyvirus genome expression or replication and an eIF(iso)4E function involved in host defense. If the eIF(iso)4E-VPg interaction was part of a defensive mechanism, then loss of eIF(iso)4E function would have increased susceptibility to TuMV rather than decreased susceptibility, as was observed. Loss of susceptibility resulting from loss of eIF(iso)4E in the *lsp1* mutants, therefore, is most compatible with a model in which eIF(iso)4E functions directly to promote genome expression or replication through an interaction with VPg.

The known functions or activities of eIF(iso)4E and other cap binding isoforms of eIF4E from plants and other eukaryotes suggest that eIF(iso)4E may play sev-

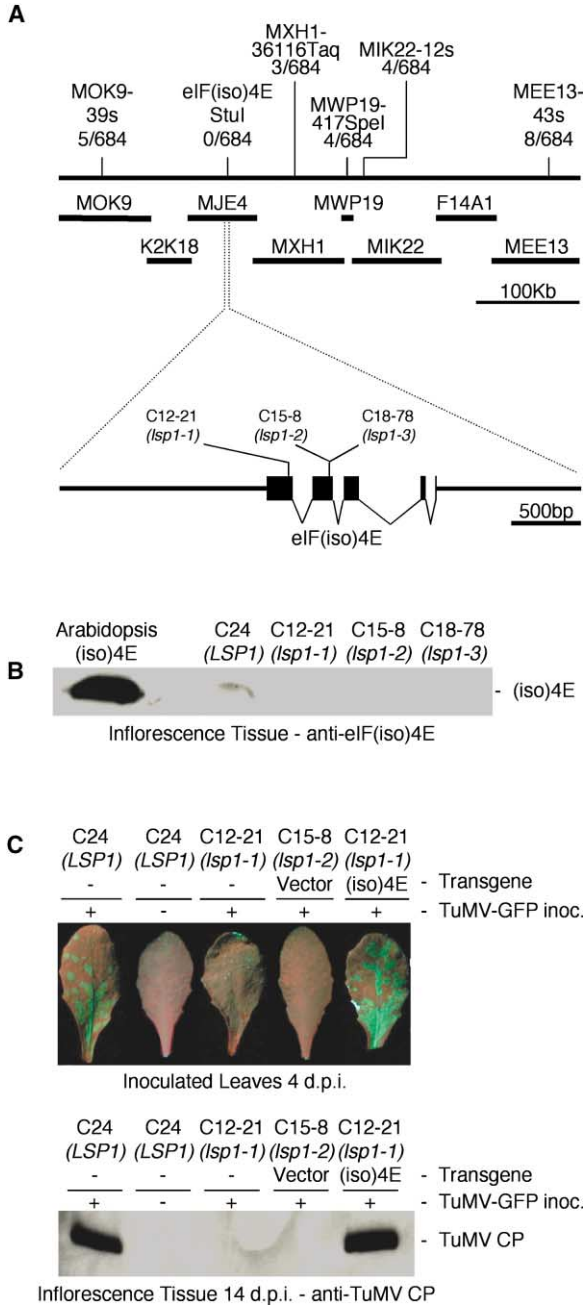

Figure 4. Cloning of *LSP1*

(A) Crosses between mutant C12-21 (*lsp1-1* allele) and ecotype La-*er* (functional *LSP1* allele) were done, and approximately 5,000 F2 generation plants were screened for TuMV susceptibility after three inoculations. Homozygous *lsp1-1/lsp1-1* plants were identified as those that lacked both TuMV-induced symptoms and TuMV CP in inflorescence tissue (by ELISA) after 18 days. Genomic DNA was isolated from inflorescence tissue of 342 TuMV-nonsusceptible *lsp1-1/lsp1-1* plants as described [24]. The PCR-based markers nga63, nga128, nga111, AthSO392, nga1145, nga1126, nga162, nga6, nga172, AthBio2b, nga8, nga151, and AthSO191 were used in preliminary mapping experiments. The Cereon *Arabidopsis* polymorphism collection (http://www.arabidopsis.org/Cereon/index.html) was used to develop simple sequence-length polymorphic markers MOK9-39s, MEE13-43s, MIK22-12s, MPO12-48s, MFO20-42s, MJB21-13s, the CAPS marker MXH1-36116 Taq, and the dCAPS marker MWP19-417 SpeI in chromosome V. eIF(iso)4E StuI is a dCAPS marker that is specific for the eIF(iso)4E allele in the *lsp1-1* mutant. The markers that flank or cosegregate with *LSP1* are indicated at the top. The number of recombination events per meiotic event scored is given beneath each marker. A BAC/P1 contig spanning *LSP1* is shown (solid lines). The genomic fragment used to complement *lsp1-1* is shown in the expanded diagram. The positions of nonsense mutations in C12-21 *(lsp1-1)*, C15-8 *(lsp1-2)*, and C18-78 *(lsp1-3)* mutants are shown.

(B) Immunoblot assay of inflorescence tissue from parental and mutant plants using anti-eIF(iso)4E serum. Recombinant eIF(iso)4E protein was analyzed as a positive control. Equivalent amounts of extract were subjected to SDS-PAGE (5% stacking/12.5% or 15% resolving gel) and immunoassay [1] using anti-eIF(iso)4E serum. Both purified eIF(iso)4E and anti-wheat eIF(iso)4E were gifts from Dr. Karen Browning, University of Texas.

(C) Transgenic complementation using the wild-type eIF(iso)4E-encoding gene. An *Arabidopsis* C24 genomic fragment consisting of the eIF(iso)4E coding region, 1478 nucleotides upstream of the eIF(iso)4E start codon and 827 nucleotides downstream of the stop codon, was amplified using Pfu Turbo (Stratagene) polymerase and was inserted into the binary vector pSLJ755I5, yielding pSLJ755I5-eIF(iso)4E. Both pSLJ755I5 and pSLJ755I5-eIF(iso)4E were mobilized into *Agrobacterium tumefaciens*. Plants from the M3 generation of mutants C12-21 and C15-8 were transformed using the vacuum infiltration method as described [4, 25], and seeds were collected. Transgenic plants in the next generation were selected in the presence of glufosinate ammonium solution (0.0025% in water) for 3 weeks. The presence of the wild-type eIF(iso)4E-encoding transgene was confirmed by allele-specific marker analysis, and seeds from positive plants were collected. Transgenic plants from the following generation were tested for susceptibility to TuMV-GFP. Noninoculated parental C24 leaves (C24−) and TuMV-GFP-inoculated leaves of parental *Arabidopsis* plants (C24+), the C12-21 (*lsp1-1*) mutant, transgenic C15-8 (*lsp1-2*) containing an empty vector, and transgenic C12-21 (*lsp1-1*) containing an eIF(iso)4E-encoding transgene are shown in the top panel under UV illumination. Empty vector-transformed C12-21 plants had infection phenotypes identical to empty vector-transformed C15-8 plants (data not shown). Immunoblot assay of inflorescence (systemic) tissue from the same plants using anti-TuMV CP serum PVAS 134 (ATCC) is shown in the bottom panel.

eral potential roles in virus infection. First, given that eIF(iso)4E is a translation initiation factor [12–14], it is logical to propose a function in translation initiation on viral RNA (Figure 5A). In effect, the VPg could function like a typical 5′ cap structure to recruit translation initiation factors and 40S ribosomal subunits. It should be noted, however, that the potyviral 5′ nontranslated region lacking a VPg structure contains information to direct cap-independent translational enhancement in vivo and in vitro [15]. There may be multiple or redundant signals and structures to initiate translation of a functional potyvirus genome.

Second, eIF(iso)4E may provide stability to the potyvirus genome through interaction with the VPg (Figure 5B). Interaction between eIF4E and the 5′ cap promotes mRNA stability in yeast by protecting against DCP1p (decapping enzyme)- and XRN1p (exonuclease)-mediated RNA degradation [16, 17]. *Arabidopsis* has genes encoding proteins that resemble DCP1p and the XRN family of exonucleases [18], although the function of these plant proteins in mRNA degradation is not clear. Conferral of translation initiation and genome stability functions would explain why potyviruses, but not TCV (which lacks a 5′-linked VPg protein), requires eIF(iso)4E. And third, eIF(iso)4E may provide functions required for intracellular localization and transport of potyviral RNA

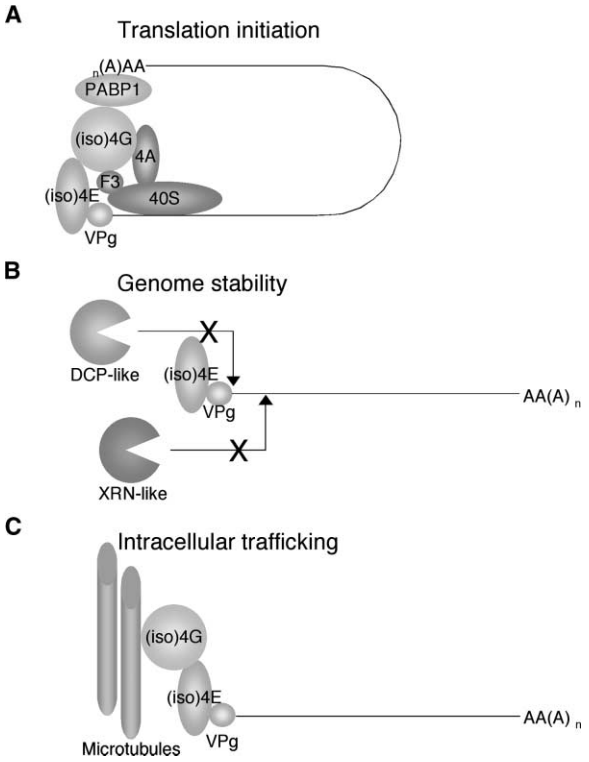

Figure 5. Potential Roles for eIF(iso)4E during Potyvirus Infection

(A) Translational initiation. eIF(iso)4E could provide a role during translation initiation through interaction with the genome-linked protein VPg. The eIF(iso)4E-VPg interaction could anchor viral genomic RNA to translation initiation complexes. Initiation factors (PAPB1, 4A, F3) and ribosomal subunits (40S) are shown for illustrative purposes.

(B) Genome stability. Interaction between eIF(iso)4E and VPg could protect viral RNA from DCP1p-like "decapping" enzymes, thereby blocking degradation of the viral genome by XRN-like host-encoded exonucleases.

(C) Intracellular trafficking. eIF(iso)4E interacts with eIF(iso)4G, a protein with microtubule binding activity and a kinesin-like domain. Interaction between VPg and eIF(iso)4E may engage the potyviral genome with the intracellular trafficking machinery.

(Figure 5C). The *Arabidopsis* eIF(iso)4E protein normally interacts with the eIF(iso)4G protein [13, 19]. The eIF (iso)4G protein from wheat interacts with microtubules in reconstitution assays [20]. Thus, potyviral genomes may interact with microtubules through a VPg-eIF(iso)4E-eIF(iso)4G interaction.

Finally, the potential importance of interaction of positive-strand RNA virus genomes with cellular RNA metabolic factors should not be underestimated. The critical role of translation factors in virus-specific functions has now been demonstrated through genetic routes using diverse positive-strand RNA virus-host systems [21]. Interactions of viral RNA genomes and such factors are likely critical for controlled routing of viral RNAs for translation, replication, transport, and degradation.

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