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Functional Dissection of Naturally Occurring Amino Acid Substitutions in eIF4E That Confers Recessive Potyvirus Resistance in Plants [W] [OA]

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Naturally existing variation in the eukaryotic translation initiation factor 4E (*eIF4E*) homolog encoded at the *pvr1* locus in *Capsicum* results in recessively inherited resistance against several potyviruses. Previously reported data indicate that the physical interaction between *Capsicum*-eIF4E and the viral genome-linked protein (VPg) is required for the viral infection in the *Capsicum-Tobacco etch virus* (TEV) pathosystem. In this study, the potential structural role(s) of natural variation in the eIF4E protein encoded by recessive resistance alleles and their biological consequences have been assessed. Using high-resolution three-dimensional structural models based on the available crystallographic structures of eIF4E, we show that the amino acid substitution G107R, found in many recessive plant virus resistance genes encoding eIF4E, is predicted to result in a substantial modification in the protein binding pocket. The G107R change was shown to not only be responsible for the interruption of VPg binding in planta but also for the loss of cap binding ability in vitro, the principal function of eIF4E in the host. Overexpression of the *Capsicum*-eIF4E protein containing the G107R amino acid substitution in *Solanum lycopersicum* indicated that this polymorphism alone is sufficient for the acquisition of resistance against several TEV strains.

INTRODUCTION

Precise molecular interactions between host and pathogen occur when disease develops or successful host defense is initiated. At one end of this spectrum, these interactions, which may be direct or indirect, allow plants to activate a defense response that is often mediated by resistance genes (Dangl and Jones, 1998, 2001; Nimchuk et al., 2003). For disease development to occur, on the other hand, the invading pathogen must establish a specific molecular relationship with the host plant that provides an environment supportive of pathogen infection and its life cycle.

Because viruses are obligate intracellular parasites lacking the components necessary for their own independent survival, they rely upon an array of host factors to support their life cycle. The identification of host factors required for viral disease has provided useful information concerning virus infection. For instance, it has been observed that geminiviruses use host factors, such as the proliferating cell nuclear agent, a DNA polymerase accessory factor, NAC domain protein (SINAC1), a transcription factor, and plant homologs of retinoblastoma protein, to reprogram the host's cell cycle to allow replication of their own genome (Gutierrez, 2000; Gutierrez et al., 2004; Rojas et al., 2005). Likewise, several host factors for *Tomato bushy stunt virus*, a tombusvirus, and *Brome mosaic virus*, a bromovirus, have been thoroughly investigated using yeast as an artificial host system (Janda and Ahlquist, 1993; Duggal and Hall, 1995; Ishikawa et al., 1997; Noueiry and Ahlquist, 2003; Panavas et al., 2005; Mas et al., 2006; Serviene et al., 2006).

The physical interaction between host eukaryotic initiation factor eIF4E or eIF(iso)4E and the viral genome-linked protein (VPg) is critical for viral infection by several members of the genus potyvirus (Wittmann et al., 1997; Leonard et al., 2000; Grzela et al., 2006; Miyoshi et al., 2006; Robaglia and Caranta, 2006). The major role of eIF4E in the host cell is initiating protein translation by allowing recognition and interaction with the cap structure of cellular mRNA. However, it has been shown that the viral protein VPg can interfere with cap binding ability of eIF4E or its isoform eIF(iso)4E, which likely is responsible for the interruption of the formation of the translation initiation complex (Miyoshi et al., 2006). Additionally, a number of recent studies have demonstrated that eIF4G, which functions as an adapter connecting the translation initiation complex with the 40S

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ribosomal subunit, and the leader sequence of the *Tobacco etch virus* (TEV) RNA genome are also involved in the formation of the complex including eIF4E and VPg (Khan et al., 2006; Michon et al., 2006; Ray et al., 2006; Nicaise et al., 2007), which further demonstrates the involvement of the host translation initiation machinery in the initial steps of viral infection. Interestingly, the involvement of host translation initiation machinery during the viral infection process is not unique to the plant/potyvirus pathosystem. Several animal viruses, such as adenovirus, influenza, and poliovirus, are known to manipulate host translation machinery by inactivating or interrupting certain translation initiation processes (Sonenberg, 1987; Sonenberg and Pelletier, 1989; Mathews, 1990; Garfinkel and Katze, 1992; Cuesta et al., 2000; Gale et al., 2000).

Because viruses rely upon host factors for their own survival during the infection process, interruption of infection could result when a host factor is modified or eliminated such that infection could no longer progress (Kasschau and Carrington, 1998; Carrington et al., 2001; Baulcombe, 2004; Kang et al., 2005b; Soosaar et al., 2005). The efficacy of this resistance, which typically manifests as recessive inheritence, is evidenced by the prominence of recessive resistance to plant viruses relative to that of other pathogen classes (Provvidenti and Hampton, 1992; Keller et al., 1998; Robaglia and Caranta, 2006). This defense strategy appears to be particularly widely observed at the *eIF4E* locus where resistance to multiple RNA phytopathogenic viruses occurs in numerous plant families, including *pvr1* in *Capsicum* (Ruffel et al., 2002; Kang et al., 2005a), *mo1* in lettuce (*Lactuca sativa*; Nicaise et al., 2003), *sbm1* in pea (*Pisum sativum*; Gao et al., 2004), *pot*-1 in tomato (*Solanum lycopersicum*; Ruffel et al., 2005), *rym4/5* in barley (*Hordeum vulgare*; Stein et al., 2005), and *nsv* in melon (*Cucumis melo*; Nieto et al., 2006). In addition to these naturally existing genes, *lsp1* and *cum1* resistance alleles created via mutagenesis in *Arabidopsis thaliana* also encode *eIF4E* (Lellis et al., 2002; Yoshii et al., 2004). Thus, it is clear that genetic resistance against many viruses may arise when a plant expresses a gene encoding an altered eIF4E that interrupts its role in viral infection. Previous studies have shown that the physical interaction between eIF4E and VPg is correlated with viral infection (Wittmann et al., 1997; Lellis et al., 2002; Kang et al., 2005a). In the case of recessively inherited resistance against potyviruses in *Capsicum*, natural variation in *eIF4E* encoded by recessive resistance alleles are responsible for nonconservative amino acid changes that disrupt interaction with TEV VPg (Kang et al., 2005a).

This article aims to address the functional significance of natural amino acid variation in recessive resistance alleles with respect to the disease resistance phenotype. Mutational dissection of eIF4E defined the potential structural role(s) of each substitution in recessive resistance assayed at the organismal and at the protein levels. Examination of the eIF4E proteins containing dissected alterations allowed us to determine the significance of each substitution with respect to changes in affinity with their viral counterpart VPg in planta. Biological function of critical amino acid changes was determined by assessing the phenotypic outcome of transgenic tomato plants expressing an eIF4E allele containing a single substitution. Based on these results, we conclude that a Gly-to-Arg change at position 107 (G107R) in the eIF4E protein encoded by the *pvr1* allele defines the most significant position for host resistance to potyviruses. The amino acid change at this position is observed in multiple recessive potyvirus resistance genes from diverse plants, including pepper (*Capsicum annuum*), lettuce, and pea. Elucidation of the importance of this particular substitution as the molecular basis for the resistance phenotype opens the way to further detailed study of the resistance phenotype, the range of viral genotypes controlled, and the durability of resistance in nature.

RESULTS

Potyvirus Resistance Is Controlled by Amino Acid Changes in eIF4E Encoded at the *pvr1* Locus

Single nucleotide polymorphisms within the coding region of *eIF4E* encoded by multiple recessive resistance alleles at the *pvr1* locus, *pvr1*, *pvr1*^{1}, and *pvr1*^{2}, compared with the susceptible allele *Pvr1*$^+$ result in nonconservative amino acid changes in the eIF4E protein. These single nucleotide polymorphisms causing amino acid changes in the eIF4E protein determine both the range of potyviral isolates controlled by the resistance alleles and the degree to which viral titer and symptoms are reduced (Kang et al., 2005a). The expression level of eIF4E protein was examined in various pepper genotypes before and after inoculation of the highly aphid-transmissable (HAT) strain of TEV to determine whether potyvirus resistance is a result of differences in protein expression or accumulation. There were no obvious correlations between the eIF4E protein level and viral resistance in all pepper genotypes tested in this study (Figure 1A). Accumulation of TEV coat protein in susceptible genotypes was confirmed by an immunoblot using an antibody for TEV coat protein. Additionally, no differences in total RNA level were observed among genotypes included in this study (data not shown). These results support the prevailing hypothesis that phenotypic differences in potyviral infection among these genotypes are determined by the amino acid changes themselves, rather than by other components regulating expression or accumulation of eIF4E protein.

The naturally occurring amino acid substitutions in eIF4E encoded by resistance alleles at the *pvr1* locus are indicated in Figure 1B. The eIF4E protein encoded by the *pvr1* allele (eIF4E-*pvr1*) contains amino acid changes T51A, P66T, and G107R, eIF4E-*pvr1*^{1} contains amino acid changes V67E and L79R, and eIF4E-*pvr1*^{2} contains amino acid changes V67E, L79R, and D109N compared with eIF4E-*Pvr1*$^+$ (Ruffel et al., 2002; Kang et al., 2005a). To understand the biochemical effect of each amino acid substitution in eIF4E, we generated novel alleles of eIF4E, which contain each amino acid substitution separately, as shown in Figure 1C. The eIF4E constructs containing each substitution, eIF4E-T51A, eIF4E-P66T, eIF4E-G107R, eIF4E-V67E, eIF4E-L79R, and eIF4E-D109N, were applied in further assays.

The G107R Amino Acid Change in the *pvr1* Allele Is Responsible for Abolishing eIF4E–VPg Interaction in Yeast

The physical interaction between eIF4E encoded by resistance alleles at the *pvr1* locus and VPg from different TEV strains has

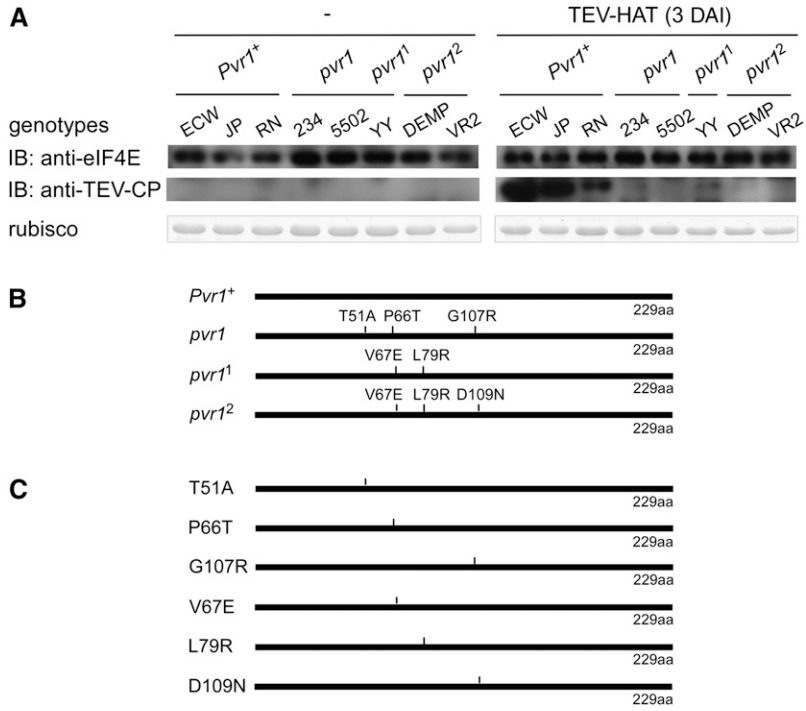

Figure 1. Amino Acid Changes in eIF4E Protein Encoded at the *Capsicum pvr1* Locus Are Responsible for Gain of Resistance.

(A) Pepper genotypes homozygous at the *pvr1* locus are expressing eIF4E protein at similar levels. The expression level of eIF4E protein encoded by the *pvr1* locus was examined in various *C. annuum* and *C. chinense* genotypes before and after TEV-HAT inoculation. Crude protein extracts obtained from pepper leaf tissue were loaded on a 12% SDS-PAGE gel. Immunoblot assay was performed using antibodies for *Capsicum*-eIF4E (top) and coat protein of TEV (middle). The amount of the loaded protein extract was shown by Coomassie blue staining (bottom). Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.

(B) Diagram indicating amino acid changes naturally occurring in the resistance alleles at the *pvr1* locus. eIF4E-*pvr1* contains T51A, P66T, and G107R amino acid substitutions; eIF4E-*pvr1*^{1} contains V67E and L79R amino acid substitutions; and eIF4E-*pvr1*^{2} contains V67E, L79R, and D109N amino acid substitutions compared with eIF4E-*Pvr1*$^+$.

(C) Diagram showing eIF4Es containing individual substitution separately: eIF4E-T51A, eIF4E-P66T, eIF4E-G107R, eIF4E-V67E, eIF4E-L79R, and eIF4E-D109N. Site-directed mutagenesis was performed to create single point mutations in eIF4E-*Pvr1*$^+$.

been investigated previously using both yeast two-hybrid and glutathione *S*-transferase pull-down assays (Kang et al., 2005a). eIF4E encoded by the susceptible allele (eIF4E-*Pvr1*$^+$) interacted strongly with VPg proteins from different TEV strains. However, VPg interaction was not detected for eIF4E-*pvr1*, eIF4E-*pvr1*^{1}, or eIF4E-*pvr1*^{2}, which implies that the T51A, P66T, and G107R amino acid changes in eIF4E-*pvr1* as well as the V67E and L79R amino acid changes in both eIF4E-*pvr1*^{1} and eIF4E-*pvr1*^{2} are responsible for the interruption of the physical interaction between eIF4E and VPg in yeast.

To examine the effect of each amino acid change on the physical interaction between eIF4E and VPg, yeast two-hybrid assays using eIF4E-T51A, eIF4E-P66T, eIF4E-G107R, eIF4E-V67E, eIF4E-L79R, and eIF4E-D109N with the VPg protein encoded by four different TEV strains: TEV-HAT, TEV-NW, TEV-N, and TEV-Mex21 (see Methods), were performed (Figure 2A). eIF4E-*Pvr1*$^+$ interacted very strongly with all VPg proteins from four different TEV strains; this interaction served as a positive control for each TEV strain. The proteins eIF4E-T51A, eIF4E-P66T, and eIF4E-D109N all interacted with VPg, showing no visible difference from results obtained in the positive control for each TEV strain. By contrast, eIF4E-V67E and eIF4E-L79R, each of which contain a single substitution observed in both eIF4E-*pvr1*^{1} and eIF4E-*pvr1*^{2}, showed inconsistent interaction with VPg protein from four TEV strains. In the case of eIF4E-V67E, the interaction with VPg from TEV-NW was noticeably reduced, but the weaker interaction was less evident with VPg proteins from the other TEV strains. eIF4E–VPg interaction was greatly diminished for eIF4E-L79R when TEV-N and TEV-Mex21 were used. These results suggest that the complete loss of VPg binding ability of eIF4E encoded by the *pvr1*^{1} and *pvr1*^{2} alleles described in the previous study (Kang et al., 2005a) is the result of an additive effect of the V67E and L79R changes. It is striking that eIF4E-G107R existing in eIF4E-*pvr1*, which displays the broadest resistance spectrum among resistance alleles at the *pvr1* locus, did not interact with VPg proteins from any of the four different TEV strains. Together, these results indicate that the loss of VPg binding ability of eIF4E-*pvr1* is caused by the single change G107R because the two other changes in eIF4E-*pvr1*, T51A and P66T, did not affect eIF4E–VPg interaction. Reliable

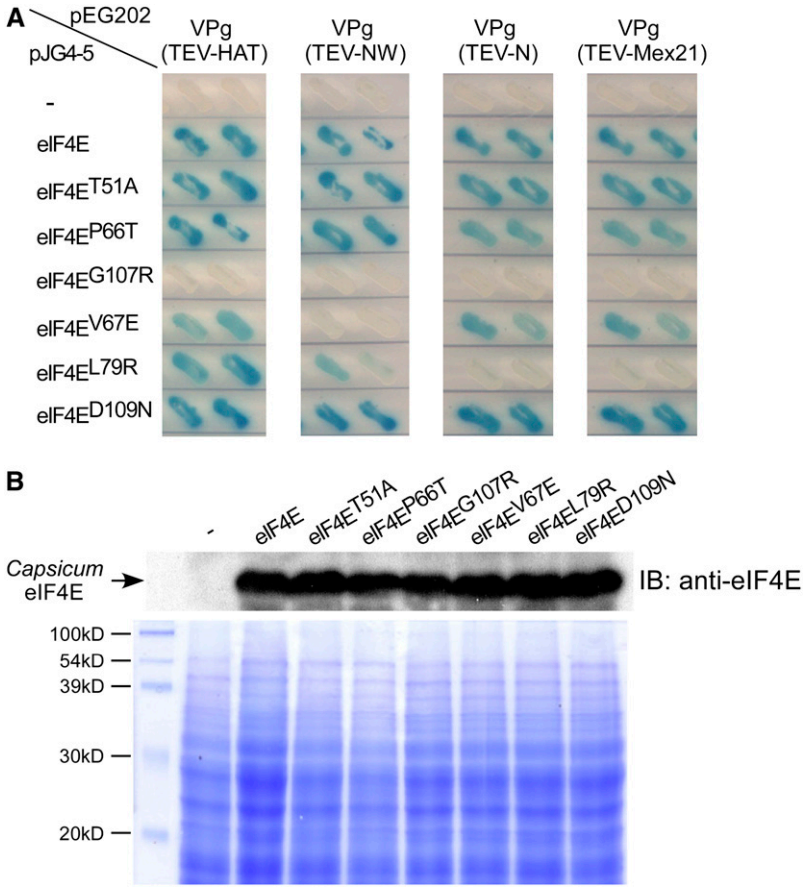

Figure 2. Interaction between eIF4E Protein Containing a Single Amino Acid Change and VPg from Four Different TEV Strains Was Examined via Yeast Two-Hybrid Assay.

(A) X-galactosidase assay of yeast two-hybrid interaction between TEV-VPg and eIF4Es. Bait plasmid pEG202 was used to express the fusion protein VPg from TEV-HAT, TEV-NW, TEV-N, or TEV-Mex21, while the prey plasmid pJG4-5 was used to express eIF4E containing T51A, P66T, G107R, V67E, L79R, or D109N. The empty vector pJG4-5 served as a negative interaction control. Yeast cells containing known interactors pEG202:VPg and pJG4-5:eIF4E-*Pvr1*$^+$ served as a positive interaction control.

(B) Expression of eIF4E proteins in yeast cells was examined via immunoblot (IB) assay. Yeast cells containing empty vector (lane 1) or eIF4E fusion gene in pJG4-5 vector (lanes 2 to 8) were grown on selection medium lacking Leu and using galactose as a carbon source. Proteins were fractionated on 12% SDS-polyacrylamide gels and immunoblotted with antibody for *Capsicum*-eIF4E (top panel). The eIF4E fusion proteins ~48 kD were detected in all yeast cells containing various eIF4E genes, as indicated by the arrow, but not in the cells containing empty vector. Coomassie blue–stained gel showing equal amounts of protein extract loaded on the gel (bottom panel).

expression of eIF4E protein for each yeast cell used in yeast two-hybrid assays was confirmed by immunoblot analysis (Figure 2B).

The G107R Amino Acid Change Interrupts eIF4E–VPg Interaction in Planta

The yeast two-hybrid assay suggested that the G107R amino acid change in eIF4E-*pvr1* alone is sufficient to abolish the capacity of eIF4E to bind VPg. To investigate further the effect of the G107R change on the interaction with VPg, a protein–protein interaction assay in planta was performed using an *Agrobacterium tumefaciens*–mediated transient expression assay and bimolecular fluorescence complementation (BiFC) assay. The N-terminal half of yellow fluorescence protein (YFP) was fused with the eIF4E construct (YN:eIF4E) and the C-terminal half of YFP was fused with the VPg construct [YC:VPg (TEV-HAT)] (Figure 3A). Yellow fluorescent signal could be detected only when eIF4E and VPg interact or are located closely enough to allow the formation of intact YFP. As shown in Figure 3B, YN:eIF4E-*Pvr1*$^+$, YN:eIF4E-*pvr1*, or YN:eIF4E-G107R without YC:VPg (TEV-HAT) were used as a negative control. Coexpression of YN:eIF4E-*Pvr1*$^+$ and YC:VPg (TEV-HAT) showed a strong yellow fluorescence signal, while the coexpression of YN:eIF4E-*pvr1* and YC:VPg (TEV-HAT) together was indistinguishable from the negative control. Coexpression of YN:eIF4E-G107R and YC:VPg (TEV-HAT) also did not show yellow fluorescence signal. The reciprocal combination, YC:eIF4E and YN:VPg (TEV-HAT), displayed similar results (data not shown). This is consistent with

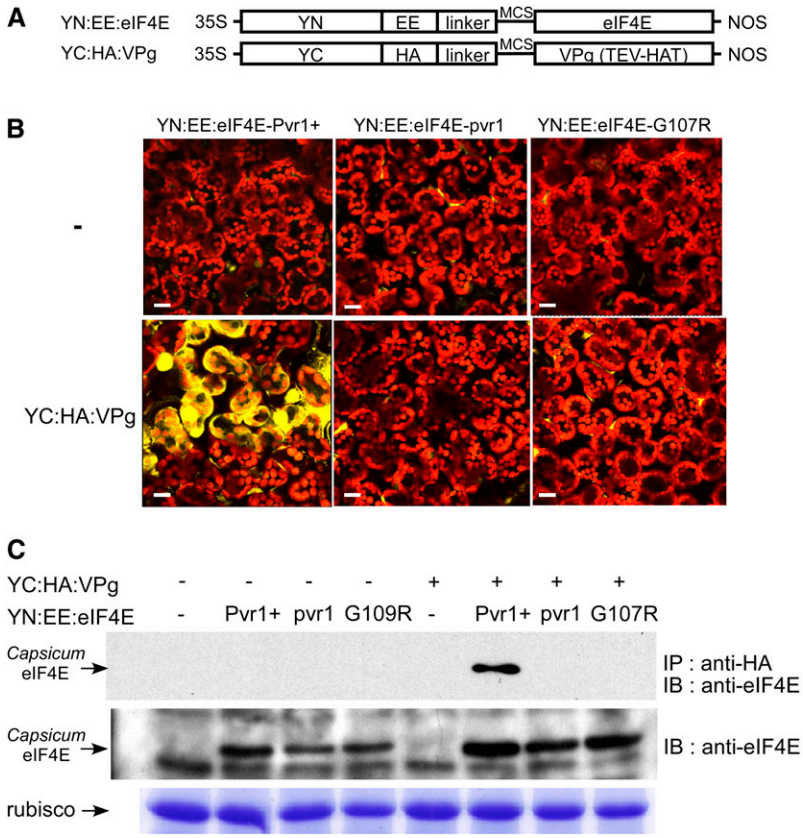

Figure 3. Loss of VPg Binding Ability Caused by the G107R Change in eIF4E Protein Was Confirmed in Planta via *Agrobacterium*-Mediated Transient Expression Assay in *N. benthamiana*.

(A) Diagram showing the constructs used in protein–protein interaction assays in planta. The N-terminal half of YFP was fused with eIF4E constructs (YN:eIF4E), and the C-terminal half of YFP was fused with the VPg construct [YC:VPg (TEV-HAT)].

(B) Confocal microscopy images from BiFC assay using *N. benthamiana* mesophyll cells. eIF4E proteins fused with YN (YN:EE:eIF4E-*Pvr1*$^+$, YN:EE:eIF4E-*pvr1*, and YN:EE:eIF4E-G107R) were transiently expressed in *N. bethamiana* leaf tissue with and without VPg protein fused with YC [YN:HA:VPg (TEV-HAT)]. Yellow fluorescent signal generated by the protein–protein interaction was detected 60 h after infiltration. Chloroplast autofluorescence is shown in red. Bars = 10 μm.

(C) Immunoblot image from coimmunoprecipitation assays. Total protein extracts were pulled down with anti-HA agarose beads and immunoblotted with antibody for *Capsicum*-eIF4E. *Agrobacterium*-mediated transient expression of eIF4E protein in *N. benthamiana* plants was evaluated with the antibody for *Capsicum*-eIF4E.

the yeast two-hybrid assay and suggests that the G107R amino acid change is also responsible for abolishing VPg binding ability in planta.

Immunoprecipitation using HA tag and Glu-Glu (EE) tag, which were fused downstream of YC and YN, respectively (Figure 3A), was performed to confirm the results of the BiFC assay (Figure 3C). Total protein extracts were immunoprecipitated with anti-HA agarose beads and immunoblotted with an antibody for *Capsicum*-eIF4E. A strong interaction between YN:EE:eIF4E-*Pvr1*$^+$ and YC:HA:VPg was detected, whereas YN:EE:eIF4E-*pvr1* and YN:EE:eIF4E-G107R were not pulled down with YC:HA:VPg, consistent with the results from the BiFC assay.

Agrobacterium-mediated transient expression of eIF4E protein in *Nicotiana benthamiana* plants was evaluated with an antibody for *Capsicum*-eIF4E (Figure 3C). Similar protein accumulation levels were detected for all tissues containing YN:EE:eIF4E-*Pvr1*$^+$, YN:EE:eIF4E-*pvr1*, or YN:EE:eIF4E-G107R. Slightly increased protein levels were detected when YN:EE:eIF4E was coexpressed with YC:HA:VPg. Similarly, accumulation of YC:HA:VPg was elevated by coexpression with YN:EE:eIF4E. Interestingly, when a physical interaction occurs between eIF4E and VPg, YC:HA:VPg accumulated to even higher levels (data not shown). VPg interaction activity in planta for eIF4E proteins encoded by other resistance alleles at the *pvr1* locus, *pvr1*^{1} and *pvr1*^{2}, are shown in Supplemental Figure 1 online.

The G107R Substitution Abolishes Cap Binding Ability of eIF4E in Vitro

In a eukaryotic cell, eIF4E recognizes and interacts with the cap structure of cellular mRNA to recruit it into the translation

initiation complex. Previously, a cap binding assay using recombinant *Capsicum*-eIF4E proteins produced in *Escherichia coli* demonstrated that eIF4E-*pvr1* does not show cap binding activity (Kang et al., 2005a). By contrast, eIF4E-*pvr1*^{1} and eIF4E-*pvr1*^{2} maintained cap binding ability while resulting in host resistance (Kang et al., 2005a). The cap binding assay using *E. coli*–produced recombinant protein is a standard method to assess the cap binding ability of a protein; however, it is possible that the recombinant protein failed to retain the characteristics of the native protein produced by host cells. To determine the effect of the variations encoded by resistance alleles on cap binding ability with better resolution and to confirm these results, a cap binding assay using pepper leaf extracts was performed in this study (Figure 4A).

In this study, eIF4E-*Pvr1*$^+$ encoded by two different pepper genotypes, *C. annuum* JP and RN, appeared to bind strongly to the m^7-GTP sephadex columns. By contrast, eIF4E-*pvr1* encoded by *C. annuum* 5502 and *Capsicum chinense* 234 displayed much reduced affinity for the m^7-GTP sephadex columns, consistent with previously published cap binding assays (Kang et al., 2005a). However, both eIF4E-*pvr1*^{1} and eIF4E-*pvr1*^{2} also showed a weakened interaction with the cap analog when compared with eIF4E-*Pvr1*$^+$. The results from this assay support the idea that eIF4E-*pvr1*, eIF4E-*pvr1*^{1}, and eIF4E-*pvr1*^{2} all show reduced or compromised cap binding ability relative to eIF4E-*Pvr1*$^+$, although eIF4E-*pvr1*^{1} and eIF4E-*pvr1*^{2} appear to maintain greater cap binding ability than eIF4E-*pvr1*.

To identify the single amino acid substitutions responsible for the observed disruption of cap binding activity in *Capsicum*-eIF4E encoded by the recessive resistance alleles at the *pvr1* locus, a cap binding assay was performed with eIF4E-T51A, eIF4E-P66T, eIF4E-G107R, eIF4E-V67E, eIF4E-L79R, or eIF4E-D109N (Figure 4B). Recombinant proteins from each construct containing a single substitution were expressed and extracted from *E. coli* BL21(DE2)pLysS and assayed for binding activity using m^7-GTP cap analog columns. The immunoblot shown in Figure 4B shows that all of the eIF4E proteins were reliably expressed after induction using 20 μM isopropylthio-β-galactoside at 20°C and that all constructs showed strong cap binding activity except for eIF4E-G107R. These results indicate that the single amino acid change G107R observed in eIF4E-*pvr1* and shown to interrupt the eIF4E-VPg interaction is also responsible

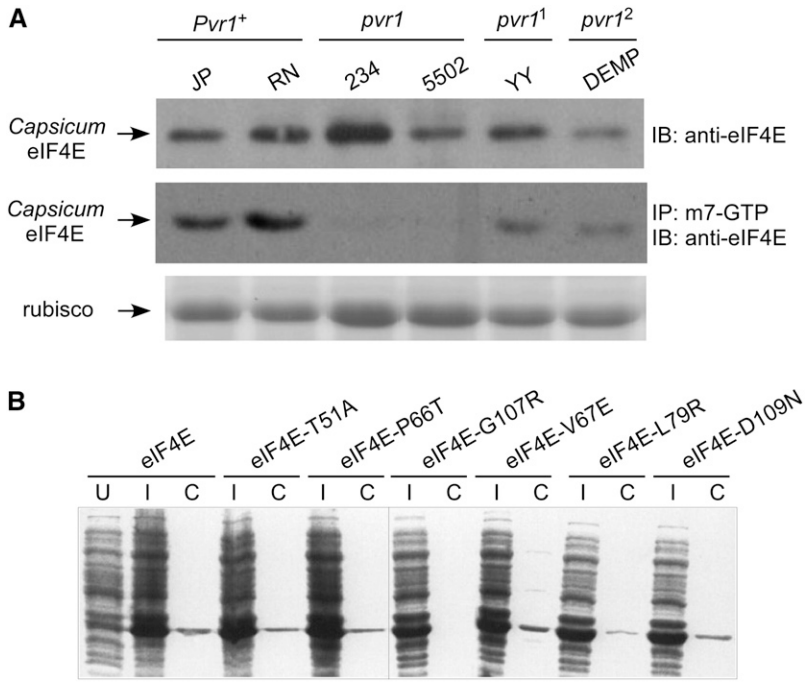

Figure 4. The Cap Binding Ability of *Capsicum* eIF4E Proteins Containing Single Amino Acid Changes Was Examined via an in Vitro Cap Binding Assay.

(A) Cap binding assay using protein extracts from six different pepper plants. Immunoblot assay was performed using an antibody for *Capsicum*-eIF4E before (top panel) and after cap binding assay (middle panel) using the m^7-GTP-Sepharose affinity column. The amount of the loaded protein extract was shown by Coomassie blue staining (bottom panel).

(B) Cap binding assay using recombinant eIF4E proteins expressed and extracted from *E. coli* (DE3). Total cell lysates of *E. coli* before (U) and after (I) induction by the addition of 20 μM isopropylthio-β-galactoside and proteins showing cap binding activity that are eluted from m^7-GTP-Sepharose affinity chromatography (C) were loaded on 12% SDS-PAGE gel and stained with Coomassie blue. The recombinant protein was induced in all *E. coli* cells containing the eIF4E gene with each substitution. The soluble fraction of bacterial cell lysates containing recombinant eIF4E protein was applied to the m^7-GTP-Sepharose column. After washing steps, the protein was eluted from the column using m^7-GTP. The eIF4E proteins containing T51A, P66T, V67E, L79R, or D109N were bound to m^7-GTP-Sepharose. eIF4E-G107R did not show cap binding activity. U, uninduced; I, induced; C, recombinant eIF4E proteins after m^7-GTP-Sepharose affinity chromatography.

for the loss of cap binding ability of eIF4E in vitro. Additionally, L79R, present in both eIF4E-*pvr1*^{1} and eIF4E-*pvr1*^{2}, negatively affected cap binding ability, although the effect was less pronounced than the complete disruption observed with eIF4E-G107R.

Predicted Protein Models for Mutated eIF4E

Three dimensional models of the *Capsicum*-eIF4E protein and the approximate location of the amino acid substitutions were predicted in a previous study based on the murine eIF4E crystal structure (Kang et al., 2005a). This analysis indicated that most of the amino acid substitutions encoded by recessive resistance alleles *pvr1*, *pvr1*^{1}, and *pvr1*^{2} are located in the binding pocket of eIF4E. In addition, the amino acid changes are located in close proximity to highly conserved residues that are known to be involved in binding cap or eIF4G. To provide a better understanding of the role of these natural variations at the amino acid level with respect to structural properties of the protein, pairwise structural alignments of the available crystallographic structures of eIF4E from mouse (Protein Data Bank [PDB] code: 1EJ1, monomer A; 190 residues), human (PDB code: 1IPB, monomer A; 217 residues), and yeast (PDB code: 1AP8; 213 residues) were obtained using the Combinatorial Extension method (Shindyalov and Bourne, 1998) and used to optimize the alignment between these protein sequences and that of *Capsicum*-eIF4E (Figure 5A). The structures of human and mouse eIF4E are quite similar. Optimal superposition of the structures was obtained by aligning 182 residues with no gaps, leading to a root mean square deviation (rmsd) of 1.0 Å for the C^{α} atoms and a sequence identity of 99.5%. The optimal superposition of the structures of yeast and mouse eIF4E was obtained by aligning 159 residues (with 11 gaps), resulting in an rmsd of 3.3 Å for the C^{α} atoms and 25.8% sequence identity. Similarly, the optimal superposition of the structures of yeast and mouse eIF4E was obtained by aligning 173 residues (with 12 gaps), resulting in a final rmsd of 3.7 Å for the C^{α} atoms and 30.1% of sequence identity.

The alignment shown in Figure 5A was used to generate all-atom models for the *Capsicum*-eIF4E protein using the program MODELLER (Sali, 1995; Sali et al., 1995; Sanchez and Sali, 2000). The sequence identity between the final models of *Capsicum*-eIF4E and the human, mouse, and yeast proteins are 41.2, 35.3, and 35.8%, respectively. Figure 5B shows the molecular surface area of a three-dimensional (3D) model of *Capsicum*-eIF4E where the amino acid substitutions T51A, P66T, G107R, V67E, L79R, and D109N have been mapped onto that surface. To highlight the location of the binding pocket region, a 7-methyl-GDP molecule (in magenta) is also displayed. In all three resistance alleles, substituted residues occurred within the protein binding pocket with the exception of T51A. Based on this analysis, it seems plausible that the substitutions P66T, G107R, V67E, L79R, and D109N perturb the binding site region, thus affecting cap binding. However, the effects of each substitution on the eIF4E–VPg interaction and cap binding ability appear to be quite different.

The position of several critical substitutions, G107R in eIF4E-*pvr1* and V67E and L79R in both eIF4E-*pvr1*^{1} and eIF4E-*pvr1*^{2}, on the protein binding pocket of eIF4E protein and the positional relationship among these substitutions were estimated using the high-resolution 3D structural model (Figure 5C). The G107R transition in eIF4E-*pvr1* appears to be entirely responsible for the loss of in vitro cap binding ability in addition to the interruption of the eIF4E–VPg interaction. To explain how this single amino acid substitution could have such a dramatic effect, we first considered the nature of the substitution. Electrostatic calculations performed with the program GRASP (Nicholls et al., 1991) on a 3D model of *Capsicum*-eIF4E showed that the region of the molecular surface defining the cap binding slot in *Capsicum*-eIF4E is covered in its center by low values of electrostatic potential, while the surrounding walls are very positive (data not shown). By changing G107 into Arg, an additional positive charge and a rather large side chain are introduced that may produce strong electrostatic repulsion and steric hindrance with adjacent positively charged residues.

The reduced effects of the V67E and L79R changes found in eIF4E-*pvr1*^{1} and eIF4E-*pvr1*^{2} observed for both the VPg–eIF4E interaction and the cap binding relative to the G107R change can be explained as follows. As shown in Figure 5C, residues V67 and L79 lie at both ends of a loop region that lines the binding pocket. This loop contains a key residue, W75, equivalent to murine W56, which was known to be essential for cap binding (Ueda et al., 1991; Marcotrigiano et al., 1997). It appears that the structure can tolerate the presence of a single substitution, either V67E or L79R, because eIF4E-V67E binds to 7-methyl-cap at a similar level to eIF4E-*Pvr1*$^+$, and eIF4E-L79R can still bind the cap with reduced efficiency. The coexistence of these two substitutions is likely to disrupt the conformation of the loop containing W75 and alter the binding properties of the site. This hypothesis is further supported by the detection of the additive effect of these two changes on the interruption of the eIF4E–VPg interaction.

Transgenic Tomatoes Overexpressing eIF4E-G107R or *eIF4E*-pvr1+pvr1^2 Gained Resistance against Several TEV Strains

Ectopic overexpression of *Capsicum*-eIF4E-*pvr1* in *S. lycopersicum* resulted in gain of viral resistance (Kang et al., 2007). Overexpression of eIF4E-*pvr1* in susceptible tomato plants switches the phenotypic outcome from susceptibility to resistance against several potyviruses (Kang et al., 2007). To test the hypothesis that the G107R change in eIF4E-*pvr1*, which abolishes the physical interaction between eIF4E and VPg in planta, is responsible for gain of resistance, transgenic tomato plants overexpressing eIF4E-G107R were generated and evaluated. Additionally, transgenic plants overexpressing eIF4E-*pvr1*+*pvr1*^{2} containing all six substitutions existing in the *pvr1* and *pvr1*^{2} alleles, T55A, P66T, V67E, L79R, G107R, and D109N, were tested for enhancement of resistance and broadened spectrum of resistance.

The full-length open reading frames of eIF4E-G107R and eIF4E-*pvr1*+*pvr1*^{2} were cloned into the plant transformation vector pBI121 in sense orientation relative to the 35S promoter. Plasmids containing engineered eIF4E constructs were introduced into the experimental tomato variety Micro-Tom via *Agrobacterium*-mediated transformation (Meissner et al., 1997). Successful transformation was primarily detected by expression of kanamycin resistance. The existence of the transgene was confirmed via PCR screening using two primer sets: one based

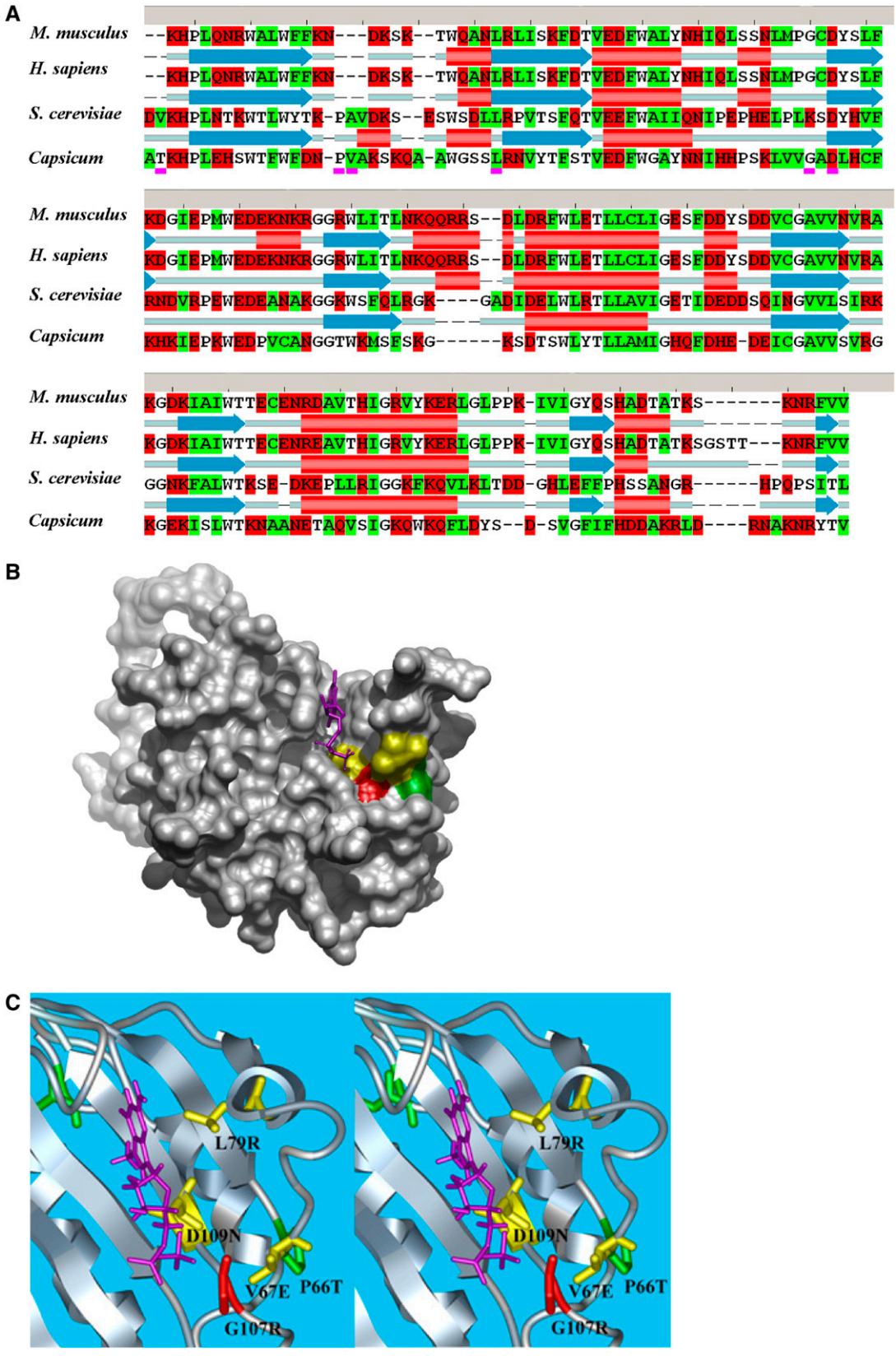

Figure 5. A 3D Structural Model of *Capsicum*-eIF4E Protein Showing the Location of Natural Variations in Recessive Virus Resistance Alleles.

on the neomycin phosphotransferase II (*NPTII*) gene and another based on the 35S promoter/*eIF4E* sequence (data not shown). Progenies derived from self-fertilization of the primary transformants (T0 plants) that segregated in a ratio of 3:1 for resistance/sensitivity to kanamycin, implying that the transgene was integrated at a single locus, were selected for further assays. All T0 plants that contained *eIF4E*-G107R or eIF4E-*pvr1*+*pvr1*^{2} in a single copy appeared normal by visual observation. T1 transgenic progenies obtained by self-pollination from T0 plants were again screened for kanamycin resistance and PCR amplifications. The *eIF4E* transcript accumulation was assessed in T1 plants by RNA gel blot analysis to provide a relative estimation of the transcription level of the transgene (Figure 6A). All transgenic plants showed *eIF4E* expression greater than that observed for endogenous *eIF4E* RNA accumulation. We were not able to detect significantly increased total eIF4E protein in transgenic plants relative to the nontransformed or empty vector–transformed plants via an immunoblot assay using *Capsicum*-eIF4E antibody. This observation suggests that *Capsicum*-eIF4E antibody was not capable of distinguishing *Capsicum*-eIF4E from endogenous *Solanum*-eIF4E. It is also possible, however, that the levels of eIF4E protein in the transgenic plants are regulated posttranscriptionally. eIF4E is known to be a crucial rate limiting factor for the translation initiation process (Sonenberg and Gingras, 1998); therefore, overexpression may have detrimental effects for the transgenic plants. In fact, transgenic plants containing more than one ectopic copy tended to display reduced growth, although further studies need to be performed to better understand this observation.

Untransformed plants and T1 plants containing eIF4E-G107R or eIF4E-*pvr1*+*pvr1*^{2} were used for screening with TEV-HAT, TEV-N, and TEV-Mex21 (Figures 6B and 6C). Three leaves per plant were inoculated with each virus at the four to six leaf stage, and four individual plants representing each genotype were tested. Plants were observed daily for symptom development for more than a month after inoculation. Typical systemic TEV symptoms (terminal leaflet cupping, stunting, and petioles bent downward) developed in all positive control plants, including untransformed plants and T2 plants containing an empty vector, ~10 d after inoculation (DAI). By contrast, T1 plants carrying eIF4E-G107R or eIF4E-*pvr1*+*pvr1*^{2} showed no visible symptoms (Figure 6B). A month after inoculation, T1 plants carrying eIF4E-G107R or eIF4E-*pvr1*+*pvr1*^{2} were indistinguishable from their corresponding uninoculated controls. The accumulation of the viral coat protein was assessed using indirect ELISA in uninoculated tissue to detect systemic infection of TEV in the transgenic plants (Figure 6C). The results from ELISA clearly confirmed that T1 plants carrying eIF4E-G107R or eIF4E-*pvr1*+*pvr1*^{2} gained complete resistance against all three TEV strains tested: TEV-HAT, TEV-N, and TEV-Mex21. It is interesting to note that while eIF4E-*pvr1* transgenic plants showed susceptibility against TEV-Mex21, in accordance with the natural resistance spectrum of the *pvr1* allele in pepper (Kang et al., 2007), eIF4E-G107R gained resistance to TEV-Mex21. This suggests that the other two amino acid changes in the engineered resistance allele relative to the wild type, T51A and P66T, actually compromise the potyviral resistance gained by G107R. Transgenic tomatoes containing only T51A and P66T were generated and screened with TEV-HAT, TEV-N, and TEV-Mex21. These transgenic tomatoes remained susceptible to all isolates considered (data not shown). Additionally, transgenic tomatoes carrying eIF4E-*pvr1*^{1} containing both V67E and L79R displayed delayed susceptibility (see Supplemental Figure 2 online), which is consistent with the observation from pepper genotypes homozygous for the *pvr1*^{1} allele (Kang et al., 2005a). By contrast, transgenic tomatoes containing the amino acid substitutions from both *pvr1* and *pvr1*^{2} (eIF4E-*pvr1*+*pvr1*^{2}) resulted in an increased resistance spectrum to include all resistances conferred by either allele alone.

DISCUSSION

The Naturally Occurring Amino Acid Substitution G107R in the *pvr1* Allele Appears to Be Responsible for Conformational Changes Leading to Modification of the Cap Binding Pocket

Several lines of evidence indicate that the Gly-to-Arg change at position 107 (G107R) in the *Capsicum*-eIF4E protein encoded by the *pvr1* allele is a critical substitution causing the loss of interaction with TEV-VPg and alone is sufficient for the gain of resistance against potyvirus infection. This observation that the change from a Gly to an Arg appears to be responsible for the disruption of the cap binding pocket has several possible explanations. First, a neutral Gly residue is replaced with a positively charged residue with a relatively large side chain. Amino acid 107 is adjacent to Arg-171, an amino acid that interacts directly with the negative charge of the cap phosphate group and is known to be important for cap binding (Marcotrigiano et al., 1997). The

Figure 5. (continued).

(A) Structural alignment of eIF4E proteins from mouse, human, and yeast based on the experimental structures available from PDB (PDB codes 1EJ1, 1IPB, and 1AP8, respectively). The amino acid residues in the sequence are represented by single letters with the background color used to describe the hydrophobic/hydrophilic properties of each particular amino acid. The following convention was used: red = hydrophilic; green = hydrophobic; white = neutral. The assignment of the residues to the secondary structure elements is shown below the sequence: blue arrows are used to indicated β-strands, red rectangles indicate α-helices, and thick gray lines indicate the loop or disordered regions of the chain. The bottom line shows the alignment of the *Capsicum*-eIF4E sequence encoded by a susceptible allele, *Pvr1*$^+$, to the experimental structures that were used to generate the 3D models. The mutated residues in the sequence are indicated with a magenta mark below the corresponding letter.

(B) View of the molecular surface area of a model of *Capsicum*-eIF4E with the amino acid substitutions colored as follows: T51A and P66T in green; G107R in red; V67E, L79R, and D109N in yellow; and 7-methyl-GDP in magenta.

(C) The image zoomed into the hot area of the model (stereoview). The protein is represented using a ribbon model with mutated residues shown using a stick representation. The color codes used are the same as in **(B)**.

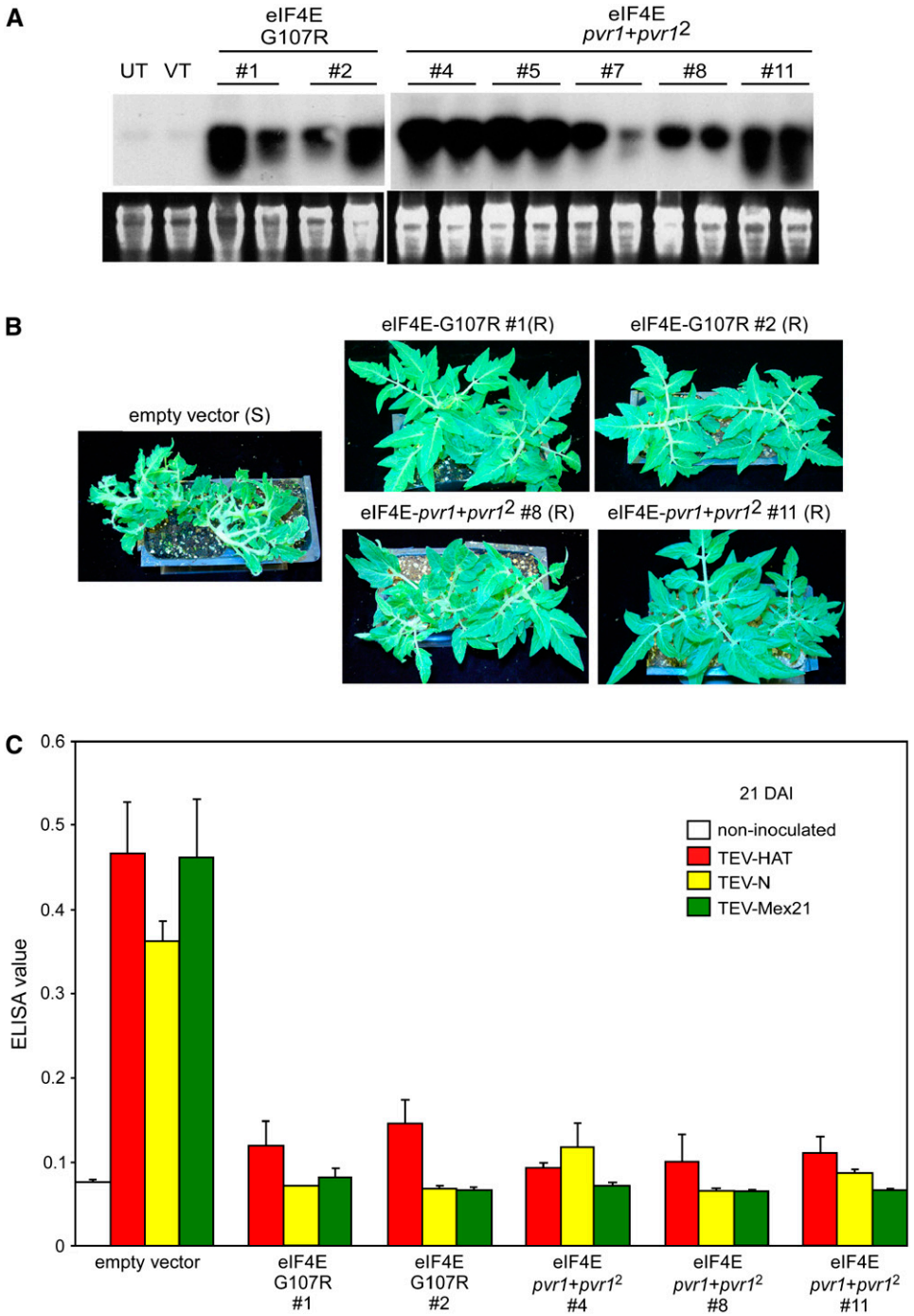

Figure 6. TEV-Screening Results from *S. lycopersicum* MicroTom T1 Plants Expressing eIF4E-G107R or eIF4E-*pvr1*+*pvr1*^{2} for TEV-HAT, TEV-N, and TEV-Mex21.

(A) Expression of *eIF4E* via RNA gel blot analysis in T1 plants. Total RNA was isolated from each plant and analyzed by RNA gel blot hybridization using pepper *eIF4E* cDNA labeled with ^{32}P-dCTP as a probe. Ethidium bromide–stained rRNA served as a loading control.

(B) Image of T1 plants containing empty vector, eIF4E-G107R, or eIF4E-*pvr1*+*pvr1*^{2} after TEV-N inoculation. Representative phenotypic differences between resistant and susceptible plants are shown. Images were taken at 30 DAI.

(C) ELISA results for T1 plants containing empty vector, eIF4E-G107R, or eIF4E-*pvr1*+*pvr1*^{2} after inoculating with TEV-HAT, TEV-N, and TEV-Mex21. Accumulation of TEV coat protein determined by ELISA of tissue sampled from upper uninoculated leaves at 21 DAI. Uninoculated MicroTom plants served as negative controls, and empty vector–containing transgenic plants served as positive controls for viral infection.

addition of another positive charge in this region is likely to produce repulsion not present in the wild-type protein, causing the location of this important residue to shift. Additionally, this region of the cap binding pocket is rather constrained and may be unable to accommodate the presence of a large residue, such as Arg. The length of the Arg side chain is 50% greater than the distance between the predicted arrangement of G107 and the bound cap; thus, the binding region may be inaccessible due to steric hindrance. In summary, it is plausible that the G107R change in the eIF4E protein could cause a strong electrostatic repulsion with adjacent positively charged residues and/or steric hindrance that interrupts the ability of the protein to bind both cap and VPg.

Further analysis of the nuclear magnetic resonance (NMR) structure of yeast, human, and mouse eIF4E provides better indication as to why the G107R change in *Capsicum* can be so disruptive. The residue G88, both in mouse and human eIF4E, is the equivalent of G107 in *Capsicum*-eIF4E. In the available x-ray structures, this G88 residue has been forced into a conformation ($\phi \sim +105$, $\psi \sim -10$) in the Ramachandran plot (Ramachandran and Sasisekharan, 1968) that is rarely occupied by any other amino acid residue. However, based on the NMR structure of yeast eIF4E in complex with 7-methyl-GDP (PDB code: 1AP8), the equivalent position to G107 in *Capsicum*-eIF4E is occupied by a Lys residue (K90) that is oriented toward the region of the active site where one of the phosphate groups of the 7-methyl-GDP is docked. The K90 residue is observed for eIF4E proteins in *Saccharomyces cereveviae* and *Schizosaccharomyces pombe*, implying that an additional positive charge in that particular position in eIF4E can be accommodated in yeast eIF4E. In the NMR structure of yeast eIF4E, where Gly is substituted by Lys (K90), the conformation of residue K90 ($\phi = +46$, $\psi = +25$) has shifted toward the α_L region of the Ramachandran plot from the highly unfavorable region occupied by G88 in mouse and human eIF4E. However, the stress in the loop region seems to be distributed among a few neighboring residues, including L89 (with $\phi = +36$, $\psi = -158$) and P88 (with $\phi = -78$, $\psi = +42$), that have been forced into energetically less-favorable regions of the conformational map (Karplus, 1996) and may act to relieve the stress on the loop caused by K90. In the case of the G107R substitution in eIF4E-*pvr1*, there is no such residue to perform a similar role. This comparative analysis supports the idea that the G107R substitution alone in *Capsicum*-eIF4E introduces an additional stress that neighboring residues cannot easily accommodate, causing conformational changes that lead to a substantial modification in the cap binding pocket.

Despite these significant consequences of the G107R change, the impact appears to be localized to the VPg and cap binding regions. Overall folding of the mutant protein appears to proceed normally because it maintains the ability to bind to eIF4G (Kang et al., 2007). In many instances, the plant factors that play a role in virus infection are also vitally important to the host. Accordingly, evolution of resistance may be constrained to modifications that disrupt some protein interactions while maintaining others. In fact, selective consequences of single amino acid changes in eIF4E have been explored statistically by measuring a nonsynonymous-to-synonymous nucleotide substitution rate ratio (ω) (J.R. Cavatorta and A.E. Savage, unpublished results). The results indicate that most sites of the eIF4E coding sequence are under purifying selection or neutral evolution, as expected due to the pleiotropic function of eIF4E in translation initiation. However, a small subset of amino acid sites at the eIF4E locus, including position 107, show a molecular signature of strong positive selection that is nonrandomly distributed with respect to sites involved in resistance against viruses.

The VPg Binding and Cap Binding Abilities of eIF4E Are Structurally Related

Two conserved Trp residues in the mammalian eIF4E protein are known to be directly responsible for cap binding of eIF4E by enhancing π-π stacking enthalpy and allowing the localization of the cap structure in the cap binding slot of eIF4E (Marcotrigiano et al., 1997, 1999). It has been noted that the eIF4E proteins encoded by the *pvr1* allele from pepper and the *sbm1* allele from pea, which both contain the G107R substitution, show reduced cap binding (Gao et al., 2004; Kang et al., 2005a). Recent studies have suggested that the mRNA cap structure and potyvirus VPg at least partially share the protein binding pocket structure of the eIF4E protein or its isoform eIF(iso)4E (Michon et al., 2006; Miyoshi et al., 2006).

We wished to examine the relationship between cap binding and VPg binding regions using the natural variations found in the *Capsicum*-eIF4E resistance alleles. The cap binding ability of eIF4E is disrupted when these two Trp residues are substituted by Lys residues (Marcotrigiano et al., 1997); therefore, we induced two mutations in wild-type *Capsicum*-eIF4E, W75L and W121L, and assayed for VPg binding ability. Based on the results from both yeast two-hybrid and BiFC assays in planta, we were able to detect that the VPg binding ability was also lost for *Capsicum*-eIF4E containing W75L and W121L mutations (data not shown). We also have shown that eIF4E-*pvr1*, eIF4E-*pvr1*^{1}, and eIF4E-*pvr1*^{2} produced in pepper have impaired in vitro cap binding ability relative to wild-type eIF4E. These results strongly suggest that the VPg binding domain overlaps with the cap binding domain, although this cap binding assay has not been performed in vivo.

Further evidence that the VPg and cap binding domains overlap is provided by the observation that the amino acid change G107R alone was sufficient to disrupt cap binding as well as the interaction with VPg. The effects of V67E and L79R, however, both found in eIF4E-*pvr1*^{1} and eIF4E-*pvr1*^{2}, are quite different. These substitutions strongly affect VPg binding while decreasing cap binding only slightly or not at all. Therefore, it appears that these amino acids, which are located at either end of a loop structure, may be in a region involved in VPg binding but do not directly associate with the mRNA 5′ cap.

The results from several independent eIF4E-VPg protein interaction assays and the information from a 3D structural model of *Capsicum*-eIF4E, in addition to this cap binding assay, allow us to better understand the roles of these natural variations in the acquisition of disease resistance and maintenance of host function. The fact that a single amino acid change, G107R, results in loss of both VPg and cap binding supports the contention that these two binding domains overlap in the eIF4E protein. The observation that other substitutions affect one interaction but not

the other suggests that this overlap between binding domains is not complete.

The G107R Change Conferring Potyvirus Resistance Has Evolved Independently in Recessive Resistance Genes Encoding eIF4E within Various Plant Families

We have shown that the Gly-to-Arg change at position 107 (G107R) in the *Capsicum*-eIF4E protein appears to be responsible for the gain of resistance conferred by the *pvr1* allele originally from *C. chinense*. In plants, a number of alleles at the *eIF4E* locus conferring resistance against multiple viruses in the family Potyviridae and at least one virus in the family Tombusviridae have been discovered recently. These include *pvr1* in pepper (Ruffel et al., 2002; Kang et al., 2005a), *mo1* in lettuce (Nicaise et al., 2003), *sbm1* in pea (Gao et al., 2004), *pot*-1 in tomato (Ruffel et al., 2005), *rym4/5* in barley (Stein et al., 2005), and *nsv* in melon (Nieto et al., 2006). It is striking to note that the critical amino acid substitution in eIF4E-*pvr1*, G107R, also exists at the homologous sites in several other recessive resistance genes: *mo1* and *sbm1* in lettuce and pea, respectively. Additionally, similar substitutions at or near position 107 are observed in various recessive potyvirus resistance genes encoding eIF4E, including *pvr1*^{2} (D109R) from *C. annuum*, *mo1*^{1} (G107H) from lettuce, and *pot1* (M106I) from tomato. Thus, it appears that a G107R change or a very similar change is present in recessive resistance genes *pvr1*, *pvr1*^{2}, *sbm1*, *mo1*^{1}, and *pot1*. The fact that similar substitutions on the eIF4E gene exist quite frequently in various host plant species suggests that phytopathogenic viruses have historically placed at least moderate if not strong selective pressures on diverse hosts and that evolution of resistance at this locus has been an important source of host plant resistance.

The defense strategy whereby a modified version of host eIF4E results in resistance against phytopathogenic viruses appears to have evolved multiple times, providing a highly durable protection from viral infection. Although viral invasion could presumably be disrupted at a number of essential steps, resistance via modification of the eIF4E protein appears to have arisen independently in numerous plant families. eIF4E has important pleiotropic functions in cap binding during initiation of protein translation that must be maintained in virus resistant genotypes. Several possibilities may account for the evolution of resistance alleles with compromised cap binding ability. One factor that may contribute to maintenance of resistance is functional redundancy of cap binding proteins, indicated in *Arabidopsis* mutational studies of eIF(iso)4E (Duprat et al., 2002). Additionally, eIF(iso)4E encoded at the *pvr6* locus is also known to be involved in recessive resistance to *Pepper veinal mottle virus* in pepper (Ruffel et al., 2006), which also suggests functional redundancy of eIF4E and eIF(iso)4E in this case. Functional redundancy may therefore free eIF4E of its pleiotropic role in protein translation and allow for the evolution of resistance.

The eIF4E proteins encoded by recessive resistance alleles *pvr1*, *pvr1*^{2}, *mo1*^{1}, and *pot1* contain several amino acid substitutions in addition to G107R. In all cases, these accompanying substitutions occur proximal to position 107 based on the 3D structure model, which suggests that these substitutions may act to relieve the electrostatic and steric hindrances predicted to be introduced by G107R. The presence of additional substitutions in *eIF4E* resistance alleles may represent an additional mechanism to minimize the costs associated with resistance. Although these amino acids are always present with G107R, they have not been observed to increase the resistance spectra of the associated alleles.

Transgenic Approaches Using Engineered eIF4E Based on Natural Variation May Define a Widely Applicable Strategy for Developing Durable, Broad-Spectrum Resistance

Recessive resistance genes are widespread in plants, generally highly durable through decades of deployment across continents and now represent a new source of dominant genetic variation for transgenic deployment. In this article, we show that overexpression of an engineered version of a recessive resistance allele encoding eIF4E in transgenic tomato plants results in a highly resistant phenotype showing dominant inheritance. It has been shown previously that ectopic expression of eIF4E containing natural variations derived from a resistant *Capsicum* plant perturb interactions required for viral susceptibility in a heterologous system without obvious adverse effects to the host (Kang et al., 2007).

The shift to dominant inheritance suggests that resistant versions of eIF4E may interfere with host factors required for viral infection (Kang et al., 2007). It has been shown that naturally occurring resistance alleles retain an eIF4G binding domain (Kang et al., 2007), implying that eIF4G may be effectively sequestered from the virus. It is also plausible that high levels of overexpressed ectopic eIF4E cause downregulation of native eIF4E. The possibility of posttranscriptional and/or posttranslational regulation of the final expression level of a translation initiation factor has been previously suggested in transgenic *Arabidopsis* lines overexpressing eIF1A that show tolerance to salt stress (Rausell et al., 2003). This explanation raises the possibility of potential pleiotropic effects of overexpressed eIF4E-G107R, which may also impair cap binding ability necessary for the host. In an attempt to address this question, we have closely monitored two self-pollinated generations of transgenic plants and have observed no visible defects in germination, vegetative growth, flowering and fruit development, or other morphological phenotypes. Consistent with this observation is the fact that the naturally existing recessive resistance alleles *pvr1*, *mo1*, and *sbm1* in pepper, lettuce, and pea, respectively, contain the G107R changes and show no perceived effects of any type. In addition, unpublished analyses from our lab show that the amino acid position 107 in eIF4E is positively selected through evolution (J.R. Cavatorta and A.E. Savage, unpublished results), a molecular signature that is typically observed in traits with positive fitness consequences. Intriguing questions remain, however, about whether mRNA translation efficiency of the host cell is affected when engineered eIF4E with impaired cap binding ability is overexpressed in transgenic plants. We are currently focusing on differentiating native eIF4E and ectopically expressed eIF4E in the transgenic plants and are also interested in understanding possible pleiotropic effects of the transgenic events.

Viral isolates overcoming the predominant resistance genes have been identified, implying that the viruses coevolved to compensate for the interruption in the VPg–eIF4E interaction (Moury et al., 2004; Kang et al., 2005b). The accumulation of existing variations in resistance alleles encoding eIF4E into one eIF4E construct may stimulate broader-spectrum resistance with increased durability. The mechanism by which the eIF4E–VPg interaction is disrupted may differ in a transgenic system relative to naturally occurring recessive resistance. However, it is likely that transgenic plants expressing appropriate *eIF4E* alleles would also show stability and durability through large-scale deployment in agriculture.

METHODS

Plant and Virus Materials

Solanum lycopersicum MicroTom seeds used for transformation were obtained from Ball Horticultural Company. *Capsicum annuum* NuMex RNaky (RN), Early Cal Wonder (ECW), Florida VR2 (VR2), Yolo Y (YY), and breeding line 5502 were obtained from Asgrow Seed Co. *C. annuum* Dempsey (DEMP) was provided by M. Deom (University of Georgia), and *C. annuum* Jupiter was provided by Syngenta Seeds. *Capsicum chinense* PI 159234 (234) was obtained from the USDA Southern Regional Plant Introduction Station. TEV-HAT and TEV-NW (nonwilting on *Capsicum frutescens* Tabasco) cultures were obtained from T. Pirone (University of Kentucky, Lexington, KY). TEV-N and TEV-Mex21 were obtained from J. Murphy (Auburn University, Auburn, AL). Each recessive resistant allele at the *pvr1* locus shows a differential resistance spectrum against these TEV strains: the *pvr1* allele is resistant to HAT, NW, and N, the *pvr1*^{1} allele is resistant to none of these TEV strains, and the *pvr1*^{2} allele is resistance to HAT, NW, and Mex21. All TEV strains were maintained on TMV-resistant *Nicotiana tabacum* Kentucky 14. *Nicotiana benthamiana* plants were used for protein–protein interaction assays in planta.

Protein Extraction Preparation and Immunoblotting Using Plant Tissue

Evaluation of protein expression in plant leaf tissue via immunoblot assay has been described previously (Lin et al., 1995; Menke et al., 2005). For immunoblots, proteins were electrotransferred onto Immun-Blot PVDF membrane (Bio-Rad), and eIF4E protein was detected with an antibody for *Capsicum*-eIF4E and peroxidase-labeled anti-rabbit antibodies using an ECL kit (GE Healthcare).

Site-Directed Mutagenesis and Plasmid Construction

Cloning of *Capsicum-eIF4E* alleles and *VPg* has been described previously (Kang et al., 2005a). The plasmid pGEM-T containing *Capsicum*-eIF4E was used for site-directed mutagenesis using the QuikChange II-E site-directed mutagenesis kit (Stratagene) following the manufacturer's instructions. Primer sets used for site-directed mutagenesis are listed in Supplemental Table 1 online. Primer sets used for subcloning processes are listed in Supplemental Table 2 online.

The plasmid construction for cap binding analysis using pET16b vector system also has been previously described in detail (Kang et al., 2005a). To generate the fusion protein with eIF4E and either the N-terminal half of YFP tagged with Glu-Glu (YN:EE) or the C-terminal half of YFP tagged with influenza hemagglutinin (HA) epitope tag (YC:HA), the pSY vector series (pSY 736, pSY 735, pSY 728, and pSY 738; provided by Nir Ohad, Tel Aviv University, Tel Aviv, Israel) was used (Bracha-Drori et al., 2004). In the case of VPg, an intragenic *Bam*HI recognition site was erased using site-directed mutagenesis. To express these fusion proteins in planta, the pCAMBIA binary vector system was used and the *Hin*dIII restriction enzyme site was selected for subcloning. Plant transformation was performed using the pBI121 system as described previously (Kang et al., 2007). The DNA sequences were confirmed for the resulting plasmids after the subcloning process.

Yeast Two-Hybrid Analysis

Yeast two-hybrid analysis was performed as previously described (Kang et al., 2005a). Yeast strains and plasmid vectors were provided by G.B. Martin (Boyce Thompson Institute, Ithaca, NY). A bait plasmid, pEG202, was used for the fusion of VPg from TEV-HAT, TEV-NW, TEV-Mex21, and TEV-N strains; a prey plasmid, pJG4-5, was used to express *Capsicum-eIF4Es* containing each substitution separately. Both bait plasmid and prey plasmid were transformed into yeast strain EGY48 containing the *lacZ* reporter plasmid pSH18-34. The equivalent expression of eIF4E protein in yeast cells was confirmed by immunoblot assay following a procedure previously described (Printen and Sprague, 1994).

Agrobacterium-Mediated Transient Protein Expression in *N. benthamiana*

The incubation, induction, and coinfiltration of *Agrobacterium tumefaciens* cultures were performed as previously described with minor modifications (Bendahmane et al., 2000). The cultures were infiltrated into the *N. benthamiana* leaves at 0.25 OD_{600}.

Fluorescence and Confocal Microscopy Imaging

Protein–protein interaction in planta was monitored 40, 60, and 80 h after *Agrobacterium* coinfiltration using BiFC assay. The signal from YFP, which is generated by the contact of YN and YC when the two target proteins are proximal, was monitored using a Leica TCS SP2 scanning confocal microscope.

Coimmunoprecipitation

Coimmunoprecipitation was performed as described previously (Moffett et al., 2002; Rairdan and Moffett, 2006). Immunoprecipitated samples were collected and separated by 10% SDS-PAGE gel and blotted onto an ImmunoBlot PVDF membrane (Bio-Rad) for immunoblot assay.

Cap Binding Assays

The cap binding assay was performed as previously described (Morino et al., 1996; Kang et al., 2005a). Expression of the recombinant proteins and purification of the proteins by m^7GTP-Sepharose 4.B (Amersham Biosciences) were performed as described previously with minor modifications (Friedland et al., 1997). After eIF4E protein was eluted using the extraction buffer containing 100 uM m^7GTP, SDS-PAGE and immunoblot analysis were performed using a *Capsicum*-eIF4E antibody (New England Biolabs) for detection of eIF4E maintaining cap binding ability in vitro.

Tomato Transformation and Transgenic Screening

The vector construction and tomato transformation followed previously described methods (Kang et al., 2007). *S. lycopersicum* MicroTom cotyledons were transformed and regenerated into whole plants as described earlier (Meissner et al., 1997). At least 10 T0 transgenic individuals were obtained for each construct. Regenerated plants were self-pollinated and T1 seeds collected. For kanamycin resistance testing, plants at the three or four leaf stage were sprayed with 200 μg/mL of

kanamycin solution more than three times. Genomic DNA was extracted using a hexadecyltrimethylammonium bromide method, and the presence of the *NPTII* gene and eIF4E constructs were verified via PCR amplification using the primers described previously (Kang et al., 2007). For RNA gel blot analysis, total RNA was isolated from tomato leaves using the RNeasy plant mini kit (Qiagen) following the manufacturer's instructions. RNA gel blots were performed according to standard methods. Probes were labeled with ^{32}P-dCTP (Amersham Biosciences) using the Prime It II kit (Stratagene).

Virus Screening Procedure

T1 plants confirmed as transgenic tomatoes were evaluated for viral infection using TEV-HAT, TEV-N, TEV-Mex21, and PepMoV. Plants were inoculated at the four to six leaf stage. After light application of carborundum, viral inoculum was applied to the three oldest leaves. Viral inoculum was produced by grinding systemically infected tobacco tissue in 50 mM potassium phosphate buffer, pH 7.5 (1 g of tissue: 20 mL of buffer). Mock-inoculated and uninoculated controls were routinely included. Treatments typically consisted of four plants. Inoculated plants were monitored for appearance of symptoms, and systemic infection was evaluated at 9 and 21 DAI using antigen plate-coating indirect ELISA as previously described (Kang et al., 2005a). Anti-viral immunoglobulins were obtained from Agdia and used according to the manufacturer's instructions.

Protein Modeling

The program MODELLER (Sali and Blundell, 1993; Sali et al., 1995; Sanchez and Sali, 2000) was used to generate the 3D models for the sequence of *Capsicum*-eIF4E. The input data for the MODELLER program were as follows: (1) a template structure corresponding to an experimentally determined structure retrieved from the PDB (Berman et al., 2000), and (2) a pairwise alignment between the particular *Capsicum* sequence and that of the template structure. MODELLER minimizes the violations of distance and dihedral-angle restraints derived from the structures used as templates. Models based on three different templates (mouse, human, and yeast proteins) were built to assess the variability of different parts of the structure, particularly parts involving loop regions where substitutions occur. To facilitate this task, a structure alignment of the experimentally determined structures of the mouse, human, and yeast eIF4E proteins was produced using the Combinatorial Extension method (Shindyalov and Bourne, 1998). Final adjustment of the alignment of the *Capsicum*-eIF4E with mouse, human, and yeast sequences was performed with the help of graphic tools included in the commercial programs ICM (MOLSOFT) and DS-Modeling (Accelrys). The final manual alignment was used in the model generation process.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AY485127, AY485129, AY485130, AY485131, and AAA48909.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Confocal Microscopy Images from Bimolecular Fluorescence Complementation Assay Using *Agrobacterium*-Mediated Transient Expression Assay in *N. benthamiana*.

Supplemental Figure 2. Accumulation of TEV Coat Protein Determined by ELISA for Transgenic Tomatoes Containing Empty Vector, eIF4E-*pvr1*, or eIF4E-*pvr1*^{1}.

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