Simultaneous mutations in translation initiation factors eIF4E and eIF(iso)4E are required to prevent pepper veinal mottle virus infection of pepper

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Capsicum resistance to Pepper veinal mottle virus (PVMV) results from complementation between the pvr2 and pvr6 resistance genes: recessive alleles at these two loci are necessary for resistance, whereas any dominant allele confers susceptibility. In line with previous results showing that pvr2 resistance alleles encode mutated versions of the eukaryotic translation initiation factor 4E (eIF4E), the involvement of other members of the eIF4E multigenic family in PVMV resistance was investigated. It was demonstrated that pvr6 corresponds to an eIF(iso)4E gene, predicted to encode the second cap-binding isoform identified in plants. Comparative genetic mapping in pepper and tomato indicated that eIF(iso)4E maps in the same genomic region as pvr6. Sequence analysis revealed an 82 nt deletion in eIF(iso)4E cDNAs from genotypes with the pvr6 resistance allele, leading to a truncated protein. This deletion was shown to co-segregate with pvr6 in doubled haploid and F2 progeny. Transient expression in a PVMV-resistant genotype of eIF(iso)4E derived from a genotype with the pvr6+ susceptibility allele resulted in loss of resistance to subsequent PVMV inoculation, confirming that pvr6 encodes the translation factor eIF(iso)4E. Similarly, transient expression of eIF4E from a genotype with the pvr2+ eIF4E susceptibility allele also resulted in loss of resistance, demonstrating that wild-type eIF4E and eIF(iso)4E are susceptibility factors for PVMV and that resistance results from the combined effect of mutations in the two cap-binding isoforms. Thus, whilst most potyviruses specifically require one eIF4E isoform to perform their replication cycle, PVMV uses either eIF4E or eIF(iso)4E for infection of pepper.

INTRODUCTION

Viruses depend on the host cellular machinery to achieve plant infection and completion of the viral cycle is the result of a complex interplay between virus-encoded and host-encoded factors. Identification of the host-encoded factors involved and understanding how they are implicated in viral genome amplification and cell-to-cell and long-distance movement represent major challenges to our knowledge of plant–virus interactions (reviewed by Whitham & Wang, 2004). Among the host factors required for viral infection, eukaryotic translation initiation factor 4E (eIF4E) has been demonstrated to play an essential role. eIF4E provides the cap-binding function during formation of translation initiation complexes of most mRNA and is associated with eIF4G, a scaffold for other components of the complex, to form the eIF4F complex. Plants contain two distinct cap-binding proteins, eIF4E and eIF(iso)4E, which assemble into different eIF4F complexes [eIF4F and eIF(iso)4F] (Browning, 1996). In Arabidopsis thaliana, three genes named eIF4E1 (At4g18040), eIF4E2 (At1g29590) and eIF4E3 (At1g29550) encode the proteins of the eIF4E subfamily and one gene (At5g35620) encodes eIF(iso)4E (http://www.arabidopsis.org/info/genefamily/eIF.html; Robaglia & Caranta, 2006). The structure of the eIF4E gene family in other plant species is not known precisely, but in all cases several loci have been identified. Overall, differences in transcription patterns, in binding affinities depending on the 5’ mRNA structure and/or the presence of a cap and in ability to rescue the growth of a yeast mutant in which the eIF4E gene was disrupted strongly suggest that isoforms encoded by eIF4E1 and eIF(iso)4E have complementary biological roles (Gallie & Browning, 2001; Rodriguez et al.,
1998). It remains to be determined whether elf4E2 and elf4E3 encode proteins with translation initiation function. Arabidopsis mutants lacking either transposon- or ethyl methanesulfonate-induced null alleles for the elf(iso)4E gene have been shown to be resistant to infection by several viruses from the genus Potyvirus including Turnip mosaic virus (TuMV), Lettuce mosaic virus (LMV) and Tobacco etch virus (TEV) (Duprat et al., 2002; Lellis et al., 2002). More recently, it was demonstrated that the knockout mutation of elf4E1 prevented Clover yellow vein virus (ClYVV) replication, indicating that potyviruses selectively use elf4E isoforms (Sato et al., 2005). Potyviral RNA differs from host mRNAs in that it lacks a 5′ GpppX cap structure and is instead covalently linked at its 5′ end to a viral-encoded protein, VPg (viral protein genome-linked) (Urcuqui-Inchima et al., 2001). Experiments using the yeast two-hybrid system and an ELISA have shown that the VPg (or its precursor, Nla) interacts with elf4E or elf(iso)4E (Léonard et al., 2000; Schaad et al., 2000; Wittmann et al., 1997). Loss of interaction of VPg with elf4E is correlated with a loss of infectivity of the virus, suggesting that the interaction is critical for virus production (Léonard et al., 2000; Schaad et al., 2000).

Interestingly, the molecular characterization of naturally occurring recessively inherited resistance loci also converged towards identification of the translation initiation factor 4E (reviewed by Robaglia & Caranta, 2006). Four recessive resistance genes against potyviruses have been shown to encode defective forms of elf4E: pvr2 and pvr1 from pepper (recently shown to be alleles of the same gene; Kang et al., 2005; Ruffel et al., 2002, 2004) and its orthologue, pot-1, from tomato for Potato virus Y (PYV) and TEV resistance (Ruffel et al., 2005), m1 for lettuce resistance to LMV (Nicaise et al., 2003) and sbm-1 for pea resistance to Pea seed-borne mosaic virus (PSbMV) (Gao et al., 2004). Resistance resulted from a small number of amino acid changes in the elf4E proteins encoded by the recessive resistance alleles. Moreover, in the four species, most of the resistance-related changes corresponded to non-conservative amino acid substitutions and were clustered in two neighbouring regions and at the surface of the predicted elf4E 3D structure (Gao et al., 2004; Nicaise et al., 2003; Ruffel et al., 2005). Recently, the rym4/5 gene for barley resistance to strains of the Barley yellow mosaic virus complex from the genus Bymovirus was also demonstrated to correspond to elf4E (Stein et al., 2005).

Numerous recessive resistance genes characterized by an important diversity of resistance spectra and resistance levels against potyviruses have been described in pepper, making this species a useful model for the study of host factors required for viral infection. In addition to pvr2 resistance against PYV and TEV mediated by mutations in elf4E, two other recessive resistance loci have been identified. pvr3, from Capsicum annuum cv. Avelar, confers resistance to long-distance movement of Pepper mottle virus (Guerini & Murphy, 1999). pvr6, from C. annuum cv. Perennial, was demonstrated to control resistance to Pepper vein mottle virus (PVMV) only when combined with the pvr2 or pvr6 resistance alleles, whereas pvr2 and pvr6 do not separately confer any resistance to PVMV and pvr6 alone has no detectable effect on the potyvirus infection process (Caranta et al., 1996).

In the present study, we investigated the involvement of the second cap-binding isoform, elf(iso)4E, in pepper resistance against PVMV. Our data showed that pvr6 corresponds to elf(iso)4E and that simultaneous mutations in two genes belonging to the elf4E family are required to prevent the PVMV infectious cycle. Therefore, while PVY and TEV require one specific elf4E isoform to achieve pepper infection, PVMV can use both elf4E and elf(iso)4E isoforms.

**METHODS**

**Plant material.** The C. annuum homozygous lines used were Yolo Wonder (YW), Yolo Y (YY), FloridaVR2 (F), Perennial (P) and DH801. YW contains the pvr2 dominant allele for susceptibility to potyviruses and pvr6, which does not confer resistance to PVMV in combination with pvr2 alleles; YY has the recessive allele pvr2 for complete resistance to PVY pathotype 0 and the pvr6 allele; F has the recessive allele pvr2 for complete resistance to PVY pathotypes 0 and 1 and to TEV and the pvr6 allele; P has the recessive allele pvr2 for partial resistance to PVY and the pvr6 allele. YY, YY, F and P are susceptible to PVMV. DH801 is a doubled haploid (DH) line homozygous for pvr6 (from P) and pvr2 (from F). It is completely resistant to PVMV (Caranta et al., 1996) this study; the pvr6 segregating population was composed of 22 DH lines obtained by another culture from an F1 hybrid between P and YW. The procedure to determine the allele at the pvr6 locus has been described by Caranta et al. (1996). Briefly, the genotype at the pvr6 locus was inferred through PVMV resistance evaluation of 22 F2 progeny plants (200 plants per F2) obtained by crossing the 22 DH lines with F1, homozygous for pvr2. The F2 progeny (182 plants) segregating for both pvr2 and pvr6 were generated from the F1 hybrid between DH218 (known to be homozygous for pvr6; Caranta et al., 1996) and F (homozygous for pvr2). This F2 progeny was assayed for PVMV resistance. The same F2 plants were also genotyped for delel at elf(iso)4E and for alleles at the pvr2 elf4E locus. The tomato introgression line (IL) population was used to map elf(iso)4E. The IL population was composed of 75 Lycopersicon esculentum cv. M82 lines, each containing a single genomic fragment introgressed from the wild species Lycopersicon pennellii LA716 (Liu & Zamir, 1999).

**Virus strain and detection of virus accumulation.** Inoculation experiments were carried out under growth chamber conditions (22°C, 12 h photoperiod). Experiments were performed using the PVMV-IC isolate from the Ivory Coast, provided by J. C. Thouvenel (IRD, Montpellier, France). PVMV-IC was maintained on susceptible pepper cultivar YW and transferred every 4 weeks. Inoculum was prepared from 1 g (fresh weight) infected foliar tissues ground with 4 ml 0·03 M potassium phosphate buffer (pH 7) containing 0·2 % diethyldithiocarbamate, 80 mg active charcoal and 80 mg Carbomunudum (400 mesh). For inoculation, leaves of 4-week-old plants (two- to three-leaf stage) were rubbed manually with inoculum extract and rinsed with water for 5 min after rubbing. Purity was monitored regularly with differential host index tests. PVMV-IC accumulation in inoculated and apical non-inoculated pepper leaves was checked by double-antibody sandwich ELISA (DAS-ELISA) and RT-PCR. Polyclonal antibodies raised against the PVMV coat
protein (CP) were supplied by R. Nono-Wondim (AVRDC, Arusha, Tanzania). Samples were considered to be virus-positive when the ELISA absorbance value was at least three times greater than the mean value of the healthy controls. RT-PCR for PVMV was performed with primers specific for the coding sequences of Nib (forward, 5'-GGIAARGCNCCTAYAT-3') and CP (reverse, 5'-CGCGCTTAATGACATATCGG-3') (Moury et al., 2005).

Restriction fragment length polymorphism (RFLP) analysis. Plant genomic DNA isolation and RFLP were done as described previously with hybridization at 65 °C (Caranta et al., 1996). The tomato elf(iso)4E cDNA probe for RFLP mapping on tomato and pepper progenies was obtained by RT-PCR using primers 5'-TGGACTGCCTACTGACGAG-3' (forward) and 5'-GCCAGAACGGGAAAAAGTG-3' (reverse), which amplified an 882 bp elf(iso)4E sequence (TIGR accession no. TC126316).

Amplification, cloning and sequencing of elf(iso)4E cDNA from pepper. Total RNA from YW, YY, F, P and DH801 plants was isolated from 100 to 200 mg leaf tissue using TRI Reagent (Sigma-Aldrich). The 3' end of YW elf(iso)4E cDNA was obtained using the Gibco-BRL Life Technologies 3' RACE system (version 2.0). The primer 5'-AATGGACTGTGACGTACACG-3' specific for the elf(iso)4E gene was designed from alignment of A. thaliana (GenBank accession no. Y10547), Lactuca sativa (GenBank accession no. AF530163) and Lycopersicon esculentum (TIGR accession no. TC126316) elf(iso)4E cDNAs and used together with the adapter primer (AUAP) in the kit. Full-length YW elf(iso)4E cDNA was amplified by RT-PCR with a primer designed from the sequence of the 3'RACE product (5'-ATTGCTGGAACTTGGGGAGGG-3') and a primer designed from the 5' untranslated region of TIGR accession no. TC126316 (5'-AAAAAATGGCAGCAGCAAG-GCA-3'). All amplifications were performed with High Fidelity Platinum Taq polymerase (Gibco-BRL Life Technologies). The pGEM-T Easy vector system (Promega) was used to clone cDNAs after PCR amplification. At least three independent positive clones were sequenced from both ends by Genome Express (Grenoble, France). The Genetics Computer Group (Madison, WI, USA) software package was used for nucleic and protein sequence analysis.

PCR markers. Total RNA was extracted from the leaves of genotypes P, F and YW, from the 22 DH lines segregating for pvr6 and from the 182 F2 progeny (DH218 × F) segregating for both pvr2 and pvr6 as described above. The elf(iso)4E cDNA of 527 or 609 bp, depending on the presence/absence of the 82 bp deletion, was amplified by RT-PCR with the primers 5'-ATGGCAGCTGACTCAGGAG-3' (forward) and 5'-TCACACGGTTGTATCGGC-3' (reverse). The elf(iso)4E cDNA corresponding to the pvr2 locus was amplified with the primers 5'-AAAAAATGGCAGCAGCAAG-GCA-3' (forward) and 5'-TTTCCGACATTTGACAGCAAG-3' (reverse). A cleaved amplified polymorphic sequence (CAPS) differential cleavage site revealed by the restriction endonuclease Mvnl was based on a single nucleotide polymorphism of T→G at position 236 of the cDNA that distinguishes pvr6 from the pvr22 and pvr2 alleles. After amplification of the 687 bp product with the elf(iso)4E cDNA-specific primers, products were digested with Mvnl, generating two fragments of 453 and 234 bp only for the pvr22 allele from F. PCR products were resolved by electrophoresis in a 2% (w/v) agarose gel.

Functional complementation assays. Functional complementation was performed using a Potato virus X (PVX)-mediated transient expression assay as described by Ruffel et al. (2002). elf(iso)4E ORFs from YW and F were cloned into pPVX201 (provided by D. C. Baulcombe, Sainsbury Laboratory, UK; Baulcombe et al., 1995) to produce plasmids pPVXeYW and pPVXeF, respectively. Similarly, elf(iso)4E ORFs from YW and P were cloned into pPVX201 to produce plasmids pPVX(iso)eYW and pPVX(iso)eP. Plasmids were then manually inoculated to Nicotiana benthamiana; at 10 days post-inoculation (p.i.), N. benthamiana-inoculated leaves were used as an inoculum source for a transient expression assay in pepper. In three independent experiments, pepper genotypes YW (homozygous for pvr22 and pvr6 alleles) and DH801 (homozygous for both pvr22 and pvr6 resistance alleles) were inoculated with PVX plasmids and 10 days later, the same leaves were inoculated with PVMV-IC. PVX and PVMV accumulation in inoculated pepper leaves was checked at 10 days p.i. by DAS-ELISA and RT-PCR. A sample was considered to be positive for virus when the ELISA absorbance value at 405 nm was at least three times greater than the mean value of the healthy controls. RT-PCR for PVX was performed with primers 5'-CCGATCTCAGCAGCAG-3' (forward) and 5'-CTCAGAGCCTTGCGGAAGATTG-3' (reverse) specific for either side of the cloning site to check the stability of the constructs.

RESULTS

Detection of PVMV-IC in C. annuum genotypes with distinct allelic combinations at the pvr2 and pvr6 loci

All YW, YY, F and P plants inoculated with PVMV-IC presented mosaic symptoms in apical, non-inoculated leaves (referred to as apical leaves) and exhibited high DAS-ELISA values at 15 days p.i., whereas no systemic infection was observed and no virus CP was detected by DAS-ELISA in inoculated or apical leaves of DH801 plants carrying both pvr22 and pvr6 resistance alleles (Table 1). To determine precisely how infection progresses in the various host genotypes and to check whether pvr2 or pvr6 resistance alleles alone had any detectable effect on PVMV accumulation in inoculated leaves, further virus detection on inoculated leaves was performed by RT-PCR and DAS-ELISA at 2, 3, 4 and 7 days p.i. Viral RNA and CP accumulation were detected in inoculated leaves of YW, YY, F and P plants challenged with PVMV-IC at 2, 3, 4 and 7 days p.i., but were not detected in inoculated leaves of DH801 at any time point during two independent experiments (Table 1). Thus, these data, together with previous genetic analysis (Caranta et al., 1996), indicate that resistance to PVMV, expressed as undetectable accumulation of the virus in inoculated leaves, results from the combined effect of the pvr22 and pvr6 alleles.

eIF(iso)4E maps in the same genomic region as the pvr6 locus

Genetic mapping of elf(iso)4E was conducted as a first step in tomato because of the availability of IL progeny and expressed sequence tags that facilitate mapping of multiloci components. The tomato elf(iso)4E probe generated from TIGR accession no. TC126316 identified RFLPs between Lycopersicon pennellii LA716 and Lycopersicon esculentum M82 with HindIII- and XbaI-digested DNA. For both restriction enzymes, an RFLP was assigned to a genomic region of 236 cM spanning ILs 9.3 to 9.3.2 with overlapping introgression fragments (Fig. 1).

The syntetic relationship between tomato and pepper genomes makes it possible to infer the location of a pepper
locus on the tomato genome (Livingstone et al., 1999). In pepper, the pvr6 locus was localized in a genomic region of chromosome P3 defined by RFLP markers CT220, TG591 and CD008, 9-4 CM from TG591 (Caranta et al., 1996; Lefebvre et al., 2002) (Fig. 1). These three RFLP markers are linked on the tomato genetic linkage map and localize to a chromosome 9 region that overlaps the chromosomal region defined by ILs 9.3 and 9.3.2 (Liu & Zamir, 1999; Tansley et al., 1992) (Fig. 1). Taken together, these data suggested that pvr6 and the RFLP marker generated using the tomato eIF(iso)4E probe were localized to the same genomic region.

To delimit further the map location of eIF(iso)4E in comparison with pvr6, the tomato eIF(iso)4E probe was hybridized on pepper genotypes YW, F and P with distinct alleles at the pvr6 locus. With EcoRV-digested DNA, hybridization revealed an RFLP between the pvr6 and pvr6+ genotypes. This marker was mapped on the pepper progeny of 22 DH lines that were used by Caranta et al. (1996) for the demonstration of complementation between pvr2+ and pvr6 for PVMV resistance. These lines segregated for pvr6 alleles into eight pvr6+ lines and 14 pvr6 lines. Genetic co-segregation between pvr6 and the RFLP generated using the eIF(iso)4E cDNA probe was observed among the 22 DH lines, providing additional data in favour of linkage between the two loci.

**eIF(iso)4E cDNAs from pvr6 genotypes have a deletion of 82 nt**

An eIF(iso)4E cDNA was obtained from the potyvirus-susceptible pepper genotype YW using 3’RACE PCR and sequence data available for the tomato eIF(iso)4E cDNA (TIGR accession no. TC126316). A 660 nt cDNA was deduced that contained a single open reading frame of 609 nt encoding a protein of 202 aa. The closest matches obtained after a BLAST search with the full-length nucleotide sequence were the eIF(iso)4E cDNA from lettuce (GenBank accession no. AF530163, E=1·10⁻⁵⁹), pea (GenBank no. AY423377, E=2·10⁻²⁴), maize (GenBank no. AF076955, E=4·10⁻¹⁰), Arabidopsis (GenBank no. Y10547, E=6·10⁻⁸) and wheat (GenBank no. M95818, E=9·10⁻⁸). The closest matches with the predicted translation product were the eIF(iso)4E amino acid sequences from lettuce (GenBank protein no. AAP86603, E=8·10⁻⁷¹) and wheat (GenBank protein no. AAA34296, E=1·10⁻⁷⁸), confirming that the cloned cDNA corresponded to eIF(iso)4E. The pepper eIF4E (GenBank accession no. AY122052) and eIF(iso)4E coding regions were 57·5 % identical in nucleotide sequence and 48·3 % identical in amino acid sequence.

To determine whether sequence variation could be associated with pvr6 or pvr6+ alleles, nucleotide and amino acid sequences of eIF(iso)4E cDNAs from YW, YY, F, P and DH801 plants were compared. Nucleotide sequence alignment showed that the pvr6+ genotypes YW, YY and F were 100 % identical in their eIF(iso)4E nucleotide sequence. The pvr6 genotypes P and DH801 were also 100 % identical in their eIF(iso)4E nucleotide sequence but presented, in comparison with pvr6+ genotypes, a deletion from nt 89 to 170, a G→A substitution at position 268, a C→A substitution at position 483 and a C→T substitution at position 537 (Fig. 2). The complete eIF(iso)4E genomic sequence was amplified from YW and P DNA and the first exon was sequenced to confirm that the deletion occurred on genomic DNA and was not the result of a mRNA splicing event (data not shown). The 82 nt deletion modified the open reading frame by insertion of a stop codon after aa 51. Alignment of predicted eIF(iso)4E protein sequences from

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**Table 1. PVMV-IC accumulation assessed by DAS-ELISA and RT-PCR in inoculated or apical non-inoculated leaves of C. annuum genotypes with distinct alleles at the pvr2 and pvr6 loci**

<table>
<thead>
<tr>
<th>C. annuum genotype and alleles at the pvr2 and pvr6 loci</th>
<th>YW</th>
<th>YY</th>
<th>F</th>
<th>P</th>
<th>DH801</th>
</tr>
</thead>
<tbody>
<tr>
<td>prv2+/pvr2+ prv6+/prv6+</td>
<td>1·44 ± 0·18</td>
<td>1·46 ± 0·16</td>
<td>1·45 ± 0·23</td>
<td>1·56 ± 0·22</td>
<td>0·13 ± 0·02</td>
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<tr>
<td>ELISA on inoculated leaves at 15 days p.i.*</td>
<td>1·74 ± 0·20</td>
<td>1·58 ± 0·40</td>
<td>1·62 ± 0·33</td>
<td>1·56 ± 0·26</td>
<td>0·14 ± 0·01</td>
</tr>
<tr>
<td>ELISA on apical leaves at 15 days p.i.*</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RT-PCR on inoculated leaves at 2 days p.i.†</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RT-PCR on inoculated leaves at 3 days p.i.†</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>RT-PCR on inoculated leaves at 4 days p.i.†</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>RT-PCR on inoculated leaves at 7 days p.i.†</td>
<td>+</td>
<td>+</td>
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</table>

*Means ± SD of DAS-ELISA values of A₄₅₀ calculated from 10 leaves in two independent experiments. ELISA was considered to be positive when the A₄₅₀ of the sample was at least three times greater than the mean value of the healthy controls (i.e. A₄₅₀ ≥ 0·36 in these experiments). The mean A₄₅₀ of healthy plants was 0·12 ± 0·01.*

†As negative controls, RT-PCR was performed on PVMV-IC-inoculated leaves of the non-host plants *Cucumis melo* and *Vinca rosea* at 2, 3, 4 and 7 days p.i. Ten inoculated leaves were assessed for each genotype in two independent experiments. +, RT-PCR amplification of a 737 bp fragment corresponding to the Nilb/CP region of PVMV-IC (Moury et al., 2005); −, no amplification.
pvr6+ and pvr6 genotypes showed that only the N-terminal 29 aa of the 202 aa of wild-type protein were conserved (Fig. 2).

The 82 nt deletion within elf(iso)4E co-segregates with pvr6

To confirm the genetic linkage between the pepper elf(iso)4E cDNA and pvr6, the linkage between pvr6 and the 82 nt deletion in the elf(iso)4E cDNA was tested in 182 F2 progeny plants segregating for both the pvr6 and pvr2 resistance alleles. In the first step, F2 plants were phenotyped for PVMV-IC resistance. The segregation ratio observed for PVMV resistance (proportion of resistant and susceptible plants assessed by DAS-ELISA) was consistent with two recessive genes involved in resistance [observed ratio 14R : 16S; \( \chi^2 \text{df}(1R:1S) = 0.6461 \) and \( P = 42.2\% \)]. The same 182 F2 plants were then genotyped with the PCR marker based on the presence/absence of the 82 nt deletion in the elf(iso)4E cDNA and with the CAPS marker based on a single nucleotide polymorphism within the coding region of the pvr2-elf4E locus that distinguishes pvr2 from other pvr2 alleles segregating in the progeny. Among the 182 F2 plants, all of the 14 PVMV-resistant plants produced both a PCR fragment of 527 bp corresponding to the 82 nt deletion in elf(iso)4E and two fragments of 453 and 234 bp following MvnI digestion of the 687 bp PCR product obtained using elf4E cDNA-specific primers, a pattern specific for the pvr2 allele (Fig. 3a). In contrast, all PVMV-susceptible plants displayed PCR profiles producing at least one non-deleted elf(iso)4E cDNA fragment and/or at least one PCR fragment corresponding to pvr2 alleles distinct from pvr2 (Fig. 3a).

Segregation of the PCR marker based on the presence/absence of the 82 nt deletion in the elf(iso)4E cDNA was also assessed in the 22 DH lines segregating for pvr6. All of the DH lines known to be homozygous for pvr6 produced a PCR fragment of 609 bp, whereas all those homozygous for pvr6 produced a PCR fragment of 527 bp (Fig. 3b). Taken together, the observed co-segregation between pvr6 and the 82 nt deletion in the elf(iso)4E cDNA in both F2 and DH progeny strengthens the possibility that this locus corresponds to elf(iso)4E.

Transient expression in a PVMV-resistant genotype of either pvr6+ elf(iso)4E or pvr2+ elf4E restores PVMV susceptibility

Previously published results (Caranta et al., 1996) together with RT-PCR detection of PVMV RNA in inoculated leaves of C. annuum genotypes with distinct allele combinations at the pvr2 and pvr6 loci showed that complete resistance to PVMV resulted from the combined effect of the pvr6 and pvr2 resistance alleles. Genetic and sequence analysis also suggested that the pvr6 locus corresponded to elf(iso)4E and that the recessive resistance allele encoded a truncated protein lacking key domains for cap-binding activity. In order to confirm the involvement of the two cap-binding isomers in the pepper/PVMV interaction, elf(iso)4E and elf4E cDNAs from pvr6+ and pvr2+ genotypes were expressed independently in a PVMV-resistant genotype to check whether their expression could lead to loss of resistance. This was achieved through PVX-mediated transient expression of elf(iso)4E or elf4E in the resistant genotype DH801, followed by PVMV inoculation. Previous results have clearly demonstrated that PVX is an efficient expression vector in Capsicum (Ruffel et al., 2002). PVMV...
susceptibility was monitored using RT-PCR and DAS-ELISA. We first verified that PVX systemic infection of pepper genotypes DH801 and YW followed by inoculation with PVMV-IC did not modify the infectivity of PVMV-IC compared with plants inoculated with PVMV-IC alone (Table 2; Fig. 4, lanes 3 and 10).

To see whether PVMV infection of resistant DH801 plants could be supported by expression of eIF(iso)4E from the susceptible pvr6+ genotype, DH801 plants were inoculated with pPVX(iso)eYW and PVMV-IC. Of 40 double-inoculated leaves, eight displayed significant accumulation of PVMV CP and NIb-CP RNA (Table 2; Fig. 4, lane 14). Conversely, DH801 leaves inoculated with pPVX(iso)eP expressing eIF(iso)4E from resistant pvr6 plants and PVMV-IC did not support PVMV accumulation (Table 2; Fig. 4, lane 13).

To determine whether PVMV infection of resistant DH801 plants could also be supported by expression of eIF4E encoded by the pvr2+ susceptibility allele, DH801 plants were inoculated with pPVXeYW and PVMV-IC. Of 30 double-inoculated leaves, 13 displayed significant accumulation of PVMV CP and NIb-CP RNA (Table 2; Fig. 4, lane 12), whereas expression of eIF4E encoded by the pvr6 resistance allele did not support PVMV accumulation in DH801 plants (Table 2; Fig. 4, lane 11).

Thus, functional complementation experiments demonstrated that transient expression of the pvr6+-eIF(iso)4E or pvr2+-eIF4E allele in the resistant genotype DH801 resulted in loss of resistance to subsequent PVMV infection. These experiments confirmed that pvr6 corresponds to eIF(iso)4E and that both eIF4E and eIF(iso)4E wild type are susceptibility factors for PVMV infection. In three independent experiments, the expression of the resistance allele pvr2+-eIF4E or pvr6-eIF(iso)4E did not restore PVMV susceptibility, clearly indicating that loss of resistance results from amino acid changes identified in proteins encoded by resistance and susceptibility alleles.

Fig. 2. Alignment of nucleotide eIF(iso)4E open reading frame sequences (a) and predicted eIF(iso)4E protein sequences (b) from pepper genotypes homozygous for pvr6+ or pvr6 alleles. Genotypes homozygous for the pvr6+ susceptibility allele are YW, YY and F and genotypes homozygous for the pvr6 resistance allele are P and DH801. Black boxes indicate nucleotide differences observed between pvr6+ and pvr6 genotypes.
DISCUSSION

In pepper, resistance to PVMV is digenic and results from complementation between the pvr6 and pvr2 recessive resistance alleles (Caranta et al., 1996). pvr6 alone has no detectable effect on potyvirus infection, whereas pvr2 controls both PVY and TEV resistance. In line with previous results showing that resistance alleles at the pvr2 locus encode mutated versions of eIF4E, we investigated the involvement of the second cap-binding isoform, eIF(iso)4E, in PVMV resistance. Genetic mapping, sequence analysis and functional complementation assays demonstrated that pvr6 encodes eIF(iso)4E and that complete resistance to PVMV resulted from the combined effect of mutations in translation initiation factors eIF4E and eIF(iso)4E. This provides the first molecular interpretation of complementation for resistance between two naturally occurring recessive genes. The systematic analysis of alleles of the eIF4E gene family therefore appears to be a powerful means of characterizing molecularly the nature of recessive resistance genes towards poty- and related viruses.

In contrast to previously characterized naturally occurring recessive resistance genes against potyviruses, all of which depend on a discrete number of amino acid changes in eIF4E homologues, the pvr6 resistance allele was predicted to encode a truncated protein with only the N-terminal 29 aa. Because none of the features considered to be hallmarks of functional eIF4E proteins is present, including the eight conserved tryptophan residues, three of which are required for cap-binding activity (Marcotrigiano et al., 1997; Matsuo et al., 1997), we suggest that pvr6 encodes a non-functional cap-binding factor. Similarly to the Arabidopsis mutant line completely lacking both eIF(iso)4E mRNA and protein (Duprat et al., 2002), plants homozygous for pvr6 do not display any obvious growth and development phenotype, suggesting that this eIF(iso)4E activity is not essential. This is strengthened further by the fact that the pvr6 allele has been deployed in the field without any reduction in crop

Table 2. Number of PVMV-infected leaves/number of PVMV-inoculated leaves assessed by DAS-ELISA in a PVX-based transient expression assay

A leaf was considered to be infected when the DAS-ELISA absorbance value was at least three times greater than the mean value of the healthy controls (A405 ≥ 0.39 in these experiments).

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>C. annuum genotype</th>
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<tr>
<td></td>
<td>YW (pvr2+/pvr6*)</td>
</tr>
<tr>
<td>PVMV-IC</td>
<td>20/20</td>
</tr>
<tr>
<td>pPVX201* + PVMV-IC</td>
<td>20/20</td>
</tr>
<tr>
<td>pPVX(iso)eYW + PVMV-IC</td>
<td>20/20</td>
</tr>
<tr>
<td>pPVX(iso)eP + PVMV-IC</td>
<td>20/20</td>
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<tr>
<td>pPVXeYW + PVMV-IC</td>
<td>20/20</td>
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<tr>
<td>pPVXeF + PVMV-IC</td>
<td>20/20</td>
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*pPVX201, empty PVX vector; pPVX(iso)eYW, PVX with pvr6 - eIF(iso)4E cDNA from the YW genotype; pPVX(iso)eP, PVX with pvr6-eIF(iso)4E cDNA from the P genotype; pPVXeYW, PVX with pvr2 - eIF4E cDNA from the YW genotype; pPVXeF, PVX with pvr2-eIF4E cDNA from the F genotype.

Fig. 3. Genetic co-segregation between pvr6 and the B2 nt deletion in the eIF(iso)4E cDNA in two progeny pepper plants. (a) Genetic co-segregation in the F2 progeny segregating for both pvr6 and pvr2 recessive resistance alleles. The 14 PVMV-resistant plants produced a PCR fragment of 527 bp corresponding to the 82 nt deletion in eIF(iso)4E and two fragments of 453 and 234 bp following MvnI digestion of the 687 bp PCR product obtained using eIF4E cDNA-specific primers. This pattern is characteristic of pvr2 homozygous plants. PVMV-susceptible plants produced at least one non-deleted eIF(iso)4E fragment and/or a PCR pattern for pvr2-eIF4E corresponding to a homoygous susceptible or heterozygous state. (b) Genetic co-segregation in the DH progeny segregating for pvr6. A RT-PCR fragment of 609 bp was amplified in YW, F and the eight DH lines known to be homozygous for the susceptibility allele pvr6+ (6+), whereas a fragment of 527 bp was amplified in P and the 14 DH lines known to be homozygous for the resistance allele pvr6.
yield. Similarly, eIF4E1 also appears to be dispensable for normal *A. thaliana* growth (Yoshii et al., 2004). These observations reinforce the hypothesis that, despite having idiosyncratic properties, these factors can compensate for one another in cellular functions.

Interestingly, whereas stable antisense depletion of either eIF4E or eIF(iso)4E in tobacco had no obvious effect, depletion of both eIF4E and eIF(iso)4E resulted in plants with a semi-dwarf phenotype and an overall reduction in polyribosome loading, demonstrating that the two isoforms contribute additively to translation and plant growth (Combe et al., 2005). PVMV-resistant pepper genotypes probably combine a non-functional eIF(iso)4E and an eIF4E differing from the wild-type protein by three amino acid substitutions, V67E, L79R and D109N (Ruffel et al., 2002; this study). The D109N mutation affects a highly conserved residue involved in stabilization of cap binding (Marcotrigiano et al., 1997). The fact that PVMV-resistant plants are phenotypically normal in every respect suggests that point mutations at pvr62-eIF4E have no functional consequences for translation initiation *in planta*. This hypothesis is supported by the recent demonstration that pvr62-eIF4E mutations did not abolish *in vitro* cap-binding activity (Kang et al., 2005). However, amino acid changes in eIF4E proteins encoded by other resistance alleles against potyviruses in pea and pepper have been demonstrated to abolish cap-binding activity (Gao et al., 2004; Kang et al., 2005). Taken together, these observations are of particular interest for the management of eIF4E resistance factors in breeding programmes. The diversity of mutations in several eIF4E factors could be exploited for broad-spectrum resistance against poty- and related viruses if the cellular function of one or the other cap-binding isoforms is preserved.

Recent analysis of *Arabidopsis* mutant lines demonstrated that potyviruses differ in their ability to use eIF4E isoforms from a given host plant. The knockout mutation of the eIF(iso)4E gene prevents infection by TuMV, TEV and LMV (Duprat et al., 2002; Lellis et al., 2002) without preventing infection by CIYVV (Sato et al., 2005). Conversely, the knockout mutation of eIF4E1 allows TuMV but not CIYVV replication (Sato et al., 2005), indicating that potyviruses selectively use either eIF4E1 or eIF(iso)4E to infect *Arabidopsis*. This study therefore identifies a new situation regarding the specificity of use of eIF4E isoforms by potyviruses. PVMV, unlike other potyviruses, can use either eIF4E or eIF(iso)4E for pepper infection, indicating that the two isoforms are functionally interchangeable in this particular plant/potyvirus pair. The molecular mechanism that determines this specificity remains to be elucidated. From the well-documented ability of the potyviral Vpg to bind to eIF4E proteins (Schaad et al., 2000; Wittmann et al., 1997) and published data supporting a key role for the eIF4E–Vpg interaction with respect to the outcome of viral infection (Kang et al., 2005; Léonard et al., 2000), a simple hypothesis would be that this specificity relies on differential binding affinities between the potyviral Vpg and eIF4E isoforms. Yeast two-hybrid assays and *in vitro* assays of Vpg–eIF4E interactions support this hypothesis. In *Arabidopsis*, eIF(iso)4E is required for TuMV infection and eIF(iso)4E interaction with the TuMV Vpg is stronger than with eIF4E (Léonard et al., 2000). Similarly, in tomato, resistance against TEV is controlled by eIF4E (Ruffel et al., 2005) and yeast two-hybrid assays have identified interactions between the TEV Vpg and eIF4E and a failure to bind to eIF(iso)4E (J. L. Gallois & C. Caranta, unpublished data). However, in another system where eIF4E is known to be the resistance gene, no interaction between the viral avirulent determinant Vpg of PSBMV and eIF4E from a susceptible pea genotype was demonstrated (Gao et al., 2004). It is likely
that interaction of eIF4E and VPg in planta are influenced by interactions with other plant or viral proteins and nucleic acids. Taken together with our current results, these observations illustrate the diversity of molecular mechanisms underlying recessive resistance against potyviruses mediated by cap-binding factors.

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