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Molecules and Cells

Double Mutations in eIF4E and eIFiso4E Confer Recessive Resistance to *Chilli Veinal Mottle Virus* in Pepper

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To evaluate the involvement of translation initiation factors eIF4E and eIFiso4E in *Chilli veinal mottle virus* (ChiVMV) infection in pepper, we conducted a genetic analysis using a segregating population derived from a cross between *Capsicum annuum* 'Dempsey' containing an *eIF4E* mutation (*pvr1*^{2}) and *C. annuum* 'Perennial' containing an *eIFiso4E* mutation (*pvr6*). *C. annuum* 'Dempsey' was susceptible and *C. annuum* 'Perennial' was resistant to ChiVMV. All F_1 plants showed resistance, and F_2 individuals segregated in a resistant-susceptible ratio of 166:21, indicating that many resistance loci were involved. Seventy-five F_2 and 329 F_3 plants of 17 families were genotyped with *pvr1*^{2} and *pvr6* allele-specific markers, and the genotype data were compared with observed resistance to viral infection. All plants containing homozygous genotypes of both *pvr1*^{2} and *pvr6* were resistant to ChiVMV, demonstrating that simultaneous mutations in *eIF4E* and *eIFiso4E* confer resistance to ChiVMV in pepper. Genotype analysis of F_2 plants revealed that all plants containing homozygous genotypes of both *pvr1*^{2} and *pvr6* showed resistance to ChiVMV. In protein-protein interaction experiments, ChiVMV viral genome-linked protein (VPg) interacted with both eIF4E and eIFiso4E. Silencing of *eIF4E* and *eIFiso4E* in the VIGS experiment showed reduction in ChiVMV accumulation. These results demonstrated that ChiVMV can use both eIF4E and eIFiso4E for replication, making simultaneous mutations in *eIF4E* and *eIFiso4E* necessary to prevent ChiVMV infection in pepper.

INTRODUCTION

Potyviruses are very destructive to crop production and account for approximately 30% of all known plant viruses. Among the potyviruses, five species commonly infect peppers (*Capsicum* spp.): *Potato virus Y* (PVY), *Pepper mottle virus* (PepMoV), *Tobacco etch virus* (TEV), *Pepper veinal mottle virus* (PVMV), and *Chilli veinal mottle virus* (ChiVMV) (Kyle and Palloix, 1997). ChiVMV is one of the most predominant viruses of peppers in Asia (Green et al., 1999; Siriwong et al., 1995; Wang et al., 2006). Surveys done by the Asian Vegetable Research and Development Center (AVRDC) in 16 Asian countries have shown that 30% of pepper crops are affected by this disease (www.avrdc.org). Recent molecular analysis of ChiVMV isolates revealed high amino acid and nucleotide identity of the coat protein (CP) among the ChiVMV isolates in Asia (Tsai et al., 2008). The group of ChiVMVs also includes the previously identified *Pepper vein banding virus* (PVBV) and *Chilli vein-banding mottle virus* (CVbMV) (Tsai et al., 2008). ChiVMV is distinct from PVMV (Green et al., 1999; Moury et al., 2005), but a conserved region has been identified among the CP of the two viruses (Tsai et al., 2008).

RNA virus infection requires the interaction of host factors with viral proteins and RNA for replication and systemic spread (Ahlquist et al., 2003; Kushner et al., 2003). During the last decade, several host factors for plant viruses have been identified and characterized (Robaglia and Caranta, 2006). Mutations in these host factors often result in recessive resistance to potyvirus in nature (Kang et al., 2005a). However, only translational initiation factors of the eIF4E and eIF4G family have been found to be involved in natural recessive virus resistance (Kang et al., 2005b; Robaglia and Caranta, 2006). Naturally occurring mutations in eIF4E are responsible for resistance to *Lettuce mosaic virus* (LMV) by *mo1* in lettuce, *Pea seed-borne mosaic virus* (PSbMV) by *sbm1* in pea (Gao et al., 2004), *Barley yellow mosaic virus* by *rym4/5* in barley (Stein et al., 2005), and *Melon necrotic spot virus* (MNSV) by *ncv* in melons (Nieto et al., 2007). In pepper, a mutation of eIF4E at the *pvr1* (*pvr2*) locus confers resistance to several TEV strains and PVY (Kang et al., 2005b; Murphy et al., 1998; Yeam et al., 2007). Simultaneous mutations of eIF4E and eIFiso4E (at the *pvr1*^{2} and *pvr 6* alleles) relate to PVMV resistance (Ruffel et al., 2006). Physical interactions between host factors and the viral genome-linked proteins are required for the virus infection and the disruption of the

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Table 1. PCR markers used for *pvr1* and *pvr6* genotyping in this study

Locus	Marker name	Primer sequences (5′ → 3′)	PCR product size (bp)	Restriction enzyme	Fragment size after digestion	Expected genotype	References
pvr1	pvr1-R2 (CAPS)	GGGCTAAAATACGCTCATCTCCCTTC GGCTCAATTTTATGCTTGAAACAATGTAAGC	412	*Hind*III	32+380 412	*pvr1*^{2} non *pvr1*^{2}	Yeam et al. (2005)
pvr6	Pvr6 (SCAR)	GGTGAAACAGCCACATAAGC GCCACCATTAGCGCACTCAGG	420 or 500	-	500 420	*Pvr6*$^+$ *pvr6*	This study

interactions result in failure of virus infection many potyviruses (Charron et al., 2008; Grzela et al., 2006).

Our objective of this study was to test for the involvement of mutations of translation initiation factor eIF4E and/ or eIFiso4E in resistance to ChiVMV in pepper. We also examined interactions between ChiVMV viral genome-linked protein (VPg)/ eIF4E and ChiVMV VPg/eIFiso4E to determine if ChiVMV VPg could use both proteins.

MATERIALS AND METHODS

Plant materials

C. annuum 'Dempsey' (DEMP) was provided by M. Deom, University of Georgia (USA). *C. annuum* 'Perennial' (PER), an Indian hot pepper line, has been widely used in multiple disease resistance studies in pepper. *C. annuum* 'Jeju' (JEJU), a Korean local variety, was provided by NongWoo Bio (Yeoju, Korea). DEMP contains *pvr1*^{2}, which confers resistance to potyvirus TEV-HAT, PVY (0), but remains susceptible to PepMoV-FL (Yeam et al., 2005). PER contains *Pvr1*$^+$ and *pvr6* (Ruffel et al., 2006). JEJU contains *Pvr1*$^+$ and *Pvr6*$^+$. Preliminary ChiVMV screening showed that DEMP and JEJU were susceptible and PER was resistant to ChiVMV (data not shown). An intraspecific cross between DEMP and PER was produced and used to develop a segregating population of ChiVMV resistance. Individual plants of DEMP (n = 5), PER (n = 5), F_1 (n = 3), and the segregating F_2 population (n = 187) were used for initial phenotypic screening of resistance to ChiVMV. Genomic DNA was extracted from these plants for genotypic assay by standard DNA extraction protocols for solanaceous crops (Prince et al., 1997). Cosegregation analysis was also carried out for the segregating F_2 population. To gain further insight into the relationship between ChiVMV resistance and the two genes of interest in the segregating population, cosegregation analysis was also carried out for 329 F_3 individuals generated from 17 F_2 individuals.

Virus isolation and inoculation

ChiVMV was obtained from ChiVMV-infected pepper plants in Indonesia. Virus isolation, maintenance, and inoculation were carried out in the experimental station of Nongwoo Bio in Indonesia by standard procedures. ChiVMV was maintained in JEJU. The presence of ChiVMV was re-confirmed by DAS-ELISA (Agdia, USA). Plants were inoculated at the five to six leaf stage of development. ChiVMV was collected from infected leaves ground in 50 mM potassium phosphate buffer (pH 7.5). Mechanical inoculation was carried out by applying virus inoculum onto the two oldest leaves with light carborundum dusting. Plants were monitored daily and classified as resistant or susceptible to ChiVMV by the absence or presence of visual virus symptoms, respectively.

PCR markers for *pvr1*^{2} and *pvr6* genotyping

An allelic-specific cleaved amplified polymorphic sequence (CAPS) marker, pvr1-R2, was used for genotyping. This previously reported marker was specifically designed to distinguish the resistance allele *pvr1*^{2} from other *pvr1* alleles based on point mutations at the *pvr1* locus encoding eIF4E on chromosome 3 in pepper (Yeam et al., 2005). Primers, restriction enzymes, and experimental procedures followed Yeam et al. 2005 (Table 1). In brief, 25 μl PCR reactions were prepared with 1 μl of genomic DNA template (25-50 ng), 2 μl of dNTPs, 2.5 μl of 10× PCR buffer (Roche, IN), 1 μl each of forward and reverse primer, 0.2 μl of Taq DNA polymerase, and 17.3 μl of ddH_2O. DNA was amplified in a thermal cycler (MyCycler, Bio-Rad) under the following conditions: 94°C 5 m; 30 cycles of 94°C 1 m, 59°C 1 m, 72°C 1 m; and 72°C 5 m. Restriction enzymes were applied according to the manufacturer's instructions (Roche, USA). Gel electrophoresis was carried out on 2.2% agarose gels in 1× TAE buffer.

We designed a sequence characterized amplified region (SCAR) marker to genotype the *pvr6* locus. An 82 nt deletion in eIFiso4E corresponds to the resistance genotype at the *pvr6* locus (Ruffel et al., 2006). Full length cDNA of eIFiso4E corresponding to the *pvr6* locus was recovered from DEMP and PER by RT-PCR as described by Ruffel et al. (2006). Sequences of the cloned cDNA were obtained (NICEM, Seoul National University). Nucleotide alignment showed 100% similarity between DEMP and the published *Pvr6*$^+$ allele of *C. annuum* cultivars Florida VR2, Yolo Y, and Yolo Wonder (GenBank accession no. DQ022082.1, DQ022081.1, and DQ 022080.1, respectively). The sequence of the *pvr6* allele of PER was confirmed (GenBank accession no. DQ022083.1) to have an 82 nt deletion and three point mutations at positions 268, 483, and 537 as compared with the *Pvr6*$^+$ allele. We used Intron Finder for Solanaceae ESTs (SOL Genomic Networks, www.sgn.cornell.edu) to identify the locations of expected introns of *Pvr6*$^+$ at positions 203, 372, 498, and 561. Primers were designed from the exon regions flanking the 82 nt deletion to allow differentiation between the *pvr6* and *Pvr6*$^+$ alleles (Table 1). PCR reaction and conditions for the SCAR marker for the *pvr6* locus were similar to those described above except for an annealing temperature of 55°C for PCR and 1.5% agarose gels for electrophoresis.

Yeast two-hybrid analysis

Yeast two-hybrid analysis was performed as described previously (Kang et al., 2005b). Gene-specific primers were designed to introduce restriction enzyme sites (Table 2). The eIF4E (4E) and eIFiso4E (iso4E) coding sequences were amplified from PER and DEMP, and VPg was amplified from ChiVMV. PCR products were cloned into the pCR®2.1- TOPO® vector (Invitrogen, USA) and all constructs were confirmed by sequencing. Coding sequences were then subcloned in-frame with the GAL4 activation domain or the GAL4 binding domain into the pGADT7 or pGBKT7 vectors, respectively (Clontech, USA).

The Matchmaker GAL4 two-hybrid system 3 (Clontech, USA)

Table 2. Primer sets used for vector construction in this study

Primer	Sequence (5′ → 3′)
eIF4E-EcoRI-F	GAATTCATGGCAACAGCTGAAATGGA
eIF4E-XhoI-R	CTCGAGCTATACGGTGTAACGATTCTTG
eIFiso4E-NdeI-F	CATATGGCCACCGAAGCACCACC
eIFiso4E-XhoI-R	CTCGAGCTCGAGTCACACGGTGTATCGGCTC
TRV2:*eIF4E*-F	ATGGCAACAGCTGAAATGGA
TRV2:*eIF4E*-R	CTATACGGTGTAACGATTCT
TRV2:*eIFiso4E*-F	ATGGCCACCGAAGCACCACC
TRV2:*eIFiso4E*-R	TCACACGGTGTATCGGCTCT
*Bra.*eIFiso4E*-EcoRI-F	GAATTCATGGCGACAGAGGATGTGAAC
*Bra.*eIFiso4E*-BamHI-R	GGATCCTCAGACAGTGAACC
ChiVMV VPg-EcoRI-F	GAATTCGCAATGGAAACAGTCATGCATC
ChiVMV VPg-BamHI-R	GGATCCTTCATGCTCAACTCCTTCTCTTGAT
TEV VPg-NdeI-F	CATATGATGGGGAAGAAGAATCAGA
TEV VPg-EcoRI-R	GAATTCCTATTCAAACGTCAAGTCCT
TuMV VPg-NdeI-F	CATATGATGGCGAAAGGCAAGAGGCA
TuMV VPg-SacI-R	GAGCTCCTATCGTGGTCCACTGGGAC

*Bra.: *Brassica rapa*

was used according to manufacturer's protocols. pGADT7:*4E* (or pGADT7:*iso4E*) and pGBKT7:*VPg* constructs were transformed into AH109 yeast strains. After yeast transformation, colonies were picked and resuspended in 100 μl sterile water, and 10 μl aliquots were spotted onto various selective media lacking leucine and tryptophan (SD-LW); leucine, tryptophan, and histidine (SD-LWH); and leucine, tryptophan, histidine, and adenine (SD-LWHA). Plates were incubated at 30°C and growth was checked 3 to 5 days after inoculation. Empty vectors pGADT7 and pGBKT7 were used as negative controls. Interaction between murine p53 and SV40 large T antigen (controls from the Matchmaker GAL4 two-hybrid system 3) was used as a positive control. In addition, TEV-HAT VPg and *Brassica rapa* eIFiso4E (iso4E^{Bra}) were used as interaction controls. Protein interactions were also quantified by α-galactosidase (α-gal) assays. In the quantitative α-gal assay, the catalytic activity of α-galactosidase was monitored colorimetrically by measuring the rate of hydrolysis of the chromogenic substrate, *p*-nitrophenyl-α-d-galactoside (PNP-α-gal), according to the manufacturer's protocols.

Virus-induced gene silencing (VIGS)

The *Agrobacterium tumefaciens* strain GV2260 transformed with the *Tobacco rattle virus* 1 (TRV1) and with the *Tobacco rattle virus* 2: *phytoene desaturase* (TRV2:*PDS*) plasmids was kindly provided by S.P. Dinesh-Kumar (Yale University, USA). A full length of *Capsicum eIF4E* and *eIFiso4E* open reading frame were amplified by RT-PCR with gene-specific primers (Table 2). The PCR products were recombined into pCR8/GW/TOPO plasmids, and the entry clones containing *Capsicum eIF4E* and *eIFiso4E* were combined with TRV2 using gateway system (Invitrogen, USA). The TRV2:*eIF4E* and TRV2:*eIFiso4E* vectors were subsequently introduced into *Agrobacterium* GV2260. The VIGS experiment was performed as described previously (Liu et al., 2002), with minor modifications. *Agrobacteria* containing TRV1 and TRV2:*PDS* or TRV2:*eIF4E* and TRV2:*eIFiso4E* were incubated overnight at 30°C with the appropriate antibiotics, 10 mM of MES, and 20 μM of acetosyringone at pH 5.7. The *Agrobacterium* cells were harvested and resuspended in VIGS agroinfiltration medium (10 mM $MgCl_2$, 10 mM MES, pH 5.7, 150 μM acetosyringone) to a final OD_{600} of 0.5, and incubated at room temperature with shaking for 4 to 6 h. Cells with the TRV1 and with the TRV2:*PDS* or TRV2:*eIF4E* and TRV2:*eIFiso4E* plasmids were mixed in a 1:1 ratio and infiltrated into the cotyledons of 4 to 6-day-old JEJU seedlings using a 1-mL syringe without a needle. The infected seedlings were incubated at 16°C for 24 h and grown in the growth chamber for 3-4 weeks.

Silenced plants were used for ChiVMV inoculation. Virus inoculation and ELISA experiment were performed as described previously (Kang et al., 2005b). ChiVMV accumulation was tested in inoculated leaves at 6 days after inoculation.

RESULTS

Genetic analysis of ChiVMV resistance

The phenotypic ChiVMV screening of the parental lines DEMP and PER and the F_1 and F_2 segregating populations derived from the intra-specific crosses is shown in Table 3. The parental lines PER and DEMP were 100% resistant and 100% susceptible, respectively. All tested F_1 plants were resistant to ChiVMV, indicating that resistance is a dominant trait conferred by PER. The segregation data of the F_2 populations fit a 13:3 ratio of resistant to susceptible ($\chi^2 = 6.5$; $P < 0.01$) suggesting a two-gene-model of dominant and recessive epistatic interactions for ChiVMV resistance.

Genotyping of F_2 and F_3 plants using eIF4E- and eIFiso4E-specific markers

The pvr1-R2 primers for the *pvr1* locus amplified a 412 bp fragment. Because the CAPS marker was designed to specifically target the single nucleotide polymorphism (SNP) (G- > A) at 325 in the *eIF4E* allele, only the *pvr1*^{2} genotype was cleaved by *Hin*dIII, producing a 380 bp (32 bp) fragment; the fragment remains uncleaved for the non *pvr1*^{2} genotype (Yeam et al., 2005). Heterozygous genotypes show both bands of 412 bp

Table 3. Phenotypic screening and genetic analysis of ChiVMV resistance

Plant material	Resistance	Susceptible	Model	χ^2
Perennial (PER)	5	0	-	
Dempsey (DEMP)	0	5	-	
F_1	3	0	-	
F_2	166	21	13:3	6.5** (P < 0.01)

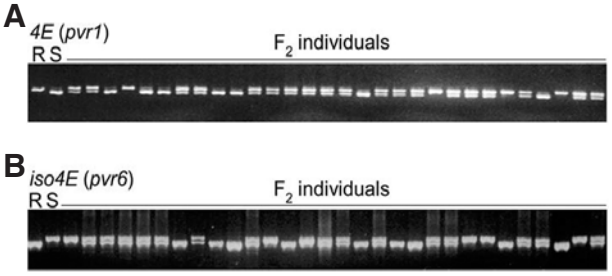

Fig. 1. PCR marker analysis of the F_2 population derived from a cross between PER and DEMP using *pvr1²*- and *pvr6*-specific primers. (A) Analysis of the *pvr1²* specific marker. Application of the dCAPS marker resulted in digestion of 412 bp PCR products from the *pvr1²* allele. (B) Analysis of the *pvr6* specific marker. Due to an 80 bp deletion in the first exon of the *pvr6* allele, PCR products from *pvr6* were 82 bp shorter than *Pvr6⁺*. PCR product size of *pvr6* is 420bp and *Pvr6⁺* is 502 bp. Lanes R and S indicate PER and DEMP, respectively.

and 380 bp (Fig. 1A). DEMP and PER were homozygous for the *pvr1²* and non *pvr1²* alleles, respectively, confirming the expected genotypes of the parental lines. The primers for the SCAR marker for the *pvr6* locus, a pvr6-exon1, amplified a 420 bp fragment in the *pvr6* genotype and a 502 bp fragment in the *Pvr6⁺* genotype (Fig. 1B).

Cosegregation analysis of ChiVMV resistance and eIF4E/eIFiso4E polymorphisms

Our hypothesis was that *eIF4E* and/ or *eIFiso4E* conferred recessive resistance to ChiVMV. Genetic analysis, however, demonstrated the involvement of dominant gene(s). To avoid the influence of dominant resistance, we selected individuals susceptible to ChiVMV from the F_2 segregating population and used them to generate the F_3 segregating population. We closely monitored the 75 F_2 plants and 268 F_3 individual plants from 12 lines to evaluate the involvement of *pvr1* (*eIF4E*) and *pvr6* (*eIFiso4E*) in ChiVMV resistance. These plants, with phenotypic screening data recorded for ChiVMV resistance, were genotyped for *pvr1²*- and *pvr6*-specific markers (Tables 4 and 5).

Two individuals in the F_2 segregating population that contained homozygous genotypes of both *pvr1²* and *pvr6* were resistant to ChiVMV (Table 4). This observation was consistent with our cosegregation analysis of the F_3 populations. A total of 11 individuals of four F_3 families showed the presence of both recessive alleles (*pvr1²pvr1²* and *pvr6pvr6*, Table 5); all of these plants were resistant to ChiVMV. In order to confirm these results, we screened another batch of F_2 population and obtained 6 F_2 plants with *pvr1²pvr1²* and *pvr6pvr6* genotype. These plants were self-pollinated and plants for each F_3 family were tested for virus resistance. All the F_3 individuals were resistant (Fig. 2). These results indicate that simultaneous mutations of

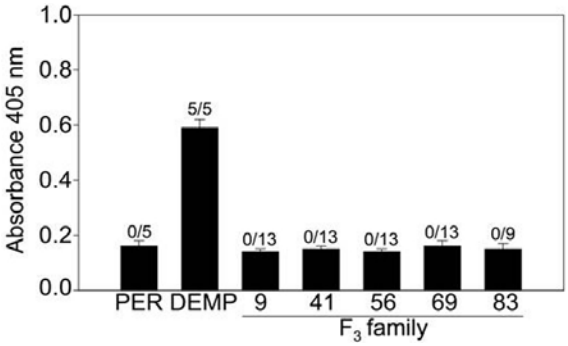

Fig. 2. Infectivity test of F_3 plants containing homozygous genotypes of *pvr1²* and *pvr6*. Accumulation of ChiVMV coat protein in systemic leaves of pepper plants was determined by DAS-ELISA. Virus accumulation was tested at 21 dpi. Numbers above each bar represent susceptible plants over total number of plants tested.

both *eIF4E* and *eIFiso4E* at the *pvr1* and *pvr6* loci confer resistance to ChiVMV.

Physical interaction of ChiVMV VPg with eIF4E and eIFiso4E

According to our genetic analysis results, we expected ChiVMV VPg to interact with both wild type eIF4E and eIFiso4E, but not with the mutated forms. To test the physical interactions between ChiVMV VPg and eIF4E or eIFiso4E, we performed a yeast two-hybrid analysis. For the yeast two-hybrid experiment, the positive controls of murine p53 and SV40 large T antigen showed strong interactions. As previously shown, the eIF4E protein ($4E^{PER}$) from PER, which has the *Pvr1⁺* allele interacted strongly with VPg proteins from TEV-HAT (Fig. 3A). The eIFiso4E protein ($iso4E^{Bra}$) from *B. rapa* that is susceptible to *Turnip mosaic virus* (TuMV) also interacted strongly with TuMV VPg (Fig. 3A). Yeast cells transformed with $4E^{PER}$ and ChiVMV VPg were grown on two different selection media (-LWH and -LWHA), indicating that $4E^{PER}$ and ChiVMV VPg interact strongly. In contrast, the eIF4E protein translated from DEMP ($4E^{DEMP}$) failed to interact with ChiVMV VPg, demonstrating that mutations in $eIF4E^{DEMP}$ prevented interaction with ChiVMV VPg (Fig. 3A). When yeast cells were transformed with eIFiso4E ($iso4E^{DEMP}$) from DEMP and ChiVMV VPg, the transformed cells grew only on the selection medium lacking leucine, tryptophan, and histidine, demonstrating that the interaction of $iso4E^{DEMP}$ and ChiVMV VPg was weaker than that of $iso4E^{Bra}$ and TuMV VPg. However, the eIFiso4E protein ($iso4E^{PER}$) from PER failed to interact with ChiVMV VPg as expected (Fig. 3A). The 82 nt deletion in the eIFiso4E from PER results in a nonsense mutation. A quantitative α-gal assay was performed to confirm the observed yeast two-hybrid results, and showed a trend of protein interactions (Fig. 3B). These observations suggest that susceptibility to ChiVMV is associated with the interactions of ChiMV VPg with eIF4E or eIFiso4E.

Silencing of *eIF4E* and *eIFiso4E* suppresses ChiVMV accumulation

To investigate the functions of *eIF4E* and *eIFiso4E* in ChiVMV replication, VIGS was performed. Full-length pepper *eIF4E* or *eIFiso4E* gene was cloned into TRV2 vector and infiltrated JEJU plants with *Agrobacterium* containing TRV2:*eIF4E* or TRV2:*eIFiso4E.* Plants infiltrated with TRV2:*PDS* were taken as a positive control in the VIGS experiment. TRV2:*PDS* infected plants showed typical photo-bleaching phenotype (Fig.

Table 4. Cosegregation analysis of the ChiVMV resistant phenotype and genotype at the *pvr1* and *pvr6* loci for the F_2 segregating population.

Phenotype		Genotype								No. of plants simultaneous *pvr6*/*pvr6* and *pvr1²*/*pvr1²*	Phenotype of simultaneous *pvr6*/*pvr6* and *pvr1²*/*pvr1²* plants
		pvr1 locus				*pvr6* locus					
Resistant	Susceptible	*Nonpvr1²* *Nonpvr1²*	*Nonpvr1²* *pvr1²*	*pvr1²* *pvr1²*	nd	*Pvr6⁺* *Pvr6⁺*	*Pvr6⁺* *pvr6*	*pvr6* *pvr6*	nd		
34	41	21	34	14	6	17	35	15	8	2	R

Table 5. Cosegregation analysis of the ChiVMV resistant phenotype and genotype at the *pvr1* and *pvr6* loci for the F_3 segregating population and corresponding F_2 individuals.

F_2		F_3													
			Phenotype			Genotype									
						pvr1 locus				*pvr6* locus					
Plant ID	Genotype*	No. of F_3 plants	R	S	nd	*no-r1²* *no-r1²*	*no-r1²* *r1²*	*r1²* *r1²*	nd	*r6⁺* *r6⁺*	*r6⁺* *r6*	*r6* *r6*	nd	No. of plants simultaneous *r6*/*r6* and *rf²*/ *r1²* genotype	Phenotypes of plants having *r6*/*r6* and *rf²*/ *r1²* genotype
4	*no-r1²*/*no-r1²* *r6*/*r6*	22	1	21	0	22	0	0	0	0	0	0	22	0	-
13	*no-r1²*/*no-r1²* *r6⁺*/*r6*	41	0	41	0	40	0	0	1	17	12	12	0	0	-
15	*r1²*/*r1²* *r6⁺*/*r6*	14	4	10	0	0	0	14	0	6	5	3	0	3	R
21	*no-r1²*/ *r1²* *r6⁺*/*r6*	33	0	33	0	5	19	9	0	33	0	0	0	0	-
25	*no-r1²*/*r1²* *r6⁺*/*r6*	14	10	4	0	10	3	1	0	9	0	5	0	0	-
34	*no-r1²*/*r1²* *r6⁺*/*r6*	32	2	30	0	8	20	4	0	8	16	8	0	1	R
36	*no-r1²*/*r1²* *r6⁺*/*r6*	22	5	17	0	7	10	5	0	19	0	3	0	0	-
46	*no-r1²*/*r1²* *r6⁺*/*r6*	37	4	33	0	10	21	6	0	12	14	7	3	2	R
57	*no-r1²*/*no-r1²* *r6⁺*/*r6*	7	0	7	0	7	0	0	0	2	5	0	0	0	-
61	*no-r1²*/*r1²* *r6⁺*/*r6*	10	8	2	0	3	5	1	1	2	3	5	0	1	R
63	*no-r1²*/*no-r1²* *r6⁺*/*r6⁺*	36	2	33	1	0	0	35	1	35	0	0	1	0	-
64	*no-r1²*/*r1²* *r6⁺*/*r6*	23	7	16	0	4	13	6	0	2	0	20	1	4	R

**no-r1²*: *Pvr1⁺*, *r1²*: *pvr1²*, *r6⁺*: *Pvr6⁺*, *r6*: *pvr6*

4A) and reduced *PDS* mRNA accumulation after 3 weeks post infiltration (Fig. 4B).

To examine the effects of VIGS on endogenous expression of *eIF4E* and *eIFiso4E*, we performed semiquantitative RT-PCR using the total RNA extracted from the upper leaves of TRV infiltrated plants. Analyses of RT-PCR products showed that mRNA of endogenous *eIF4E* and *eIFiso4E* had no change in TRV:*PDS* infected plants compared with that in uninoculated control plants (Fig. 4B, lanes 1 and 2); significantly reduced expression of both genes was shown in both TRV:*eIF4E* and TRV:*eIFiso4E* infiltrated plants (Fig. 4B, lanes 3 and 4).

At 21 days after *Agrobacterium* infiltration, the upper leaves of silenced plants were challenged with ChiVMV together with non-silenced susceptible control plants. Systemic ChiVMV symptom started to develop from 6 dpi in the upper leaves of control and silenced plants. No obvious symptomatic differences could be observed between control and *eIF4E* or *eIFiso4E* silenced plants (data not shown). In order to test whether *eIF4E* or *eIFiso4E* silenced plants show reduced virus accumulation, DAS-ELISA experiment was performed using the inoculated leaves of ChiVMV. Silencing of *eIF4E* reduced about 50% of ChiVMV accumulation compared with control plants (Fig. 4C). *eIFiso4E* silenced plants showed more ChiVMV accumulation than those of *eIF4E* silenced plants, however, significantly less (80%) than that observed in control plants (Fig. 4C). Our results confirmed that ChiVMV can use either eIF4E or eIFiso4E for infection and silencing either *eIF4E* or *eIFiso4E* can reduce ChiVMV accumulation in pepper.

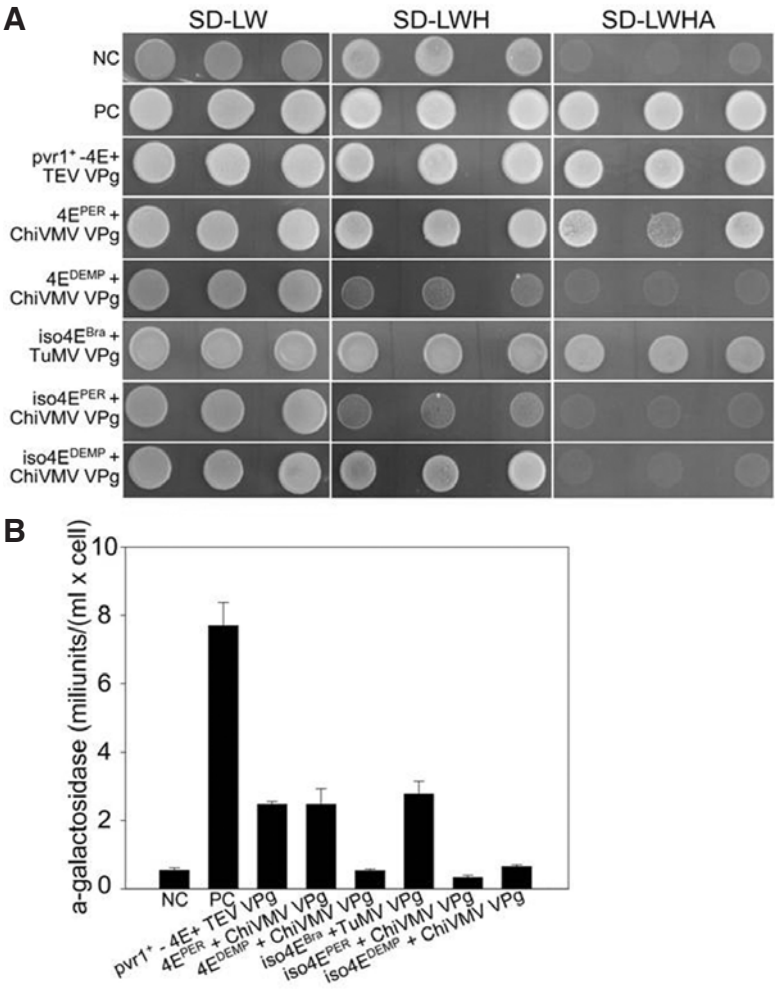

Fig. 3. Interaction assay between eIF4E and/or eIFiso4E and VPg of ChiVMV using a yeast two-hybrid system. (A) Bait plasmid pGBKT7 was used to express the fusion protein VPg from TEV and ChiVMV, while the prey plasmid pGADT7 was used to express eIF4E and eIFiso4E. Yeast transformants expressing both 'bait' and 'prey' recombinant proteins were obtained by control plates (SD/-LW) and then cultured on two selective media lacking leucine, tryptophan, and histidine (SD/-LWH) or leucine, tryptophan, histidine, and adenine (SD/-LWHA). Each plasmid combination was tested in triplicate. Positive and negative controls from the Matchmaker GAL4 two-hybrid system 3 were used. Yeast transformants of TEV-HAT VPg and eIF4E $^{pvr1+}$ were also used as interaction controls and yeast transformants of TuMV VPg and Brassica *eIFiso4E* were used as an eIFiso4E interaction controls for the yeast two-hybrid assay. (B) The α-gal quantitative assay of yeast two-hybrid interactions between ChiVMV VPg and eIF4E or eIFiso4E from PER and DEMP.

DISCUSSION

Simultaneous mutation of both *eIF4E* and *eIFiso4E* at *pvr1* and *pvr6* confers resistance to ChiVMV

Functional analysis suggested that the physical interaction between the host factor eIF4E at *pvr1* in pepper and VPg in *Tobacco etch virus* (TEV) is required for viral infection. Recessive mutation of the *pvr1*^{2} allele, which interrupts the binding ability of VPg, causes loss of function of eIF4E in the host and thus acquisition of resistance against TEV (Kang et al., 2005b; Yeam et al., 2007). Unlike the *Capsicum*-TEV pathosystem, we found that mutation of eIF4E at *pvr1* alone was not enough to confer resistance to ChiVMV in pepper. We also investigated a second locus, *pvr6*, which encodes eIFiso4E in pepper. Interestingly, we observed that the homozygous co-occurrence of both mutated alleles (*pvr1*^{2} and *pvr6*) restored resistance to ChiVMV. Consequently, simultaneous mutation of both eIF4E and eIFiso4E at *pvr1* and *pvr6* was required to obtain the resistance phenotype. Our observation is consistent with previous work on PVMV resistance in pepper (Ruffel et al., 2006).

ChiVMV VPg is able to interact with both eIF4E and eIFiso4E

Because most plant viruses encode less than 10 genes, viruses require many host factors to complete their infection cycle. Previous studies on recessive resistance genes in natural populations have shown that eukaryotic translation initiation factor *eIF4E* (or *eIFiso4E*) and its scaffold protein *eIF4G* (or *eIFiso4G*) are common host factors for infection of many plant RNA viruses (Kang et al., 2005a; Robaglia and Caranta, 2006). Mutations of these host factors often result in disruption of interactions of the host factors with viral proteins, and consequently lead to potyvirus resistance (Charron et al., 2008; Grzela et al., 2006). In this study, we demonstrated that mutations of *eIF4E* and *eIFiso4E* result in malfunctional proteins which are not able to interact with ChiVMV VPg in a yeast two-hybrid analysis. These results together with the genetic analysis indicate that simultaneous mutations in eIF4E and eIFiso4E confer recessive resistance to ChiVMV. Although Ruffel et al. (2006) found a similar result using the PVMV system, this study is the first to show that potyviral VPg is able to interact physically with both eIF4E and eIFiso4E, thereby affecting viral infection. Depending on the virus, eIF4E or eIFiso4E is selectively used for virus infection. Sato et al. (2005) showed selective involvement of eIF4E family members in potyvirus infection in *Arabidopsis*. *Clover yellow vein virus* (ClYVV) and LMV require eIF4E, while TEV and TuMV require eIFiso4E to infect *Arabidopsis*. Furthermore, in the case of TEV, eIF4E is used to infect tomato and pepper, but eIFiso4E is used to infect *Arabidopsis*. Despite these observations, the molecular mechanisms of these selectivity are largely unknown. The selective recruitment of

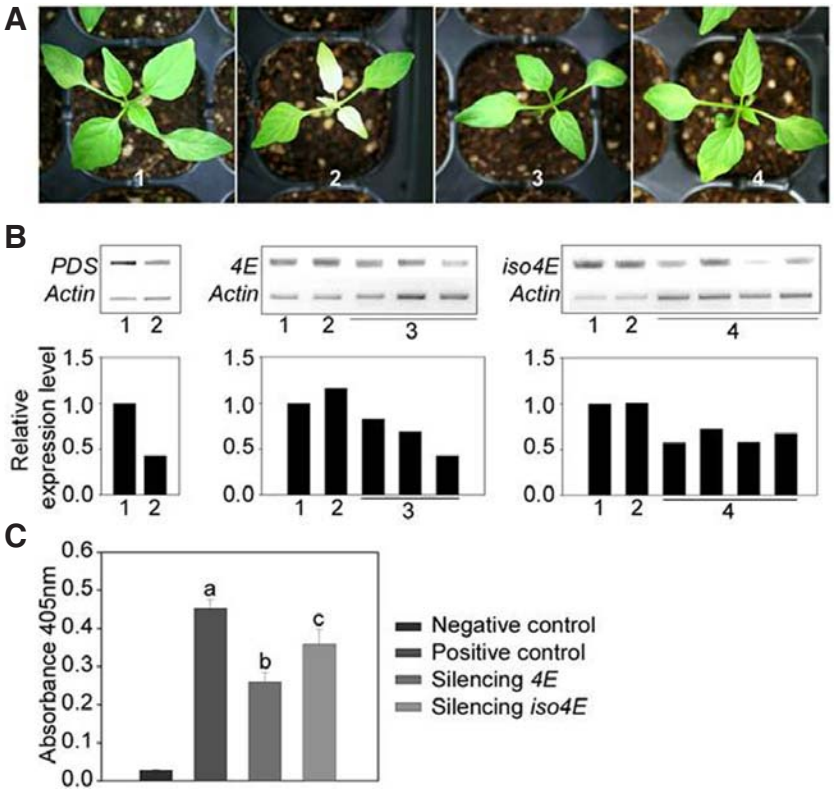

Fig. 4. Silencing *eIF4E* and *eIFiso4E* in *C. annuum* Jeju. (A) VIGS *eIF4E* and *eIFiso4E* in *Capsicum*. The plants infected with TRV1 and TRV2 or TRV2: *PDS* were used as negative (1) and positive (2) controls, respectively. Plants were infected with TRV2: *eIF4E* (3) and with TRV2:*eIFiso4E* (4). Silenced plants were photographed at 21 dpi. Six plants were used for each infection. This experiment was independently repeated 2 times with the same results. (B) Expression of *Capsicum eIF4E*, *eIFiso4E* and *PDS* transcripts in the silenced plants. Expression levels were examined by RT-PCR. Relative levels of *eIF4E* and *eIFiso4E* transcripts were shown in the underside. Relative amounts are recalculated by using the level of *Actin*, a standard control. (C) Accumulation of ChiVMV in the inoculated leaves of silenced plants. Virus accumulation in inoculated leaves was tested at 6 dpi. Four independent leaf samples were used for ELISA analysis. These data are the averages with standard errors of four replicates.

these proteins by different potyviruses in different hosts may be due to different binding affinities of VPg proteins to eIF4E or eIFiso4E, as noted previously (Sato et al., 2005). We observed that the binding affinity of ChiVMV VPg to both proteins was quite unique, and anticipate that the ChiVMV/eIF4E and eIFiso4E systems will be used to elucidate the molecular mechanisms of the host specificity of potyviruses. Our yeast two-hybrid experiments showed that ChiVMV VPg has higher binding affinity to eIF4E than to eIFiso4E. Silencing experiments supported the yeast two-hybrid experiments: ChiVMV virus accumulation was reduced more in *eIF4E*-silenced plants than in *eIFiso4E*-silenced plants, indicating that eIF4E is preferentially used for virus replication. Further research is required to reveal how ChiVMV evolved and gained the ability to use both eIF4E and eIFiso4E.

Broad spectrum disease resistance

Unlike dominant gene resistance, which is mostly conferred by NB-LRR genes (groups of resistance genes with nucleotide binding sites and leucine rich repeats) (Chisholm et al., 2006; Dangl and Jones, 2001; Michelmore and Meyers, 1998), recessive gene resistance that is specifically contributed by host factor-related genes is more durable and provides broad spectrum resistance (Kang et al., 2005a; 2007). Furthermore, we found that the co-occurrence of two gene mutations, *pvr1*^{2} and *pvr6*, conferred cross-species resistance to ChiVMV and PVMV (Ruffel et al., 2006). The shared resistance mechanism in pepper against ChiVMV and PVMV may be explained by the conserved genome identity of the two viruses (Tsai et al., 2008). In this study, we used ChiVMV isolates from Indonesia for the phenotypic analysis to test the effectiveness of resistance conferred by *pvr1*^{2} and *pvr6* within the ChiVMV group. We plan to include other ChiVMV isolates in future investigations (Green et al., 1999; Tsai et al., 2008).

Marker-assisted selection (MAS) of ChiVMV

In this study, we used the pvr1-R1 and pvr6-exon1 markers to genotype the *pvr1* and *pvr6* loci, respectively. These two gene-specific PCR-based markers should be very useful in applied breeding programs (Ruffel et al., 2006; Yeam et al., 2005). Because the ChiVMV resistance we demonstrated involved double recessive genes, simultaneous transfer of both recessive alleles would be necessary to restore resistance, a feat difficult to achieve by conventional breeding methods. Instead, the two CAPS and SCAR markers suggested in this study could be used to transfer *pvr1*^{2} and *pvr6* alleles to recurrent breeding materials through marker-assisted-selection (MAS) (Ruffel et al., 2006; Yeam et al., 2005), allowing the development of multiple potyvirus resistance peppers against TEV, PVY, PVMV, and ChiVMV.

Resistance to ChiVMV is complicated in pepper

Although we have successfully demonstrated that the simultaneous presence of homozygous recessive genotypes of two genes conferred resistance to ChiVMV, we cannot ignore other alternative mechanisms for ChiVMV resistance. For instance, genetic analysis of F_1 plants in this study indicated that the dominant gene(s) contributed by PER could be involved in ChiVMV resistance, which is not surprising since dominant resistance to other potyviruses has been demonstrated (Grube et al., 2000a; 2000b; Kyle and Palloix, 1997). Furthermore, genetic analysis of ChiVMV resistance in the F_2 population derived from the cross between DEMP and PER showed a very complicated segregation pattern. This finding may be due to a mixed segregation of two recessive genes (*pvr1*^{2} and *pvr6*), or PER may contain quantitative trait loci (QTL) for resistance to ChiVMV (Caranta et al., 1997; Kyle and Palloix, 1997; Moury et al., 2005). Further investigation will be necessary to delineate all the resistance genes (both dominant and recessive) involved

in resistance to ChiVMV in pepper. Nevertheless, by exploiting the involvement of simultaneous mutations of both *eIF4E* and *eIFiso4E* at *pvr1* and *pvr6* in resistance to ChiVMV, we have established a solid foundation for further research.

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