Allele mining in the pepper gene pool provided new complementation effects between \textit{pvr2-eIF4E} and \textit{pvr6-eIF(iso)4E} alleles for resistance to pepper veinal mottle virus

Manuel Rubio,† Maryse Nicolas, Carole Caranta and Alain Palloix

Correspondence
Alain Palloix
alain.palloix@avignon.inra.fr

INRA, Centre d’Avignon, UR1052, Unité de Génétique et Amélioration des Fruits et Légumes, BP 94, 84143 Montfavet cedex, France

Molecular cloning of recessive resistance genes to potyviruses in a large range of host species identified the eukaryotic translation initiation factor 4E (eIF4E) as an essential determinant in the outcome of potyvirus infection. Resistance results from a few amino acid changes in the eIF4E protein encoded by the recessive resistance allele that disrupt the direct interaction with the potyviral protein VPg. In plants, several loci encode two protein subfamilies, eIF4E and eIF(iso)4E. While most eIF4E-mediated resistance to potyviruses depends on mutations in a single eIF4E protein, simultaneous mutations in eIF4E (corresponding to the \textit{pvr2} locus) and eIF(iso)4E (corresponding to the \textit{pvr6} locus) are required to prevent pepper veinal mottle virus (PVMV) infection in pepper. We used this model to look for additional alleles at the \textit{pvr2-eIF4E} locus that result in resistance when combined with the \textit{pvr6-eIF(iso)4E} resistant allele. Among the 12 \textit{pvr2-eIF4E} resistance alleles sequenced in the pepper gene pool, three were shown to have a complementary effect with \textit{pvr6-eIF(iso)4E} for resistance. Two amino acid changes were exclusively shared by these three alleles and were systematically associated with a second amino acid change, suggesting that these substitutions are associated with resistance expression. The availability of new resistant allele combinations increases the possibility for the durable deployment of resistance against this pepper virus which is prevalent in Africa.

INTRODUCTION

Improving the knowledge of the functional basis of resistance specificity and defence pathways provides increasing candidate sequences to explore available gene pools in cultivated plants and related species, but also enables further engineering of new resistance alleles and expansion of the gene pool for breeding (Michelmore, 2003). Moreover, functional markers that are derived from DNA polymorphisms directly responsible for the resistance phenotype provide optimal tools to track the resistance genes in breeding progenies (Andersen & Lübberstedt, 2003). However, relating phenotype variations to sequence information remains challenging when considering the major class of resistance genes encoding the nucleotide-binding sites–leucine rich repeat (NBS–LRR) proteins because of their great number in plant genomes, their organization in clusters and their sequence homology, which make the development of allele-specific amplification difficult (Hammond-Kosack & Parker, 2003).

Among resistance genes, the eukaryotic translation initiation factors eIF4E and eIF(iso)4E control conserved resistance mechanisms in several genera of RNA viruses and offer facilities for allele mining in plant gene pools and tracking targeted alleles in breeding programmes (Robaglia & Caranta, 2006). In eukaryotic cells, eIF4E binds to the m7GpppX cap as the first step in recruiting mRNA into the translation initiation complex. In plants, eIF4E belongs to a small multigenic family encoding two protein isoforms, eIF4E and eIF(iso)4E. Although these two isoforms are considered equivalent for \textit{in vitro} translation of some mRNA, they differ in their \textit{in vivo} expression pattern and show some specificity for different capped mRNAs (Gallie & Browning, 2001).

In a range of plant species, eIF4E-mediated resistance to viruses belonging to the genus \textit{Potyvirus} results from a small number of nonconservative amino acid changes in the protein encoded by the recessive resistance allele (Ruffel \textit{et al}., 2002; Robaglia & Caranta, 2006; Maule \textit{et al}., 2007 for review). These amino acid changes are clustered in two neighbouring regions at the surface of the predicted eIF4E 3D structure (Monzingo \textit{et al}., 2007). Resistance genes control multiple specificities targeted against distinct
potyviruses or pathotypes of the same virus and these specificities are explained by the occurrence of small allelic series where distinct alleles are represented as a combination of distinct amino acid substitutions. The exact mechanism by which eIF4E mutations control resistance remains to be elucidated, but protein–protein interaction studies have shown that the physical interaction between eIF4E and the potyviral protein VPg is required for viral infection, and that eIF4E mutations that cause resistance prevent VPg binding and inhibit the viral infectious cycle (Yeam et al., 2007; Charron et al., 2008). Reciprocally, the resistance breakdown by the virus results from amino acid substitutions in VPg that restore the physical interaction with the mutated eIF4E protein (Moury et al., 2004; Ayme et al., 2007; Charron et al., 2008), so that co-evolution between the two partners has led to the diversification of both genes.

The largest allelic series is found at the pvr2-eIF4E locus in pepper (Capsicum spp.), with 11 alleles of the eIF4E gene in which mutations have related resistance specificities to diverse strains of potato virus Y (PVY) and tobacco etch virus (TEV) strains (Charron et al., 2008; Kang et al., 2005a). Characterization of the molecular mechanisms underlying digenic recessive resistance to another pepper potyvirus pepper veinal mottle virus (PVMV) (Brunet et al., 1978) suggests a more complex pattern of interaction. Complete resistance to PVMV results from the combination of recessive alleles at two distinct loci: the pvr2-eIF4E locus on the pepper chromosome P4 and the pvr6 locus on the chromosome P3 that corresponds to the isoform eIF(iso)4E (Ruffel et al., 2006). The resistant allele pvr6-eIF(iso)4E differs from the susceptible one (pvr6+-eIF(iso)4E) by an 82 nt deletion and encodes a truncated protein with only the N-terminal 29 aa, suggesting a non-functional cap-binding factor. Up to now, only the pvr2-eIF4E allele, differing from the pvr2+-eIF4E allele by three amino acid substitutions, has been shown to complement pvr6-eIF(iso)4E for resistance to PVMV. The other pvr2 alleles have not been assessed for their complementary effect with pvr6 for PVMV resistance. In this context, new allele combinations would be useful, since the high prevalence of PVMV all over sub-Saharan Africa and the diversity of PVMV strains can threaten the durability of this unique digenic resistance source (Moury et al., 2005).

With the objective to look for new sources of resistance to PVMV, we generated all the possible combinations between the partially deleted pvr6-eIF(iso)4E allele and the 10 recessive pvr2-eIF4E alleles previously identified in pepper germplasm, and tested their complementation for PVMV resistance. Two new allele combinations were identified that provide alternatives to the single resistance source available up to now. Comparisons between pvr2-eIF4E protein sequences enabling or not the complementarity effect with pvr6-eIF(iso)4E brought insights into amino acid changes within the pvr2-eIF4E protein that determine PVMV resistance.

**METHODS**

**Plant material.** Eleven strains of *Capsicum* spp. were chosen for their original alleles at the pvr2-eIF4E locus (Table 1, Charron et al., 2008): *Capsicum annuum* genotypes ‘Yolo Wonder’, ‘Yolo Y’, ‘HD801’, ‘Perennial’, ‘PI322719’, ‘SC81’, ‘Marc 1’, ‘Serrano VC’, ‘PI195301’ and ‘Chile de árbol’, which are homozygous for the alleles pvr29, pvr2+, pvr2+, pvr2+, pvr2+, pvr2+, pvr2+ and pvr2+, respectively, and *Capsicum chinense* ‘PI195336’ homozygous for pvr1, which was shown to be a C. chinense original allele at the pvr2 locus (Ruffel et al., 2006; Kang et al., 2005b). All these genotypes are homozygous at the pvr6-eIF(iso)4E locus and possess the susceptible allele pvr6+, except ‘Perennial’ and ‘HD801’ which possess the recessive resistance allele pvr6.

In order to test the ability of the different pvr2-eIF4E alleles to confer resistance to PVMV when combined with pvr6-eIF(iso)4E, F2 progenies were produced between ‘Perennial’ and the genotypes representing the different pvr2-eIF4E haplotypes (Table 1). To check the combinations between pvr6-eIF(iso)4E and the C. chinense pvr1-eIF4E allele, breeding progenies previously developed to introduce PVMV resistance from HD801 into C. chinense cultivars were used: two plants, heterozygous pvr1/pvr2 and homozygous pvr6/pvr6, were selected using molecular markers described below from the F2 (‘HD801’ × ‘PI195336’) and BC1 (‘HD801’ × ‘PI195336’) × ‘HD801’ progenies, respectively (Table 1). These plants were self-pollinated to generate, respectively, F3 and BC1-II progenies segregating for the pvr1/pvr22 alleles.

**PVMV resistance evaluation.** Seeds were sown in disinfected peat and grown to the expanded cotyledon stage under greenhouse conditions and transferred into growth chambers before inoculation. Six to 20 plants for parental lines and 121–200 plants for segregating progenies were mechanically inoculated with the virus as described previously (Caranta & Palloix, 1996). The PVMV strain used originated from the Ivory Coast (PVMV-IC) (Moury et al., 2005). At 21–30 days post-inoculation (p.i.), systemic infection was assayed by the presence/absence of symptoms on non-inoculated leaves and confirmed by DAS-ELISA using specific PVMV antibodies, as described previously (Moury et al., 2005). Samples were considered positive when absorbance values were at least three times greater than the negative control.

**Amplification, sequencing and sequence analysis of eIF4E and eIF(iso)4E cDNAs and gDNAs.** Total RNA was isolated from pepper leaf tissues using the TRI-reagent protocol (Sigma-Aldrich). The eIF(iso)4E cDNA of 527 or 609 bp, depending on the presence/absence of the 82 bp deletion, was amplified by RT-PCR using the primers 5′-ATGGCCACCGAAGCACCACCGG-3′ (forward) and 5′-TCACACGGGTATCGGGTCCTTAGCT-3′ (reverse), as described by Ruffel et al. (2006).

DNA was extracted according to the micro-extraction method described by Fulton et al. (1995). Tetra-primer ARMS-PCR was used to differentiate the alleles pvr22, pvr2+, pvr2+ and pvr2+, as described by Rubio et al. (2008).

To distinguish pvr22 from pvr1, a cleaved amplified polymorphic sequence (CAPS) marker was used. The differential cleavage site revealed by MvlI was based on a single nucleotide polymorphism of T236G of cDNA that distinguishes pvr2 from the pvr1 allele [described by Ruffel et al. (2006)] after amplification of the 687 bp product with the eIF4E cDNA-specific primers (forward 5′-AAAAAGCACACAGCACCAAA-3′ and reverse 5′-TATTCGCCAGATTGC ATCAAGAA-3′).

To distinguish the allele pvr2 from the others, we analysed the sequence of the eIF4E cDNAs from the F2 ‘Perennial’ (pvr22) × ‘Serrano VC’ (pvr2), RT-PCR was performed using eIF4E...
cDNA-specific primers (forward primer 5’-AAAAGCACACACG- ACCACA-3’ and reverse primer 5’-TATTCCGACATTGCATCAA- GAA-3’). Each amplification product was sequenced by Genome- Express (Grenoble, France). Nucleotide sequences were aligned with the Chromas Lite 2.01 software (http://www.Technelysium.com.au). The nucleotide and amino acid sequences were analysed with the eIF4E

Among the seven F2 progenies, five were totally susceptible to PVMV (i.e. no resistant plants at 21 days p.i.) and only the F2 (‘Perennial’ × ‘Yolo Y’) and the F2 (‘Serrano VC’ × ‘Perennial’) segregated for resistance, with 9 and 14 plants, respectively, remaining healthy (i.e. no symptoms and ELISA-negative at 30 days p.i.). The observed ratios of 9R : 15S and 14R : 160S fitted well the ratio of 1R : 15S expected from the frequency of the double recessive resistant genotypes (\(\varphi^2=0.228\) P=0.63 and \(\varphi^2=0.958\) P=0.33, respectively). Because of the susceptibility of the parental lines, this suggested that PVMV resistance resulted from complementary effects between recessive alleles from both parental origins.

In the interspecific F3 and BC1-I1 progenies, different ratios between resistant and susceptible plants were expected, since these progenies do not segregate for the alleles at the pvr6 locus (the mother plants were homozygous for the recessive pvr6 allele), but segregate at the pvr2 locus, with the pvr2\(^6\) allele from HD801 which is already known to complement pvr6. Following the hypothesis that pvr1 does not complement with pvr6 for PVMV resistance, only the pvr2\(^6\) homozygous plants should display a PVMV-resistant phenotype resulting in a ratio of 1R : 3S. Following the hypothesis
that pvr1 and pvr6 have a complementary effect for PVMV resistance, all the plants (100%) should display a PVMV-resistant phenotype. The segregation ratio observed (Table 1) fitted the theoretical ratio of 1R : 3S for the BCI-I1 progeny (42R : 147S, χ² = 0.78, P = 0.378) but not for the F3 progeny (68R : 126S, χ² = 10.4, P = 0.0012), which showed an excess of resistant individuals. This suggests that pvr1 does not confer PVMV resistance when combined with pvr6 and that only the pvr6 × homozygous plants were resistant. The excess of resistant plants in the F3 progeny may result from skewed segregations that are commonly observed in interspecific crosses between C. annuum and C. chinense (Djian-Caporalino et al., 2006). However, the genotypes of the resistant plants remain to be assessed to confirm the hypotheses.

**Genotypes of the resistant plants**

To determine the alleles at the pvr2 and pvr6 loci that lead to PVMV resistance, all the resistant plants and samples of susceptible plants from the three progenies segregating for resistance were genotyped at these two loci.

Genotyping the pvr6 locus confirmed that all the resistant plants were homozygous for the recessive allele (pvr6/pvr6), with the characteristic 527 bp fragment that corresponds to the deleted allele of eIF(iso)4E (Fig. 1). All the individuals that were genotyped as heterozygous (pvr6/pvr6+) or homozygous (pvr6+ /pvr6+) at this locus were susceptible. A sample of these individual patterns is shown in Fig. 1. One plant (lane 7 in Fig. 1) was marked as susceptible despite its homozygous (pvr6/pvr6) genotype.

Considering the pvr2 locus, in the F2 progeny ('Perennial' × 'Yolo Y'), all of the nine resistant individuals were genotyped as homozygous for the pvr2+ allele. The individual that was marked as susceptible despite its homozygous (pvr6/pvr6) genotype (lane 7 in Fig. 1) was shown to be homozygous (pvr2+/pvr2+) (lane 11 in Fig. 2). In the F2 progeny ('Serrano VC' × 'Perennial'), the cDNA sequences from all of the 14 resistant plants were identical to the 'Serrano VC' allele pvr2 (Supplementary Fig. S1, available in JGV Online) and the amino acid sequences deduced from their cDNA sequence displayed the arginine substitution at position 79 instead of leucine for the pvr2 allele from 'Perennial'. In summary, all the PVMV-resistant plants were homozygous for the pvr6 and the pvr2+ or pvr2 alleles, whereas all the genotyped susceptible plants displayed heterozygosity at one of these loci or homozygosity for the pvr6+ or pvr2+ alleles.

Because of the unexpected segregation in the F3 interspecific progeny (‘HD801’ × ‘PI159336’), 93 individuals were randomly sampled in order to explore the frequency of their genotypes at the pvr2 locus (all the plants from this progeny are homozygous pvr6/pvr6). Thirty-seven of these individuals were resistant and 56 were susceptible. The 37 resistant individuals proved to be homozygous (pvr2+/pvr2+). Amongst the 56 susceptible individuals, 48 were heterozygous (pvr2+/pvr1) and 8 were homozygous (pvr1/pvr1). This confirmed that pvr1 did not complement pvr6 for resistance to PVMV. The genotypic ratios of 37 (pvr2+/pvr2+), 48 (pvr2+/pvr1) and 8 (pvr1/pvr1) also indicate a skewed segregation compared to the 1 : 2 : 1 ratio expected from the F3 progeny, with an excess of the resistant homozygous (pvr2+/pvr2+) genotype and a deficit in susceptible homozygous (pvr1/pvr1) genotype (χ² = 18.18, P = 0.000113).

**DISCUSSION**

Allele mining based on the knowledge of sequence diversity provided two new sources of resistance to PVMV in pepper...
and permitted the optimization of the exploitation of genetic resources for resistance traits resulting from complementation effects. This complementary effect was previously known between pvr6 from the C. annuum cultivar ‘Perennial’ and pvr2\textsuperscript{a} from ‘Florida VR2’ (Caranta et al., 1996; Ruffel et al., 2006). We extended this effect to two new gene combinations involving the same pvr6 allele and two new alleles: pvr2\textsuperscript{a} from ‘Yolo Wonder’ and pvr2\textsuperscript{b} from ‘Serrano VC’. Indeed, as 9 and 14 resistant plants, respectively, proved to be homozygous for both pvr6 and pvr2\textsuperscript{a} or pvr2\textsuperscript{b} alleles, the probability that these double-recessive genotypes do not control the target resistance is $P=\left(\frac{1}{16}\right)^9\cdot10^{-10}$ and $P=\left(\frac{1}{16}\right)^{14}\cdot10^{-16}$, respectively. The 10 pvr2 alleles tested for complementation with pvr6 were screened on the basis of their original sequence among a core collection of 25 pepper genotypes (Charron et al., 2008). This core collection itself resulted from a sampling of 25 individuals among the hundreds of strains of the INRA pepper germplasm, based on geographical origin and absence of parent relationships (Sage-Polloix et al., 2007). Looking for individual effects of these alleles, pvr1 confers resistance to the same strains of PVY and TEV as pvr2\textsuperscript{b} (Kang et al., 2005b), and may have been a good candidate, but it did not complement pvr6 for PVMV resistance. The pvr2\textsuperscript{a} allele displayed the same resistance spectrum as pvr2\textsuperscript{a}, pvr2\textsuperscript{b}, pvr2\textsuperscript{a} and pvr2\textsuperscript{b} (Charron et al., 2008) but only the former provides resistance to PVMV when combined with pvr6. Moreover, none of these alleles displays individual effects regarding PVMV resistance, since the parental lines are fully susceptible to this virus. The expected complementation effects between these alleles were not predictable from their individual effects, and a tremendous number of segregating progenies should be generated and tested to detect resistant combinations. The advantage of focussing research on alleles with distinct sequences is particularly significant in reducing the effort required to generate the allele combination for phenotype expression.

The recessive resistance allele pvr6-elf(iso)4E results from an 82 nt deletion in the elf(iso)4E cDNAs, leading to a truncated and non-functional elf(iso)4E protein (Ruffel et al., 2006). This deletion had no perceptible phenotypic effect, since the cultivar ‘Perennial’ displayed a normal phenotype, supporting the hypothesis that elf4E and elf(iso)4E proteins can compensate for one another in cellular functions (Duprat et al., 2002; Yoshii et al., 2004). However, combination with mutated forms of pvr2-elf4E might alter the plant phenotype, since simultaneous deletion of elf4E and elf(iso)4E in tobacco through antisense strategy resulted in plants with a semi-dwarf phenotype and an overall reduction in polysome loading, demonstrating that the two isoforms contribute additively to translation and plant growth (Combe et al., 2005). Such alterations were not expected in plants combining pvr6-elf(iso)4E with the C. annuum alleles pvr2\textsuperscript{a}–pvr2\textsuperscript{b} elf4E, since amino acid substitutions characterizing the proteins encoded by these elf4E alleles were shown not to impede essential elf4E functions, including those linked to mRNA translation (Charron et al., 2008). However, phenotype alterations were expected from the combination of pvr6-elf(iso)4E and pvr1-elf4E, since the amino acid substitution G107R, the signature of the pvr1-elf4E protein, abolished in vitro cap-binding activity (Kang et al., 2005b; Yeam et al., 2007). Despite the deficit in double-recessive homozygous genotypes in the F3 interspecific progeny (i.e. pvr1/pvr1 and pvr6/pvr6), the occurrence of both pvr1 heterozygous and homozygous plants indicated that the combination of both deleted isoforms is viable at the gamete level. Moreover, the plants’ phenotypes were not significantly affected, indicating that the allele combination is also viable at the zygote level. This lack of phenotype alteration may indicate that other elf4E and elf(iso)4E genes may compensate the deleted isoforms, since at least two elf4E genes and two elf(iso)4E genes were characterized in pepper (Charron, 2007). The possible number of combinations of mutated forms of elf4E and elf(iso)4E is larger than expected and a large range of allele combinations can be exploited to enlarge the spectrum and durability of resistance to potyviruses.

Compared with the elf4E protein encoded by the susceptible allele pvr2\textsuperscript{a}, only the three elf4E proteins that contribute to PVMV resistance, i.e. pvr2\textsuperscript{1}, pvr2\textsuperscript{2} and pvr2\textsuperscript{3}, share the two amino acid substitutions V67E and L79R (Table 2). V67E was also present in other elf4E proteins as a single substitution (pvr2\textsuperscript{1}) or associated with other substitutions (pvr2\textsuperscript{3}, pvr2\textsuperscript{5}), indicating that this substi-
Table 2. Amino acid substitutions observed between deduced eIF4E protein sequences from different C. annuum and C. chinense genotypes and their corresponding alleles

The resistance phenotypes of the lines were extracted from Charron et al. (2008). The extension of resistance to PVMV when the pvr2 allele is combined with the pvr6 allele is indicated in parentheses. The L79R substitution discussed in the text is indicated by bold type.

<table>
<thead>
<tr>
<th>Inbred line</th>
<th>Allele</th>
<th>Resistance phenotype</th>
<th>Position of aa substitution</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>51  66  67  68  71  73  74  79  107  109  205</td>
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<tr>
<td><strong>C. annuum</strong></td>
<td></td>
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<tr>
<td>Yolo Wonder</td>
<td>pvr21</td>
<td>Susceptible</td>
<td>T   P   V   A   K   A   A   L   G   D   D</td>
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<tr>
<td>Yolo Y</td>
<td>pvr22</td>
<td>PVY (+PVMV)</td>
<td>−   −   E   −   −   −   −   R   −   −   −</td>
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<tr>
<td>HD801</td>
<td>pvr23</td>
<td>PVY, TEV (+PVMV)</td>
<td>−   −   E   −   −   −   −   R   −   N   −</td>
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<tr>
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<td>pvr24</td>
<td>PVY</td>
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<tr>
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<td>PVY</td>
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<td>pvr26</td>
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<td>−   −   −   −   −   −   −   −   −   G   −</td>
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<tr>
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<td>pvr27</td>
<td>PVY</td>
<td>−   −   −   −   −   −   −   −   −   G   −</td>
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<tr>
<td>Serrano VC</td>
<td>pvr28</td>
<td>PVY (+PVMV)</td>
<td>−   −   E   −   −   −   −   −   −   −   G</td>
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<tr>
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<td>pvr29</td>
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<td>pvr30</td>
<td>PVY, TEV</td>
<td>A   T   −   −   R   −   −   −   R   −   −</td>
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<td><strong>C. chinense</strong></td>
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<tr>
<td>PI159336</td>
<td>pvr1</td>
<td>PVY, TEV</td>
<td>−   −   −   −   −   −   −   −   −   −   −</td>
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</table>

The resistance alone cannot be directly related to PVMV resistance. L79R was present only in the pvr21, pvr22 and pvr27 proteins but was always associated with the V67E substitution, meaning that its individual effect cannot be assessed. Both V67E and L79R are localized in region I of the core eIF4E protein, at the surface of the predicted 3D structure where several mutations were shown to disrupt the interaction with VPg from PVY and confer resistance to different strains of this virus (Robaglia & Caranta, 2006; Charron et al., 2008). The D205G substitution from pvr27, which is located in the C-terminal region of the protein and is shared with pvr21, pvr26 and pvr29, cannot be related to PVMV resistance. The D109N substitution is located in region II of the protein but is specific to the pvr22 protein. Its occurrence appears to be independent of the complementation with pvr6 for PVMV resistance. Thus, only the V67E and L79R substitutions in the pvr2 proteins can be closely associated with PVMV resistance.

The breakdown of eIF4E-mediated resistance by potyvirus alone cannot be directly related to PVMV resistance. L79R was present only in the pvr21, pvr22 and pvr27 proteins but was always associated with the V67E substitution, meaning that its individual effect cannot be assessed. Both V67E and L79R are localized in region I of the core eIF4E protein, at the surface of the predicted 3D structure where several mutations were shown to disrupt the interaction with VPg from PVY and confer resistance to different strains of this virus (Robaglia & Caranta, 2006; Charron et al., 2008). The D205G substitution from pvr27, which is located in the C-terminal region of the protein and is shared with pvr21, pvr26 and pvr29, cannot be related to PVMV resistance. The D109N substitution is located in region II of the protein but is specific to the pvr22 protein. Its occurrence appears to be independent of the complementation with pvr6 for PVMV resistance. Thus, only the V67E and L79R substitutions in the pvr2 proteins can be closely associated with PVMV resistance.

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