***Tobacco etch virus* Infectivity in *Capsicum* Spp. Is Determined by a Maximum of Three Amino Acids in the Viral Virulence Determinant VPg**

Kari Perez,¹ Inhwa Yeom,² Byoung-Cheorl Kang,³ Daniel R. Ripoll,⁴ Jinhee Kim,³ John F. Murphy,⁵ and Molly M. Jahn⁶

¹Department of Plant Breeding and Genetics, Cornell University, Ithaca, NY 14853 U.S.A.; ²Department of Horticulture and Breeding, Andong National University, Andongsi, Gyeongsangbukdo, 760-749, Republic of Korea; ³Department of Plant Science, College of Agriculture and Life Sciences, Seoul National University, Seoul 151-742, Republic of Korea;

⁴Computational Biology Service Unit, Life Sciences Core Laboratories Center, Cornell University, Ithaca, NY 14853 U.S.A.;

⁵Department of Entomology & Plant Pathology, Auburn University, AL 36849-5409, U.S.A.; ⁶Laboratory of Genetics and Department of Agronomy, University of Wisconsin-Madison, Madison 53706 U.S.A.

Submitted 24 April 2012. Accepted 21 August 2012.

Potyvirus resistance in *Capsicum* spp. has been attributed to amino acid substitutions at the *pvr1* locus that cause conformational shifts in eukaryotic translation initiation factor eIF4E. The viral genome-linked protein (VPg) sequence was isolated and compared from three *Tobacco etch virus* (TEV) strains, highly aphid-transmissible (HAT), Mex21, and N, which differentially infect *Capsicum* genotypes encoding *Pvr1*⁺, *pvr1*, and *pvr1*². Viral chimeras were synthesized using the TEV-HAT genome, replacing HAT VPg with Mex21 or N VPg. TEV HAT did not infect pepper plants homozygous for either the *pvr1* or *pvr1*² allele. However, the novel chimeric TEV strains, TEV-HAT^{Mex21-VPg} and TEV-HAT^{N-VPg}, infected *pvr1* and *pvr1*² pepper plants, respectively, demonstrating that VPg is the virulence determinant in this pathosystem. Three-dimensional structural models predicted interaction between VPg and the susceptible eIF4E genotype in every case, while resistant genotypes were never predicted to interact. To determine whether there is a correlation between physical interaction of VPg with eIF4E and infectivity, the effects of amino acid variation within VPg were assessed. Interaction between *pvr1*² eIF4E and N VPg was detected in planta, implying that the six amino acid differences in N VPg relative to HAT VPg are responsible for restoring the physical interaction and infectivity.

etch virus (TEV), the focus of this study, infect solanaceous crops such as pepper, potato, tobacco, and tomato (Provvidenti and Hampton 1992). Potyviruses comprise the largest genus of plant viruses characterized to date and are found within the Potyviridae family (Ward and Shukla 1991). The *Potyvirus* genome consists of a single strand of positive-sense RNA, approximately 10 kb in length, that encodes a polyprotein subsequently cleaved into functional proteins by three virus-encoded proteinases: P1, the helper-component proteinase (HC-Pro), and the nuclear inclusion a (NIa) (Dougherty and Carrington 1988; Dougherty and Semler 1993). Based on similarities of genome organization, *Potyvirus* spp. reside within the same class or superfamily as the Picornaviridae, which includes the polioviruses and rhinoviruses (Koonin et al. 1993).

The NIa polyprotein, also referred to as viral genome-linked protein (VPg)-Pro, includes the VPg at the N terminus and a proteinase domain at the C terminus (Carrington and Dougherty 1987; Dougherty and Parks 1991; Murphy et al. 1990). Upon cleavage from the polyprotein, VPg is covalently bound to the 5' end of the viral RNA by a tyrosine residue (Murphy et al. 1991). Mutation of this tyrosine residue in *Tobacco vein mottling virus* rendered the virus noninfectious (Murphy et al. 1996). Proposed functions of VPg in the virus infection cycle include roles in replication, translation, host gene inactivation, and potyviral genome stabilization (Gallois et al. 2010; Lellis et al. 2002; Robaglia and Caranta 2006). The involvement of VPg in either virus replication or translation has been demonstrated for both poliovirus and potyviruses (Eskelin et al. 2011; Pathak et al. 2008).

Virulence determinants provide clues into the molecular interactions that govern virus–host interactions. For several potyviruses, VPg was identified as the virulence determinant, which is responsible for determining the infectivity spectrum of the viruses (Borgstrom and Johansen 2001; Gallois et al. 2010; Huang et al. 2010; Kuhne et al. 2003; Leonard et al. 2000; Moury et al. 2004; Rajamaki and Valkonen 1999, 2002). Further, in a number of systems, potyviral VPg is known to interact with host eukaryotic translation initiation factor eIF4E or its isoform, eIF(iso)4E, proteins widely implicated in virus resistance (Bruun-Rasmussen et al. 2007; Gallois et al. 2010; Hwang et al. 2009; Jenner et al. 2010; Kanyuka et al. 2005; Kuhne et al. 2003; Leonard et al. 2000; Ling et al. 2009;

Potyviruses are an agronomically significant group of viruses which accounts for significant losses each year in many crops. A number of *Potyvirus* spp., including *Tobacco*

K. Perez and I. Yeom contributed equally to this work.

Current address for K. Perez: AAAS Science & Technology Policy Fellow, Washington, DC.

Current address for D. Ripoll: DoD Biotechnology HPC Software Applications Institute, Telemedicine and Advanced Technology Research Center, US Army Medical Research and Materiel Command, Fort Detrick, MD, U.S.A.

Corresponding author: M. M. Jahn; Telephone: +1.608.216.6304; Fax: +1.608.262.4556; E-mail: mjahn@cal.wisc.edu

*The e-Xtra logo stands for “electronic extra” and indicates three supplementary figures and two supplementary tables are published online.

Moury et al. 2004; Roudet-Tavert et al. 2007; Wittmann et al. 1997).

In the host, eIF4E functions as a component of the translation initiation complex, eIF4F, which consists of eIF4A, eIF4E, and eIF4G (Browning 2004; Gingras et al. 1999). This complex is responsible for translation initiation, which includes mRNA 5' cap recognition, mRNA unwinding, and recruitment of the 40S ribosomal subunit (Browning 1996, 2004; Gingras et al. 1999; Kawaguchi and Bailey-Serres 2002; Nicaise et al. 2007). It has been shown that naturally occurring mutations in eIF4E are correlated with plant virus resistance in a number of plant families determining infectivity in several RNA virus families, including bymoviruses, carmoviruses, and potyviruses (Gao 2004; Kang et al. 2005; Kanyuka et al. 2005; Ling et al. 2009; Nicaise et al. 2003; Nieto et al. 2006; Ruffel et al. 2002, 2005; Stein et al. 2005; Truniger and Aranda 2009). Because potyviral RNA is structurally similar to host mRNA, it is hypothesized that VPg serves as a cap mimic in viral translation (Robaglia and Caranta 2006). Alterations in eIF4E were found to inhibit its physical interaction with VPg and were correlated with potyvirus resistance (Beauchemin et al. 2007; Bruun-Rasmussen et al. 2007; Cavatorta et al. 2011; Gallois et al. 2010; Ibiza et al. 2010; Kang et al. 2005; Kuhne et al. 2003; Leonard et al. 2000; Moury et al. 2004; Piron et al. 2010; Yeam et al. 2007).

Mutational analysis of eIF4E in several studies has shown that gain of virus resistance did not always correlate with shifts in host function of eIF4E, such as cap-binding or interaction with eIF4G (German-Retana et al. 2008; Ibiza et al. 2010; Piron et al. 2010). The central domain of the VPg has been implicated in both eIF4E interaction and virus infection (Borgstrom and Johansen 2001; Bruun-Rasmussen et al. 2007; Gallois et al. 2010; Graner and Bauer 1993; Kanyuka et al. 2005; Kuhne et al. 2003; Leonard et al. 2000; Moury et al. 2004; Rajamaki and Valkonen 1999, 2002; Roudet-Tavert et al. 2007; Wittmann et al. 1997; Yambao et al. 2003). A substitution within the VPg at central position 116 determined the outcome of infection in two pathosystems: in *Arabidopsis* eIF(iso)4E or eIF(iso)4G knockouts, a Gln116Glu substitution in *Turnip mosaic virus* VPg-determined infection (Gallois et al. 2010); and, in *Pisum sativum* plants encoding eIF4E resistance gene *wlv*, an Arg116His substitution in *Bean yellow mosaic virus* VPg determined the outcome of infection (Bruun-Rasmussen et al. 2007). Nearly the same position in *Potato Virus A* (PVA) VPg, His118Tyr, determined infectivity of *Solanum commersonii* (Rajamaki and Valkonen 2002). *Potato virus Y* (PVY) infection of *S. hirsutum* plants carrying the eIF4E resistance gene, *pot1*, was determined by a single VPg amino acid substitution, Arg119His (Moury et al. 2004). *P. sativum* plants homozygous for the eIF4E resistance gene, *sbm1*¹, controlling infection by *Pea seedborne mosaic virus*, were either resistant to or susceptible to the virus, depending on VPg mutations from amino acids 105 to 107 (Borgstrom and Johansen 2001).

The role of VPg central domain as a determinant of infectivity is not limited to potyviruses. The VPg central region of bymovirus *Barley yellow mosaic virus*, determined pathogenicity in barley genotypes homozygous for the eIF4E resistance gene, *rym4* (Graner and Bauer 1993; Kanyuka et al. 2005; Kuhne et al. 2003). In addition to the VPg central domain, the VPg C terminus has also been implicated as a determinant of infection (e.g., the PVA VPg mutation, Leu185Ser, reduced the rate of systemic infection in *Nicandra physaloides*) (Rajamaki and Valkonen 2002).

TEV VPg has long been the focus of studies aimed at understanding the nature of resistance and the determinants of infectivity (Dougherty and Parks 1991; Murphy et al. 1990; Schaad et al. 1997; Schaad and Carrington 1996; Schaad et al. 1996).

Pepper plants resistant to TEV were shown to encode an eIF4E protein which did not physically interact with TEV VPg (Kang et al. 2005; Murphy et al. 1998). In pepper, eIF4E is encoded at the *pvr1* locus. Two alleles at the *pvr1* locus, *pvr1* and *pvr1*², resulted in differential infectivity with a range of TEV isolates. Pepper plants homozygous for *pvr1* were susceptible to TEV-Mex21 but resistant to TEV-highly aphid transmissible (HAT) and TEV-N (Murphy et al. 1998); pepper plants homozygous for *pvr1*² were susceptible to TEV-N (J. Murphy and M. Jahn, unpublished data) but resistant to TEV-HAT (Deom et al. 1997; Murphy et al. 1998). Results from the present study demonstrate that naturally occurring amino acid variation within TEV VPg, primarily in the protein's central domain, was sufficient to change the infectivity spectrum of the virus, clearly establishing VPg as the determinant of infectivity in this pathosystem. Further, the specific domains of eIF4E and VPg hypothesized to account for the physical interaction between these proteins were modeled and then systematically analyzed in planta to determine the nature of the physical interaction apparently required for infectivity.

RESULTS

TEV isolates with different infectivity in pepper display variations in VPg amino acid sequence.

Resistance alleles at the *pvr1* locus encoding eIF4E genes *pvr1* and *pvr1*² differ from the susceptible allele *Pvr1*⁺ by four and three distinct amino acid substitutions, respectively; however, they vary markedly with respect to the range of TEV isolates controlled (i.e., their resistance spectra) (Fig. 1A) (Deom et al. 1997; Kang et al. 2005; Murphy et al. 1998). Pepper plants homozygous for the *pvr1* allele were resistant to all TEV strains tested, except TEV-Mex21. Likewise, pepper plants homozygous for the *pvr1*² allele were resistant to all TEV strains tested, with the exception of TEV-N. TEV-Mex21, TEV-N, and TEV-HAT will be referred to hereafter as Mex21, N, and HAT. Sequence analysis of the 188 amino acids encoding TEV VPg revealed 97% amino acid similarity between HAT and either Mex21 or N (Fig. 1B). Amino acid changes were concentrated in the central and C-terminal region of the VPg. Amino acid variations between HAT and N included A53T, I111L, E112D, P113H, S117N, and D184E. Those differing between HAT and Mex21 included I107N, P113L, S115A, D184E, and T186M.

TEV-based chimeric viruses were created and employed to analyze virulence determinant.

We created two TEV-based chimeras from a full-length infectious HAT-GFP clone harboring the *green fluorescent protein* (GFP) gene fused to HC-Pro as the primary template (Fig. 2) (Dolja et al. 1992; Schaad et al. 1997). The viral coding region VPg resides within the N-terminal domain of the NIa protein and includes bases 5,532 to 5,690 of the HAT genome. The chimeric clones replaced the HAT-GFP VPg with the VPg of Mex21 or N using the megaprimer method (Perez et al. 2006) and were designated HAT^{Mex21-VPg}-GFP and HAT^{N-VPg}-GFP. The chimeras were transcribed in vitro as described previously (Perez et al. 2006; Schaad et al. 1997) and inoculated onto pepper plants containing the *pvr1* or *pvr1*² genotypes. Two resistant pepper genotypes, *Capsicum chinense* Plant Introduction number 159234 ('CA4') (*pvr1/pvr1*) and *C. annuum* 'Dempsey' (*pvr1*^{2/pvr1²), and two susceptible genotypes, *C. annuum* 'NuMex RNaky' ('RNaky') (*Pvr1*⁺/*Pvr1*⁺) and *C. chinense* 'Habanero' (*Pvr1*⁺/*Pvr1*⁺), were used for the infectivity study. These pepper genotypes will subsequently be referred to as *pvr1*, *pvr1*², or *Pvr1*⁺, depending on the *pvr1* allele each encodes.}

VPg is the virulence determinant in the *pvr1*-TEV Mex21 pathosystem.

In order to test whether the VPg region of Mex21 is sufficient to convert the infectivity spectrum of TEV-HAT, pepper plants were inoculated with HAT-GFP, Mex21, and a chimeric viral strain, HAT^{Mex21-VPg}-GFP. *C. chinense* 'CA4' was used as a homozygous *pvr1* line. The corresponding susceptible *Pvr1*⁺ genotype, *C. chinense* 'Habanero', was selected as a paired control, drawn from the same species as the resistant genotype. *C. annuum* 'Dempsey', homozygous for the resistance allele *pvr1*², was used as a negative control plant because neither HAT nor Mex21 can overcome the *pvr1*² resistance.

Visible symptom development in noninoculated upper leaves was monitored for at 10 days postinoculation (dpi) and summarized in Table 1. HAT-GFP induced systemic mosaic symptoms and mild deformation in noninoculated upper leaves of the susceptible genotype *Pvr1*⁺ ('Habanero'), consistent with systemic infection. In contrast, no symptoms were observed in

pvr1 genotypes ('CA4' and 'Dempsey') inoculated with HAT-GFP; plant development was indistinguishable from mock-inoculated and uninoculated controls. Mex21 caused systemic mosaic symptoms and mild distortion in *Pvr1*⁺ leaves, whereas HAT^{Mex21-VPg}-GFP symptoms were less severe but still apparent on *Pvr1*⁺, consisting of systemic mosaic without leaf distortion. Plants homozygous for *pvr1* inoculated with Mex21 or HAT^{Mex21-VPg}-GFP displayed systemic, mild mosaic symptoms. In contrast, no visible symptoms developed in *pvr1*² plants inoculated with Mex21 or HAT^{Mex21-VPg}-GFP.

At 30 dpi, plants inoculated with HAT-GFP, HAT^{Mex21-VPg}-GFP, and Mex21 were again visually assessed for symptom development (Supplementary Fig. S1) and sampled for enzyme-linked immunosorbent assay (ELISA) to assess systemic infection in noninoculated upper leaves (Fig. 3A). Based on ELISA, all three viruses were detected in the *Pvr1*⁺ control genotype; however, no systemic spread was detected with any of the three strains in the homozygous *pvr1*² genotype. In *pvr1* plants, HAT-GFP was not detected; however, Mex21 and HAT^{Mex21-VPg}-

Fig. 1. Amino acid variation of eIF4E and viral genome-linked protein (VPg). **A**, eIF4E amino acid alignment. Comparison of eIF4E proteins encoded by *pvr1* resistance alleles *pvr1*, *pvr1*¹, and *pvr1*² as well as the susceptibility allele, *Pvr1*⁺. The *pvr1* variation includes T51A, P66T, and G107R. The *pvr1*² variation includes VG7E, L79R, and D109N. **B**, VPg amino acid alignment. Amino acid sequence alignment of *Tobacco etch virus* (TEV)-highly aphid transmissible (HAT), TEV-Mex21, and TEV-N VPgs. Amino acid changes are concentrated in the central and C-terminal region of the VPg. Amino acid variations between TEV-HAT and TEV-N included A53T, I111L, E112D, P113H, S117N, and D184E. Those differing between TEV-HAT and TEV-Mex21 include I107N, P113L, S115A, D184E, and T186M.

GFP were detected, demonstrating that the Mex21 VPg was sufficient to establish infection.

Plants infected with GFP-tagged viruses HAT-GFP and HAT^{Mex21-VPg}-GFP were also monitored by confocal microscopy for virus accumulation. To evaluate the progression of systemic infection of the viruses, noninoculated upper leaves from at least three plants per genotype were sampled from each virus treatment between 7 and 10 dpi, the period of highest GFP signal (K. Perez and M. Jahn, *unpublished results*). Leaves showing mosaic symptoms were preferentially selected when present. GFP signal was detected in all *PvrI*⁺ plants inoculated with either one of the GFP-tagged viruses. GFP signal was not observed in HAT-GFP inoculated *pvrI* plants; however, all *pvrI* plants infected with HAT^{Mex21-VPg}-GFP showed GFP accumulation throughout the plant regardless of visible symptom development (Fig. 3B). The *pvrI*² plants infected with either HAT-GFP or HAT^{Mex21-VPg}-GFP did not display GFP signal in any of the sampled leaves (data not shown). It appears that the strength of the GFP signal observed in *PvrI*⁺ leaves inoculated with HAT^{Mex21-VPg}-GFP was not as strong as the signal from plants inoculated with HAT-GFP; however, the ELISA result showed similar levels of virus accumulation for HAT^{Mex21-VPg}-GFP and HAT in *PvrI*⁺ at 30 dpi. A possible explanation for this observation could be uneven virus distribution in the systemically infected leaf tissue at the earlier time point of the infection. Even though the GFP signal was reduced in the sampled tissue infected with HAT^{Mex21-VPg}-GFP, these results clearly establish that VPg is the determinant of infection in this Mex21-*pvrI* pathosystem.

VPg is the virulence determinant in the *pvrI*²-TEV-N pathosystem.

For the TEV-N virulence determinant test, pepper plants were inoculated with HAT-GFP, N, and HAT^{N-VPg}-GFP. *C. annuum* ‘Dempsey’, which is homozygous for the *pvrI*² resistance allele, was selected to compare the infectivity spectrum of HAT-GFP and HAT^{N-VPg}-GFP. ‘RNaky’ was used as a susceptible control homozygous for *PvrI*⁺ because it is from the same species, *C. annuum*. Inoculated plants were monitored for visible symptom development at 10 dpi (Table 2). HAT-GFP induced systemic mosaic symptoms and mild deformation in noninoculated upper leaves of the susceptible genotype *PvrI*⁺. *PvrI*⁺ plants inoculated with TEV-N developed severe systemic mosaic symptoms and leaf distortion. Less severe symptoms were observed for *PvrI*⁺ plants infected with HAT^{N-VPg}-GFP than with TEV-N at the same time point. N infection of *pvrI*² plants resulted in systemic mosaic symptoms with chlorosis and leaf deformation, whereas HAT^{N-VPg}-GFP induced a mild systemic mosaic symptom. Despite reduced symptom severity induced by HAT^{N-VPg}-GFP in *pvrI*² compared with symptoms induced by N, symptoms were clearly visible relative to uninoculated control *pvrI*² plants and HAT-GFP-inoculated *pvrI*² plants.

Inoculated plants of each genotype were visually assessed for symptom development (Supplementary Fig. S2) and were sampled for ELISA to assess virus accumulation at 15 dpi (Fig. 4A). All three viruses (HAT, N, and HAT^{N-VPg}-GFP) were detected in the *PvrI*⁺ genotype; none were detected in the *pvrI* genotype. In *pvrI*² plants, N was detected but HAT-GFP was

Fig. 2. Diagrammatic representation of the *Tobacco etch virus* (TEV)-green fluorescent protein (GFP) chimeric constructs used in this study. TEV-GFP consists of the TEV-highly aphid transmissible (HAT) genome with GFP inserted between P1 and HC-Pro. All chimeras were derived from the TEV-HAT GFP, where GFP is fused to the HC-Pro domain of the virus. Chimeric viruses were entirely TEV-HAT GFP, except for viral genome-linked protein (VPg) (encoded in the NIa 5' end), which was replaced by TEV-Mex21 VPg or TEV-N VPg.

Table 1. Summary of visible symptom development in uninoculated upper leaves at 10 days postinoculation^a

Genotype ^b	HAT-GFP	Mex21	HAT ^{Mex21-VPg} -GFP	Uninoculated
<i>PvrI</i> ⁺	Leaf mosaic and mild leaf deformation	Leaf mosaic and mild leaf distortion	Leaf mosaic	No symptoms
<i>pvrI</i>	No symptoms	Mild leaf mosaic	Mild leaf mosaic	No symptoms
<i>pvrI</i> ²	No symptoms	No symptoms	No symptoms	No symptoms

^a Plants were inoculated with various *Tobacco etch virus* isolates as listed, or were the uninoculated control. HAT = highly aphid transmissible and GFP = green fluorescent protein.

^b Pepper genotypes: plants encoding the *pvrI* alleles are represented by the following pepper cultivars: *PvrI*⁺, ‘Habanero’; *pvrI*, ‘CA4’; and *pvrI*², ‘Dempsey’.

not, as expected. In the case of the chimeric virus, HAT^{N-VPg}-GFP was detected in the *pvr1*² genotype, indicating that the VPg region of N is sufficient to convert the infection spectrum of HAT-GFP, clearly establishing that VPg is the determinant of infection in this pathosystem.

Plants inoculated with GFP-tagged viruses HAT-GFP and HAT^{N-VPg}-GFP were monitored by confocal microscopy for virus accumulation between 7 and 10 dpi in upper noninoculated leaves. GFP signal was detected in all *Pvr1*⁺ plants inoculated with both GFP-tagged viruses. HAT-GFP did not display GFP signal in the *pvr1*² genotype, supporting the ELISA result that HAT-GFP failed to systemically infect the *pvr1*² genotype. In contrast, GFP fluorescence was detected in *pvr1*² leaves when plants were inoculated with HAT^{N-VPg}-GFP (Fig. 4B). GFP fluorescence was observed in all cases in the leaves of *pvr1*² plants inoculated with HAT^{N-VPg}-GFP, indicating that the virus accumulated in all of the sampled leaves. However, unlike all other samples, GFP was not evenly distributed; rather, islands of GFP fluorescence were observed (data not shown). Both HAT-GFP and HAT^{N-VPg}-GFP were not detected in *pvr1* leaves, as expected. GFP fluorescence was not detected

in *pvr1* plants inoculated with HAT-GFP or HAT^{N-VPg}-GFP, as expected (data not shown).

Based on these assays, replacement of the HAT VPg with that of N or Mex21 VPg did not appear to have a qualitative effect on HAT accumulation in the susceptible host, providing evidence that the genomes behaved as expected and produced functional GFP in systemically infected tissue. Reduced symptom severity was observed for the N chimera, HAT^{N-VPg}-GFP, in both *Pvr1*⁺ and *pvr1*² compared with N. For the Mex chimera, HAT^{Mex-VPg}-GFP, symptom severity was approximately the same for *pvr1* and *Pvr1*⁺ compared with Mex21.

Three-dimensional models provide a possible mechanistic explanation for infectivity phenotypes.

To refine our hypotheses regarding the role of specific changes in VPg and eIF4E with respect to the outcome of infection, we used eIF4E and VPg models to further examine and refine our understanding of the potential and nature of possible biologically relevant protein–protein interactions by generating a three-dimensional (3D) model of a fragment of VPg sequence that is expected to interact with eIF4E (the “essential

Fig. 3. Infectivity assay using *Tobacco etch virus* (TEV)-highly aphid transmissible (HAT) green fluorescent protein (GFP), TEV-Mex21, and TEV-HAT^{Mex21-VPg} GFP in different pepper genotypes. **A**, Enzyme-linked immunosorbent assay (ELISA) result of systemic TEV infection at 30 days postinoculation (dpi). Values represent average ELISA absorbance in noninoculated leaf tissue from four to six plant samples. Standard error is indicated at the top of each bar. Samples listed as mock are healthy controls and are represented as the first sample bar for each genotype. **B**, Confocal microscopy images to monitor GFP signal caused by systemic TEV infection. GFP accumulation in noninoculated upper leaves was monitored in *pvr1* and *Pvr1*⁺ genotypes at 10 to 14 dpi. Images represent noninoculated leaf tissue or one of the two GFP-encoding viruses used in this study: TEV-HAT GFP and TEV-HAT^{Mex21-VPg} GFP. Scale bar = 100 μ m. Pepper cultivars *Capsicum chinense* ‘Habanero’ (*Pvr1*⁺/*Pvr1*⁺), *C. chinense* ‘CA4’ (*pvr1*/*pvr1*), and *C. annuum* ‘Dempsey’ (*pvr1*²/*pvr1*²) were tested.

region”). The essential region was defined in this system based on the cap docking region of the mouse eIF4E crystal structure (Protein Data Bank [PDB] code: 1EJ1, monomer A; 190 residues) informed by the variation observed in the three VPg.

Unlike eIF4E, there is no crystal structure available in the PDB for any potyviral VPg or homologous protein. A nuclear magnetic resonance structure of poliovirus VPg has been reported (Schein et al. 2006); however, poliovirus VPg (22 residues) is much smaller than TEV VPg (188 residues) and there is no evidence of structural homology. Functional assays, biochemical and biophysical analyses, and structure prediction programs all suggest that potyviral VPg exists in a natively un-

folded conformation (Grzela et al. 2008; Rantalainen et al. 2008). Therefore, we focused on locations that show variation across alleles in both eIF4E and VPg. Differences among Mex21, N, and HAT VPg sequences are observed at only nine amino acid sequence positions (Fig. 1B). Most VPg variation observed is located near or in the central region of the protein. Furthermore, six of the positions where substitutions occur are concentrated in the region between residues L106 and T118. Although the effect of the substitutions at positions 53, 183, and 186 cannot be ruled out, based on the number and on the type of the substitutions (character of the residues), we have made the assumption that the sequence fragment 106-118 is a

Table 2. Summary of the visible symptoms in uninoculated upper leaves at 10 days postinoculation^a

Genotype ^b	HAT-GFP	N	HAT ^N -VPg-GFP	Uninoculated
<i>PvrI</i> ⁺	Leaf mosaic and mild leaf deformation	Severe leaf mosaic and leaf distortion	Mild leaf mosaic symptoms and leaf distortion	No symptoms
<i>pvrI</i>	No symptoms	No symptoms	No symptoms	No symptoms
<i>pvrI</i> ²	No symptoms	Leaf mosaic with chlorosis and leaf deformation	Mild leaf mosaic	No symptoms

^a Plants were inoculated with various *Tobacco etch virus* isolates as listed, or were the uninoculated control. HAT = highly aphid transmissible and GFP = green fluorescent protein.

^b Pepper genotypes: plants encoding the *pvrI* alleles are represented by the following pepper cultivars: *PvrI*⁺, ‘RNaky’; *pvrI*, ‘CA4’; and *pvrI*², ‘Dempsey’.

Fig. 4. Infectivity assay using *Tobacco etch virus* (TEV)-highly aphid transmissible (HAT) green fluorescent protein (GFP), TEV-N, and TEV-HAT^N-VPg GFP in different pepper genotypes. **A**, Enzyme-linked immunosorbent assay (ELISA) result of systemic TEV infection at 15 days postinoculation (dpi). Values represent average ELISA absorbance in noninoculated leaf tissue from four to six plant samples. Standard error is indicated at the top of each bar. Samples listed as mock are healthy controls and are represented as the first sample bar for each genotype. **B**, Confocal microscopy images to monitor GFP signal caused by systemic TEV infection. Images were obtained from noninoculated upper leaves in *pvrI*² and *PvrI*⁺ genotypes at 10 to 14 dpi. Images represent noninoculated leaf tissue or leaf tissue inoculated with one of the two GFP-encoding viruses: TEV-HAT GFP or TEV-HAT^N-VPg GFP. Scale bar = 100 μm. Pepper cultivars *Capsicum annuum* ‘RNaky’ (*PvrI*⁺/*PvrI*⁺), *C. chinense* ‘CA4’ (*pvrI*/*pvrI*), and *C. annuum* ‘Dempsey’ (*pvrI*²/*pvrI*²) were tested.

Fig. 5. Proposed three-dimensional eIF4E–viral genome-linked protein (VPg) interaction models provide a possible explanation for susceptible phenotypes. eIF4E structures are shown as light-brown ribbons while the interacting fragment of the VPg structures is displayed as a yellow ribbon in the three panels. Relevant interacting amino acid residues are highlighted by using a stick representation (hydrogen atoms are omitted). **A**, *Pvr1*⁺ Tobacco etch virus (TEV)-highly aphid transmissible (HAT) VPg proposed interaction model. HAT VPg residue P113 sits in the pocket formed by *Pvr1*⁺ residue D109 and the conserved *Pvr1*⁺ residues W75 and W121 interacting favorably with the two aromatic tryptophan residues. HAT VPg residue L116 hydrophobic–hydrophobic interaction with *Pvr1*⁺ residue V67 contributes favorably. The net sum of interactions favors *Pvr1*⁺ binding and, thus, susceptibility to HAT. **B**, The *pvr1* TEV-Mex21 VPg proposed interaction model. By displacing the *pvr1* residue R107 side chain, Mex21 VPg residue L113 is capable of forming a large hydrophobic cluster with *pvr1* residues W75, W121, V67, and L79 as well as Mex21 VPg residue L116 that favors the bound state. **C**, The *pvr1*² TEV-N VPg proposed interaction model. The *pvr1*²-binding pocket near conserved *pvr1*² residues W75 and W121 is polar or charged in character; the walls of this pocket are lined by *pvr1*² residues N109, E67, and R79. If N VPg residue H113 sits in that pocket, the electrostatic interactions with *pvr1*² residues E67 and R79 should be favorable.

primary component of the essential region of VPg. Three of six amino acid changes in Mex21 VPg (I107N, P113L, and T186M) and one of five amino acid changes in N VPg (P113H) can be considered substitutions in which the amino acid properties at the specified site (i.e., charge and hydrophobicity) are altered substantially. According to secondary structure prediction methods, the VPg fragment 106–118 is likely to be part of an α -helical or partial α -helical conformation (i.e., a broken α -helix). Using the program ECEPPAK (Ripoll et al. 1999), a regular α -helical conformation was built for the VPg fragment, which will be referred to as VPgHX, involving residues I91 to T118.

We used an interactive 3D graphics program to explore possible modes of interaction of the eIF4E and VPg molecules (Fig. 5). Among many possible conformations for the complex, a plausible binding model follows which is consistent with our infectivity data. It is possible that VPgHX docks inside the cap-binding cleft of eIF4E, with at least two of the three negatively charged residues D108, D109, and E110 in VPg forming salt bridges with K133, K176, and R171 in eIF4E. In addition to this electrostatic interaction, the N-terminal part of VPgHX is anchored near I210 and L218 in eIF4E, whereas the C-terminal portion of VPgHX is directed toward the region of eIF4E where residues W75 and W121 are located. Conserved residue L116 in VPg provides additional favorable interaction when a hydrophobic residue occupies position 67 in eIF4E. The favorable or unfavorable interactions for all eIF4E and VPg combinations are summarized in Table 3.

The susceptible allele *Pvr1*⁺ was predicted to interact strongly with HAT, N, and Mex21 VPg (Fig. 5A; Table 3). Residues in all three VPgHX proteins (especially P113 in HAT VPg, L113 in Mex21 VPg, and H113 in N VPg) can sit in the pocket structure of *Pvr1*⁺ formed by D109 and the conserved tryptophan residues W75 and W121. Additional hydrophobic–hydrophobic interaction between conserved VPg residue L116 and *Pvr1*⁺ residues V67 and L79 contributes favorably to this interaction.

Consistent with this trend, *pvr1* was predicted to interact favorably with Mex21 VPg, which is consistent with our biological observation of *pvr1* susceptibility to Mex21 (Fig. 5B; Table 3). By displacing the side chain of *pvr1* residue R107, Mex21 VPg residue L113 is capable of forming a large hydrophobic cluster with *pvr1* residues W75, W121, V67, and L79 and Mex21 VPg residue L116 that favors the bound state. In contrast, N and HAT VPg are not predicted to have a favorable interaction, corroborating the observed resistance of *pvr1* plants to N and HAT. In the case of *pvr1* interaction with N VPg, the pocket formed by *pvr1* residues D109, W75, and W121 is likely to be occupied by *pvr1* residue R107 that is predicted to interact with *pvr1* residue D109. N VPg residue H113 does not seem to be able to displace *pvr1* residue R107 to interact with *pvr1* residue D109 and the bound state of the complex is disfavored. As in the case of N VPg, HAT VPg also does not interact favorably with *pvr1*. As shown above, the HAT VPg residue P113 does not seem to be able to displace *pvr1* residue R107, and the bound state of the complex is similarly disfavored.

The hypothesis that the predicted physical interaction of the VPg and eIF4E alleles mirrors infectivity is corroborated by the observation that models predict that *pvr1*² interacts favorably with N VPg, consistent with observed *pvr1*² susceptibility to N (Fig. 5C; Table 3). The *pvr1*² binding pocket near conserved *pvr1*² residues W75 and W121 is much more polar in character, in that the walls of this pocket are lined by *pvr1*² residues N109, E67, and R79. If N VPg residue H113 sits in that pocket, the electrostatic interactions with *pvr1*² residues E67 and R79 should be quite favorable. In contrast, models

indicated an unfavorable interaction between HAT VPg and *pvr1*². HAT VPg residue P113, which sits in the polar pocket of *pvr1*² near residues W75 and W121, should not have favorable electrostatic interactions with *pvr1*² residues E67 and R79 and will adversely affect interaction. Models also indicate an unfavorable interaction between Mex21 VPg and *pvr1*². The presence of Mex21 VPg residue L113 in the pocket is also predicted to adversely affect the electrostatic interactions between *pvr1*² residues E67 and R79.

In summary, our results from modeling studies suggest the potential for differential physical interactions as the basis for differences in observed infectivity at the organismal level. The models predicted that VPg and eIF4E would interact favorably whenever TEV infection occurred. Therefore, these models support the hypothesis that VPg–eIF4E interaction allows for virus infection.

Physical interaction between *pvr1*² and TEV-N VPg detected in planta.

To confirm these predictions, assessment of physical interaction between eIF4E and VPg was performed in planta. Previously, we showed that TEV VPg interaction with *pvr1* eIF4E in planta correlated with infectivity; however, interaction results obtained from yeast two-hybrid assays, in some cases, did not correlate with infectivity or in planta interaction (Kang et al. 2005; Yeam et al. 2007). For example, VPg from HAT, which can overcome *pvr1*¹ resistance, does not demonstrate physical interaction with *pvr1*¹ eIF4E in the yeast two-hybrid system; however, an interaction was detected in planta (Yeam et al. 2007). Therefore, we performed *Agrobacterium*-mediated transient expression and a bimolecular fluorescence complementation assay (BiFC) to visualize and confirm in planta interaction using the N and HAT VPg and *Pvr1*⁺ and *pvr1*² eIF4E.

Table 3. Amino acid residues predicted to show favorable and unfavorable interactions for each eIF4E and viral genome-linked protein (VPg) combinations via three-dimensional structural modeling using VPgHX fragments^a

VPg–eIF4E interactions	VPg residues							eIF4E residues				
N– <i>Pvr1</i> ⁺	I107	L111	D112	H113	S115	N117	T51	P66	V67	L79	G107	D109
N– <i>pvr1</i>	I107	L111	D112	H113	S115	N117	A51	T66	V67	L79	R107	D109
N– <i>pvr1</i> ²	I107	L111	D112	H113	S115	N117	T51	P66	E67	R79	G107	N109
HAT– <i>Pvr1</i> ⁺	I107	I111	E112	P113	S115	S117	T51	P66	V67	L79	G107	D109
HAT– <i>pvr1</i>	I107	I111	E112	P113	S115	S117	A51	T66	V67	L79	R107	D109
HAT– <i>pvr1</i> ²	I107	I111	E112	P113	S115	S117	T51	P66	E67	R79	G107	N109
Mex21– <i>Pvr1</i> ⁺	N107	I111	E112	L113	A115	S117	T51	P66	V67	L79	G107	D109
Mex21– <i>pvr1</i>	N107	I111	E112	L113	A115	S117	A51	T66	V67	L79	R107	D109
Mex21– <i>pvr1</i> ²	N107	I111	E112	L113	A115	S117	T51	P66	E67	R79	G107	N109

^a VPg residues are the variable residues within the VPgHX fragment for the particular VPg, and the eIF4E residues are the variable residues within the predicted cap binding region of the particular eIF4E protein. Sequences of N VPg and *Pvr1*⁺ eIF4E were used as references. HAT = highly aphid transmissible. Bold letters indicate residues in the particular variant that differ from the reference sequences N VPg and *Pvr1*⁺ eIF4E. Color coding indicates residues predicted to be favorable for the interaction (light gray) and unfavorable for the interaction (dark gray).

Fig. 6. Confocal microscope images from bimolecular fluorescence complementation assay using *Agrobacterium*-mediated transient expression assay in *Nicotiana benthamiana*. eIF4E proteins fused with YN: YN::EE::Pvr1⁺ eIF4E, YN::EE::pvr1 eIF4E, and YN::EE::pvr1² eIF4E were transiently expressed in *N. benthamiana* leaf tissue with viral genome-linked protein (VPg) fused with YC: YC::VPg (*Tobacco etch virus* [TEV]-highly aphid transmissible). Yellow fluorescent signals generated by the protein–protein interaction were detected in mesophyll cells 60 h postinfiltration. Chloroplast auto-fluorescence is shown in red. White arrows are pointing at the yellow fluorescent protein signal generated. Scale bar = 10 μm.

To perform these assays, the N-terminal portion of yellow fluorescent protein (YFP) was fused with the eIF4E construct (YN::eIF4E), and the C-terminal half of YFP was fused with the N VPg (YC::N VPg). A yellow fluorescent signal was only detected when the two tagged proteins were in close enough proximity to each other to allow for the formation of intact YFP (Bracha-Drori et al. 2004). Co-expression of YN::Pvr1⁺ eIF4E and YC::N VPg showed a strong yellow fluorescence signal (which agrees with the susceptible N-Pvr1⁺ interaction), whereas the co-expression of YN::pvr1 eIF4E and YC::N VPg together did not show detectable yellow fluorescence (which agrees with the resistant N-pvr1 interaction) (Fig. 6). Although not as strong as the Pvr1⁺ eIF4E-N VPg combination (based on intensity of fluorescence), YC::N VPg co-expressed with YN::pvr1² eIF4E showed a slight yellow fluorescence signal, consistent with ELISA data that show less accumulation for N in pvr1² than in Pvr1⁺, to the extent that these different methods can be compared to infer amount of viral accumulation. These assays confirm the predictions from the N modeling, which predicted physical interaction when infectivity occurs and is absent in the resistant interaction.

DISCUSSION

The molecular determinants of infectivity and disease in plants have been the objects of intense research for decades. With the accumulation of evidence from a wide range of plant-virus pathosystems, the highly conserved host proteins eIF4E or eIF(iso)4E have been implicated through physical interaction with the viral-encoded VPg as highly correlated with virus infectivity. Over the past nearly 20 years, genetic variation in the host and now in viral molecules allow us to define precisely, at the nucleotide level, the interactions that determine the outcome of infection in the *Capsicum pvr1*-TEV pathosystem (Borgstrom and Johansen 2001; Bruun-Rasmussen et al. 2007; Kang et al. 2005; Kuhne et al. 2003; Leonard et al. 2000; Moury et al. 2004). In this study, using chimeric clones, we have demonstrated that TEV VPg is the virulence determinant in the *pvr1*-TEV pathosystem in pepper.

To support this conclusion, we compared disease outcomes from an array of viral and host genotypes assessed visually via immunoassays and fluorescence where technically possible. In every case, visible symptom development, confirmed by the presence of coat protein antigen and GFP accumulation, clearly indicated that the N or Mex21 VPg substitution in the HAT-GFP genome was sufficient to establish infectivity in pvr1² or pvr1 pepper plants, respectively (Figs. 3 and 4; Table 1).

In addition to infectivity analysis, we examined the predicted and in planta interactions between eIF4E and VPg to determine whether there was a correlation between protein interaction and virus infection. Based on the experimentally observed outcomes of infectivity and the other biochemical data, we predicted possible modes of interaction of the eIF4E and VPg molecules using the interactive 3D graphics program ECEPPAK (Ripoll 1999). The region of the VPg containing the most variation, residues I91 to T118, was selected as the focus for our 3D modeling effort. The 3D interaction model suggests that the different eIF4E-VPg interaction outcomes were due to conformational changes caused by amino acid variation at the critical positions summarized in Table 3. Some of these predictions were confirmed experimentally. A signal was observed in planta for pvr1² and N VPg, implying that there is an interaction between the two proteins, supporting both pvr1² infectivity data and the model prediction. The low signal in the assay compared with the signal observed for Pvr1⁺ and N VPg could potentially be correlated to the ob-

served reduction in virus accumulation in pvr1² plants compared with Pvr1⁺ plants infected with TEV-N (Fig. 4A). A yeast two-hybrid assay was also performed using VPg mutants generated by site-directed mutagenesis to determine the individual effect of the seven amino acid changes present in Mex21 or N VPg. None of the amino acid changes was sufficient to establish interaction with pvr1 or pvr1² in this assay (Supplementary Fig. S3). This result suggests that either this experiment was not sensitive enough to detect interaction or VPg mutants containing various combinations of these single mutations should be tested in future experiments.

In addition to the role that the eIF4E-VPg interaction could play in the viral infection cycle, this interaction may alter or interfere with the normal host functions of eIF4E, possibly with adverse effects on the host. In addition to cytoplasmic host translation, eIF4E has been detected in the nucleus, where it was believed to function in nascent nuclear translation and mRNA export to the cytoplasm (Iborra et al. 2001; Lejbkiewicz et al. 1992). Interaction with VPg could inhibit these crucial cellular functions and potentially make the plant more vulnerable to viral infection or account for symptoms. For example, in pea plants infected with *Pea seedborne mosaic virus*, host mRNA and protein levels were suppressed in infected leaves, possibly due to the eIF4E-VPg interaction occurring as a result of viral infection (Wang and Maule 1995).

Determining the specific amino acids involved in a virus-host interaction could aid in the identification or development of nontransgenic or transgenic resistance alleles. Potyvirus-resistant plants containing mutations in eIF4E have been identified or developed in many pathosystems by manipulating a specific host eIF4E using mutagenesis. A recent Targeted Local Lesions in Genomes experiment in tomato identified an eIF4E1 mutant, *S.l_eIF4E1 G1485A*, which conferred resistance to PVY and *Pepper mottle virus* (PepMoV) (Piron et al. 2010). A naturally occurring resistance gene at the same locus in tomato, *pot-1*, differs from the susceptible eIF4E1 allele by just four amino acid substitutions (Ruffel et al. 2005). Although eIF4E redundancy exists in plants, these two studies confirm that alterations in a single eIF4E homolog, eIF4E1, can result in potyvirus resistance. Like pvr1, *S.l_eIF4E1 G1485A* is also impaired in in vitro cap binding ability (Piron et al. 2010). This finding increases the likelihood of impaired or nonfunctional interaction with VPg, just as we have previously shown for pvr1, which displayed both impaired ability to bind the cap analog and VPg (Kang et al. 2005; Yeam et al. 2007). Previously, we showed that overexpression of the pvr1 resistance allele in tomato conferred multiple-strain TEV resistance as well as PepMoV resistance, and overexpression of the pvr1² allele in potato conferred multiple-strain PVY resistance (Cavatorta et al. 2011; Kang et al. 2007).

In this study, we have confirmed and extended these results, showing conclusively that VPg is the virulence determinant in this system and that a VPg-eIF4E interaction can be a reliable predictor of infectivity. In a previous study, we expressed the pvr1 allele transgenically, resulting in very high levels of resistance. We have also shown that novel engineered eIF4E variants predicted to interrupt physical interaction with VPg can be expressed using an identical strategy to eliminate interaction with VPg (Cavatorta et al. 2011). In this article, we broaden and extend the use of these transgenic resistance strategies with the identification of key amino acids that interrupt eIF4E interaction with the target viral VPg. The design and synthesis of new eIF4E or eIF(iso)4E transgenic resistance alleles could be used to confer resistance to any resistance-breaking viral strain in a pathosystem where a physical interaction between these molecules is sufficient to determine the outcome of infection. Given that many resis-

tance-breaking stains encode single mutations in the central region of VPg, identification and development of eIF4E/eIF(iso)4E transgenes could now, in theory, be adapted quickly to any potyviral pathosystem, using the tools demonstrated in this study to predict the outcomes of interaction between viral VPg and eIF4E alleles. As strategies that feature engineered eIF4E are developed (Cavatorta et al. 2011), the assays described in this article could be used as a powerful predictive tool for engineering new sources of resistance to any virus encoding a VPg.

MATERIALS AND METHODS

Plants, viruses, and populations for genetic studies.

C. annuum 'RNaky' was obtained from Asgrow Seed Co. (San Juan Bautista, CA, U.S.A.). *C. annuum* 'Dempsey' was provided by C. M. Deom, University of Georgia, Athens. *C. chinense* 'CA4' was obtained from the United States Department of Agriculture Southern Regional Plant Introduction Station (Griffin, GA, U.S.A.). *C. chinense* 'Habanero' was obtained from Tomato Growers Supply Co. (Fort Myers, FL, U.S.A.).

Plant virus strains used in this study included TEV-HAT, TEV-N, and TEV-Mex21. TEV-HAT and TEV-N were obtained from T. Pirone, University of Kentucky, Lexington. TEV-Mex21 was obtained from M. Deom, University of Georgia. Each virus was maintained by mechanical passage in *Nicotiana tabacum* 'Kentucky 14'. TEV-HAT GFP was obtained from J. Carrington, Oregon State University, Corvallis.

VPg cloning and sequence alignment.

Total RNA was extracted from flash-frozen 'Kentucky 14' noninoculated leaf tissue systemically infected with the respective virus using a Qiagen RNeasy plant mini kit (Qiagen, Valencia, CA, U.S.A.), following the manufacturer's instructions. cDNA was synthesized as described previously (Kang et al. 2005) using primers TEV-Mex21 VPg reverse primer 5'-CCCTTCAAACATCACTCCTC-3' for TEV-Mex21 RNA and TEV-N VPg reverse primer 5'-CCCTTCAAACGTCACCTC-3' for TEV-N RNA. The VPg coding region was cloned using a pGEM-T Easy kit (Promega Corp., Madison, WI, U.S.A.). Clones including TEV-Mex21 VPg and TEV-N VPg as well as TEV-HAT GFP and both viral chimera, TEV-HAT^{N-VPg} and TEV-HAT^{Mex21-VPg}-GFP, were aligned using Seqman software (DNASTAR Inc., Madison, WI, U.S.A.) as described previously (Kang et al. 2005). Primers used for chimera sequencing are listed in Supplementary Table S1.

Megaprimer polymerase chain reaction to swap VPg domains.

The chimeras were synthesized using the megaprimer method and transcribed in vitro as described previously (Kang et al. 2005; Perez et al. 2006; Schaad et al. 1997). In the initial reactions, a 564-bp VPg DNA fragment was amplified. TEV-Mex21 forward primer 5'-CAAGGGAAGAAGAATCAGAAG-3' and reverse primer 5'-CCCTTCAAACATCACTCCTC-3' were used to amplify TEV-Mex21 VPg. TEV-N VPg was amplified using TEV-N forward primer 5'-CAGGGGAAGAAGAATCAGAAG-3' and reverse primer 5'-CCCTTCAAACGTCACCTC-3'. For the second step of megaprimer amplification, the manufacturer's protocol was followed for use of the Quikchange XL kit (Stratagene, La Jolla, CA, U.S.A.), with the modification of using 100 ng of VPg megaprimers, 10 ng of pTEV-HAT GFP plasmid, 2.5 U of *Pfu* DNA polymerase, and 50 µM dNTPs as described previously (Perez et al. 2006). Following this polymerase chain reaction (PCR), a digestion with 10 U of *DpnI* was per-

formed at 37°C for 1 h and transformed into *Escherichia coli*. Clones were verified for VPg replacement by PCR, restriction enzyme digestion, and DNA sequence analysis.

In vitro transcription and biolistic inoculation.

TEV-HAT GFP, TEV-HAT^{N-VPg}, and TEV-HAT^{Mex21-VPg} RNA transcripts were prepared as described previously (Kang et al. 2005; Schaad et al. 1997) and used to inoculate 'Kentucky 14' plants at the four- to six-leaf stage using a microprojectile bombardment gun (Bio-Rad, Hercules, CA, U.S.A.). The infected tobacco plants were subsequently used as sources of inoculum throughout the study. For each virus, 15 to 20 plants were inoculated by gene gun. Leaf tissue from symptomatic plants at 7 to 10 dpi served as inoculum for additional 'Kentucky 14' plants at the four- to six-leaf stage. These plants were used as inoculum for pepper plants.

Virus inoculation and resistance test.

Pepper seed were allowed to germinate at 30°C in petri dishes containing a water-saturated sterilized germination article. Germinated seed were sown in plastic trays containing the soil-less potting medium Cornell Mix (Boodley and Sheldrake 1977). Plants were inoculated when at approximately the six-leaf stage. Virus inoculum was rub inoculated onto Carborundum-dusted leaves 3 and 4. Inoculum was prepared by grinding systemically infected 'Kentucky 14' tissue in 50 mM potassium phosphate buffer, pH 7.5 (approximately 1 g of tissue to 20 ml of buffer). Mortars and pestles were chilled at -20°C prior to use and then maintained on ice throughout the inoculation process. Genotypes *pvr1*, *pvr1*², and *Pvr1*⁺ were always included. For the TEV-Mex21, TEV-HAT-GFP, and TEV-HAT^{Mex21-VPg} GFP test, *Pvr1*⁺ genotype *C. chinense* 'Habanero' was included to allow comparison with *C. chinense* 'CA4'. For the TEV-HAT GFP, TEV-N, TEV-HAT^{N-VPg}-GFP, and TEV-HAT^{Mex21-VPg} test, the *Pvr1*⁺ genotype *C. annuum* 'RNaky' was included to allow comparison with *C. annuum* 'Dempsey'. Mock-inoculated controls were always included. Plants were arranged such that each tray contained a single genotype infected with a single virus; trays were arranged to avoid plant-to-plant contact between trays and, therefore, cross contamination among viruses. Inoculated plants were monitored for visible symptom development from 7 dpi to approximately 35 dpi. Leaf tissue was tested for the presence of virus using antigen plate-coating indirect ELISA, as described previously (Kang et al. 2005). TEV polyclonal anti-viral immunoglobulins were obtained from Agdia, Inc. (Elkhart, IN, U.S.A.). At least three noninoculated upper leaves were collected from each plant and combined to represent a single sample for ELISA tests. Plants were tested at 15 and 30 dpi. Tissue was sampled in identically sized leaf punches per plant, frozen in liquid nitrogen, and subsequently ground. ELISA absorbance values were determined using a Tecan Sunrise microtiter plate reader at 405 nm (Tecan Sunrise, Sunnyville, CA, U.S.A.). Samples were considered positive if their absorbance value was above the mean of the uninoculated controls plus three standard deviations. Samples are divided in Figures 3 and 4 based on ELISA experiment.

GFP imaging.

GFP fluorescence resulting from virus infection was monitored using a Leica TCS SP2 scanning confocal microscope (Leica, Wetzlar, Germany). At 7 to 10 dpi, four to five noninoculated upper leaves were sampled from inoculated plants for viewing. At least three plants per genotype were sampled per virus and monitored for GFP accumulation. Leaves showing symptoms of infection were preferentially selected in all cases when available.

BiFC assay using *Agrobacterium*-mediated transient protein expression approach in *N. benthamiana*.

The incubation, induction, and co-infiltration of *Agrobacterium* cultures were performed as described previously with minor modification (Bendahmane et al. 2000). The cultures were infiltrated into the *N. benthamiana* leaves at an optical density at 600 nm of 0.25. Protein-protein interaction in planta was monitored 40, 60 and 80 h after *Agrobacterium* co-infiltration using a BiFC assay (Bracha-Drori et al. 2004). The YFP signal, 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 (Leica).

Modeling of the VPg and eIF4E proteins.

PSIPRED and SAM-T02 were used to develop secondary structure predictions of VPg, and the program ECEPPAK was used to build the VPgHX fragment (Ripoll 1999). The program MODELLER (Sali 1995; Sali and Blundell 1993; Sanchez and Sali 2000) was used to generate the 3D models for the sequences of the *pvr1* alleles. Final adjustment of the alignment between the *Capsicum*-eIF4E and the yeast sequence was performed manually with the help of graphic tools included in the commercial programs ICM (MOLSOFT, San Diego, CA, U.S.A.) and DS-Modeling (Accelrys, San Diego, CA, U.S.A.). The pairwise alignments of the sequences of *Capsicum* variants and the template 1AP8 were generated by using the program BLAST (Altschul 1997).

ACKNOWLEDGMENTS

K. Perez was supported by the SUNY Minority Fellows Scholarship and the National Science Foundation (NSF) Plant Genome Research Program Award DBI-0218166. I. Yeam was partially supported by the Kwangjeong Educational Foundation. This work was supported by grants from the NSF Plant Genome Research Program DBI-0218166, the United States Department of Agriculture Initiative for Future Agriculture, Food Systems Award 2001-52100-113347 and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF 2011-0014507). We thank M. Kreitinger, G. Moriarty, N. Moskal, M. Weinreich, G. Inzina, E. Gomez, B. Beeman, M. Fink, J. Jantz, N. Vail, and S. Anthony for their critical support and technical assistance; and V. Gracen, M. Mazourek, and S. Gray for their critical reviews of this manuscript.

LITERATURE CITED

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* 25:3389-3402.

Beauchemin, C., Boutet, N., and Laliberte, J.-F. 2007. Visualization of the interaction between the precursors of VPg, the viral protein linked to the genome of *Turnip mosaic virus*, and the translation eukaryotic initiation factor iso 4E in planta. *J. Virol.* 81:775-782.

Bendahmane, A., Querici, M., Kanyuka, K., and Baulcombe, D. C. 2000. *Agrobacterium* transient expression system as a tool for the isolation of disease resistance genes: Application to the *Rx2* locus in potato. *Plant J.* 21:73-81.

Boodley, J. W., and Sheldrake, R. 1977. Cornell peat-lite mixes for commercial plant growing. Cornell Univ. Coop. Ext. Div. Info. Bull. 43.

Borgstrom, B., and Johansen, I. E. 2001. Mutations in *Pea seedborne mosaic virus* genome-linked protein VPg alter pathotype-specific virulence in *Pisum sativum*. *Mol. Plant-Microbe Interact.* 14:707-714.

Bracha-Drori, K., Shichrur, K., Katz, A., Oliva, M., Angelovici, R., Yalovsky, S., and Ohad, N. 2004. Detection of protein-protein interactions in plants using bimolecular fluorescence complementation. *Plant J.* 40:419-427.

Browning, K. S. 1996. The plant translational apparatus. *Plant Mol. Biol.* 32:107-144.

Browning, K. S. 2004. Plant translation initiation factors: It is not easy to be green. *Biochem. Soc. Trans.* 32:589-591.

Bruun-Rasmussen, M., Moller, I. S., Tulinus, G., Hansen, J. K. R., Lund, O. S., and Johansen, I. E. 2007. The same allele of translation initiation

factor 4E mediates resistance against two potyvirus spp. in *Pisum sativum*. *Mol. Plant-Microbe Interact.* 20:1075-1082.

Carrington, J. C., and Dougherty, W. G. 1987. Small nuclear inclusion protein encoded by a plant potyvirus genome is a protease. *J. Virol.* 61:2540-2548.

Cavatorta, J. R., Perez, K. W., Gray, S. M., Van Eck, J., Yeam, I., and Jahn, M. 2011. Engineering virus resistance using a modified potato gene. *Plant Biotechnol. J.* 9:1014-1021.

Deom, C. M., Murphy, J. F., and Paguio, O. R. 1997. Resistance to *Tobacco etch virus* in *Capsicum annuum*: Inhibition of virus RNA accumulation. *Mol. Plant-Microbe Interact.* 10:917-921.

Dolja, V. V., McBride, H. J., and Carrington, J. C. 1992. Tagging of plant potyvirus replication and movement by insertion of beta-glucuronidase into the viral polyprotein. *Proc. Natl. Acad. Sci. U.S.A.* 89:10208-10212.

Dougherty, W. G., and Carrington, J. C. 1988. Expression and function of potyviral gene products. *Annu. Rev. Phytopathol.* 26:123-143.

Dougherty, W. G., and Parks, T. 1991. Post-translational processing of the tobacco etch virus 49-kDa small nuclear inclusion polypeptide: Identification of an internal cleavage site and delimitation of VPg and proteinase domains. *Virology* 183:449-456.

Dougherty, W. G., and Semler, B. L. 1993. Expression of virus-encoded proteinases: Functional and structural similarities with cellular enzymes. *Microbiol. Mol. Biol. Rev.* 57:781-822.

Eskelin, K., Hafren, A., Rantalainen, K. I., and Makinen, K. 2011. Potyviral VPg enhances viral RNA translation and inhibits reporter mRNA translation in planta. *J. Virol.* 85:9210-9221.

Gallois, J.-L., Charron, C., Sanchez, F., Pagny, G., Houvenaghel, M.-C., Moretti, A., Ponz, F., Revers, F., Caranta, C., and German-Retana, S. 2010. Single amino acid changes in the turnip mosaic virus viral genome-linked protein (VPg) confer virulence towards *Arabidopsis thaliana* mutants knocked out for eukaryotic initiation factors *eIF(iso)4E* and *eIF(iso)4G*. *J. Gen. Virol.* 91:288-293.

Gao, Z., Johansen, E., Eyers, S., Thomas, C. L., Ellis, T. H. N., and Maule, A. J. 2004. The potyvirus recessive resistance gene, *sbm1*, identifies a novel role for translation initiation factor eIF4E in cell-to-cell trafficking. *Plant J.* 40:376-385.

German-Retana, S., Walter, J., Doublet, B., Roudet-Tavert, G., Nicaise, V., Lecampion, C., Houvenaghel, M.-C., Robaglia, C., Michon, T., and Le Gall, O. 2008. Mutational analysis of plant cap-binding protein eIF4E reveals key amino acids involved in biochemical functions and potyvirus infection. *J. Virol.* 82:7601-7612.

Gingras, A. C., Raught, B., and Sonenberg, N. 1999. eIF4 initiation factors: Effectors of mRNA recruitment to ribosomes and regulators of translation. *Annu. Rev. Biochem.* 68:913-963.

Graner, A., and Bauer, E. 1993. RFLP mapping of the *ym4* virus resistance gene in barley. *Theor. Appl. Genet.* 86:689-693.

Grzela, R., Szolajska, E., Ebel, C., Madern, D., Favier, A., Wojtal, I., Zagorski, W., and Chroboczek, J. 2008. Virulence factor of *Potato virus Y*, genome-attached terminal protein VPg, is a highly disordered protein. *J. Biol. Chem.* 283:213-221.

Huang, T.-S., Wei, T., Laliberte, J.-F., and Wang, A. 2010. A host RNA helicase-like protein, AtRH8, interacts with the potyviral genome-linked protein, VPg, associates with the virus accumulation complex, and is essential for infection. *Plant Physiol.* 152:255-266.

Hwang, J., Li, J., Liu, W. Y., An, S. J., Cho, H., Her, N., Yeam, I., Kim, D., and Kang, B. C. 2009. Double mutations in *eIF4E* and *eIFiso4E* confer recessive resistance to *Chilli vein mottle virus* in pepper. *Mol. Cells* 27:329-336.

Ibiza, V., Canizares, J., and Nuez, F. 2010. EcoTILLING in *Capsicum* species: Searching for new virus resistances. *BMC Genomics* 11:631.

Iborra, F. J., Jackson, D. A., and Cook, P. R. 2001. Coupled transcription and translation within nuclei of mammalian cells. *Science* 293:1139-1142.

Jenner, C. E., Nellist, C. F., Barker, G. C., and Walsh, J. A. 2010. *Turnip mosaic virus* (TuMV) is able to use alleles of both *eIF4E* and *eIF(iso)4E* from multiple loci of the diploid *Brassica rapa*. *Mol. Plant-Microbe Interact.* 23:1498-1505.

Kang, B.-C., Yeam, I., Frantz, J. D., Murphy, J. F., and Jahn, M. 2005. The *pvr1* locus in *Capsicum* encodes a translation initiation factor eIF4E that interacts with *Tobacco etch virus* VPg. *Plant J.* 42:392-405.

Kang, B.-C., Yeam, I., Li, H., Perez, K. W., and Jahn, M. 2007. Ectopic expression of a recessive resistance gene generates dominant potyvirus resistance in plants. *Plant Biotechnol. J.* 5:526-536.

Kanyuka, K., Druka, A., Caldwell, D. G., Tymon, A., McCallum, N., Waugh, R., and Adams, M. J. 2005. Evidence that the recessive bymovirus resistance locus *rym4* in barley corresponds to the eukaryotic translation initiation factor 4E gene. *Mol. Plant Pathol.* 6:449-458.

Kawaguchi, R., and Bailey-Serres, J. 2002. Regulation of translational initiation in plants. *Curr. Opin. Plant Biol.* 5:460-465.

Koonin, E. V., Dolja, V. V., and Morris, T. J. 1993. Evolution and taxonomy of positive-strand RNA viruses: Implications of comparative anal-

- ysis of amino acid sequences. *Crit. Rev. Biochem. Mol. Biol.* 28:375-430.
- Kuhne, T., Shi, N., Proeseler, G., Adams, M. J., and Kanyuka, K. 2003. The ability of a bymovirus to overcome the *rym4*-mediated resistance in barley correlates with a codon change in the VPg coding region on RNA1. *J. Gen. Virol.* 84:2853-2859.
- Lejbkiewicz, F., Goyer, C., Darveau, A., Neron, S., Lemieux, R., and Sonenberg, N. 1992. A fraction of the mRNA 5' cap-binding protein, eukaryotic initiation factor 4E, localizes to the nucleus. *Proc. Natl. Acad. Sci. U.S.A.* 89:9612-9616.
- Lellis, A. D., Kasschau, K. D., Whitham, S. A., and Carrington, J. C. 2002. Loss-of-susceptibility mutants of *Arabidopsis thaliana* reveal an essential role for *eIF(iso)4E* during potyvirus infection. *Curr. Biol.* 12:1046-1051.
- Leonard, S., Plante, D., Wittmann, S., Daigneault, N., Fortin, M. G., and Laliberté, J. F. 2000. Complex formation between potyvirus VPg and translation eukaryotic initiation factor 4E correlates with virus infectivity. *J. Virol.* 74:7730-7737.
- Ling, K. S., Harris, K., Meyer, J., Levi, A., Guner, N., Wehner, T., Bendahmane, A., and Havey, M. 2009. Non-synonymous single nucleotide polymorphisms in the watermelon *eIF4E* gene are closely associated with resistance to *Zucchini yellow mosaic virus*. *Theor. Appl. Genet.* 120:191-200.
- Moury, B., Morel, C., Johansen, E., Guilbaud, L., Souche, S., Ayme, V., Caranta, C., Palloix, A., and Jacquemond, M. 2004. Mutations in *Potato virus Y* genome-linked protein determine virulence toward recessive resistances in *Capsicum annuum* and *Lycopersicon hirsutum*. *Mol. Plant-Microbe Interact.* 17:322-329.
- Murphy, J. F., Rhoads, R. E., Hunt, A. G., and Shaw, J. G. 1990. The VPg of *Tobacco etch virus* RNA is the 49-kDa proteinase or the N-terminal 24-kDa part of the proteinase. *Virology* 178:285-288.
- Murphy, J. F., Rychlik, W., Rhoads, R. E., Hunt, A. G., and Shaw, J. G. 1991. A tyrosine residue in the small nuclear inclusion protein of *Tobacco vein mottling virus* links the VPg to the viral RNA. *J. Virol.* 65:511-513.
- Murphy, J. F., Klein, P. G., Hunt, A. G., and Shaw, J. G. 1996. Replacement of the tyrosine residue that links a potyviral VPg to the viral RNA is lethal. *Virology* 220:535-538.
- Murphy, J. F., Blauth, J. R., Livingstone, K. D., Lackney, V. K., and Jahn, M. K. 1998. Genetic mapping of the *pvr1* locus in *Capsicum spp.* and evidence that distinct potyvirus resistance loci control responses that differ at the whole plant and cellular levels. *Mol. Plant-Microbe Interact.* 11:943-951.
- Nicaise, V., German-Retana, S., Sanjuan, R., Dubrana, M., Mazier, M., Maisonneuve, B., Candresse, T., Caranta, C., and LeGall, O. 2003. The eukaryotic translation initiation factor 4E controls lettuce susceptibility to the potyvirus *Lettuce mosaic virus*. *Plant Physiol.* 132:1272-1282.
- Nicaise, V., Gallois, J.-L., Chafiai, F., Allen, L. M., Schurdi-Levraud, V., Browning, K. S., Candresse, T., Caranta, C., Le Gall, O., and German-Retana, S. 2007. Coordinated and selective recruitment of eIF4E and eIF4G factors for potyvirus infection in *Arabidopsis thaliana*. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 581:1041-1046.
- Nieto, C., Morales, M., Orjeda, G., Clepet, C., Monfort, A., Sturbois, B., Puigdomenech, P., Pitrat, M., Caboche, M., Dogimont, C., Garcia-Mas, J., Aranda, M., and Bendahmane, A. 2006. An *eIF4E* allele confers resistance to an uncapped and non-polyadenylated RNA virus in melon. *Plant J.* 48:452-462.
- Pathak, H. B., Oh, H. S., Goodfellow, I. G., Arnold, J. J., and Cameron, C. E. 2008. *Picornavirus* genome replication. *J. Biol. Chem.* 283:30677-30688.
- Perez, K., Yeam, I., Jahn, M. M., and Kang, B.-C. 2006. Megaprimer-mediated domain swapping for construction of chimeric viruses. *J. Virol. Methods* 135:254-262.
- Piron, F., Nicolai, M., Minoia, S., Piednoir, E., Moretti, A., Salgues, A., Zamir, D., Caranta, C., and Bendahmane, A. 2010. An induced mutation in tomato *eIF4E* leads to immunity to two potyviruses. *PLoS ONE* 5:e11313. Published online.
- Providenti, R., and Hampton, R. O. 1992. Sources of resistance to viruses in the Potyviridae. *Arch. Virol.* 5:189-211.
- Rajamaki, M. L., and Valkonen, J. P. 1999. The 6K2 protein and the VPg of *Potato virus A* are determinants of systemic infection in *Nicandra physaloides*. *Mol. Plant-Microbe Interact.* 12:1074-1081.
- Rajamaki, M. L., and Valkonen, J. P. 2002. Viral Genome-Linked Protein (VPg) Controls Accumulation and Phloem-Loading of a Potyvirus in Inoculated Potato Leaves. *Molecular Plant-Microbe Interact.* 15:138-149.
- Rantalainen, K. I., Uversky, V. N., Permi, P., Kalkkinen, N., Dunker, A. K., and Mäkinen, K. 2008. *Potato virus A* genome-linked protein VPg is an intrinsically disordered molten globule-like protein with a hydrophobic core. *Virology* 377:280-288.
- Ripoll, D. R., Liwo, A., and Czaplewski, C. 1999. The ECEPP package for conformational analysis of polypeptides. *TASK Q.* 3:313-331.
- Robaglia, C., and Caranta, C. 2006. Translation initiation factors: A weak link in plant RNA virus infection. *Trends Plant Sci.* 11:40-45.
- Roudet-Tavert, G., Michon, T., Walter, J., Delaunay, T., Redondo, E., and Le Gall, O. 2007. Central domain of a potyvirus VPg is involved in the interaction with the host translation initiation factor eIF4E and the viral protein HcPro. *J. Gen. Virol.* 88:1029-1033.
- Ruffel, S., Dussault, M. H., Palloix, A., Moury, B., Bendahmane, A., Robaglia, C., and Caranta, C. 2002. A natural recessive resistance gene against *Potato virus Y* in pepper corresponds to the eukaryotic initiation factor 4E (*eIF4E*). *Plant J.* 32:1067-1075.
- Ruffel, S., Gallois, J. L., Lesage, M. L., and Caranta, C. 2005. The recessive potyvirus resistance gene *pot-1* is the tomato orthologue of the pepper *pvr2-eIF4E* gene. *Mol. Genet. Genomics* 274:346-353.
- Sali, A. 1995. Comparative protein modeling by satisfaction of spatial restraints. *Mol. Med. Today* 1:270-277.
- Sali, A., and Blundell, T. L. 1993. Comparative protein modelling by satisfaction of spatial restraints. *J. Mol. Biol.* 234:779-815.
- Sanchez, R., and Sali, A. 2000. Comparative protein structure modeling. Introduction and practical examples with modeller. *Methods Mol. Biol.* 143:97-129.
- Schaad, M., Lellis, A. D., and Carrington, J. C. 1997. VPg of *tobacco etch potyvirus* is a host genotype-specific determinant for long-distance movement. *J. Virol.* 71:8624-8631.
- Schaad, M. C., and Carrington, J. C. 1996. Suppression of long-distance movement of *Tobacco etch virus* in a nonsusceptible host. *J. Virol.* 70:2556-2561.
- Schaad, M. C., Haldeman-Cahill, R., Cronin, S., and Carrington, J. C. 1996. Analysis of the VPg-proteinase (NIa) encoded by *tobacco etch potyvirus*: Effects of mutations on subcellular transport, proteolytic processing, and genome amplification. *J. Virol.* 70:7039-7048.
- Schein, C. H., Oezguen, N., Volk, D. E., Garimella, R., Paul, A., and Braun, W. 2006. NMR structure of the viral peptide linked to the genome (VPg) of poliovirus. *Peptides* 27:1676-1684.
- Stein, N., Perovic, D., Kumlehn, J., Pelli, B., Stracke, S., Streng, S., Ordon, F., and Graner, A. 2005. The eukaryotic translation initiation factor 4E confers multiallelic recessive *Bymovirus* resistance in *Hordeum vulgare* (L.). *Plant J.* 42:912-922.
- Truniger V., and Aranda, M. A. 2009. Recessive resistance to plant viruses. *Adv. Virus Res.* 75:119-159.
- Wang, D., and Maule, A. J. 1995. Inhibition of host gene expression associated with plant virus replication. *Science* 267:229-231.
- Ward, C. W., and Shukla, D. D. 1991. Taxonomy of potyviruses: Current problems and some solutions. *Intervirology* 32:269-296.
- Wittmann, S., Chatel, H., Fortin, M. G., and Laliberté, J. F. 1997. Interaction of the viral protein genome linked of turnip mosaic potyvirus with the translational eukaryotic initiation factor (iso) 4E of *Arabidopsis thaliana* using the yeast two-hybrid system. *Virology* 234:84-92.
- Yambao, M. L., Masuta, C., Nakahara, K., and Uyeda, I. 2003. The central and C-terminal domains of VPg of *Clover yellow vein virus* are important for VPg-HCPro and VPg-VPg interactions. *J. Gen. Virol.* 84:2861-2869.
- Yeam, I., Cavatorta, J. R., Ripoll, D. R., Kang, B.-C., and Jahn, M. M. 2007. Functional dissection of naturally occurring amino acid substitutions in eIF4E that confers recessive potyvirus resistance in plants. *Plant Cell* 19:2913-2928.

Tables 1 and 2 originally appeared out of order. The online pdf was changed on February 1, 2013.