An eIF4E allele confers resistance to an uncapped and non-polyadenylated RNA virus in melon

Cristina Nieto1,3,‡, Monica Morales2,3,†,‡, Gisella Orjeda3,‡, Christian Clepet3, Amparo Monfort2, Benoît Sturbois2, Pere Puigdoménech4, Michel Pitrat5, Michel Caboche3, Catherine Dogimont5, Jordi García-Mas2, Miguel. A. Aranda1 and Abdelhafid Bendahmane3,*

1Centro de Edafología y Biología Aplicada del Segura (CEBAS)- CSIC, Apdo. correos 164, 30100 Espinardo, Murcia, Spain, 2Departament de Genètica Vegetal, Laboratori de Genètica Molecular Vegetal CSIC-IRTA, carretera de Cabrilts s/n, 08348 Cabrilts, Barcelona, Spain, 3Unité de Recherche en Génomique Végétale, 2, rue Gaston Crémières CP 5708, 91057 Evry Cedex, France, 4Departament de Genètica Molecular, Laboratori de Genètica Molecular Vegetal CSIC-IRTA, Jordi Girona 18-26, 08034 Barcelona, Spain, and 5INRA, Unité de Génétique et Amélioration des Plantes, BP 94, Montfavet, F-84143, France

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*For correspondence (fax +33 160874510; e-mail bendahm@evry.inra.fr).
†Present address: Department of Disease and Stress Biology and Molecular Microbiology, John Innes Center, Norwich NR4 7UH, UK. ‡These authors contributed equally to this work.

Summary

The characterization of natural recessive resistance genes and virus-resistant mutants of Arabidopsis have implicated translation initiation factors of the 4E family [eIF4E and eIF(iso)4E] as susceptibility factors required for virus multiplication and resistance expression. To date, viruses controlled by these genes mainly belong to the family Potyviridae. Melon necrotic spot virus (MNSV) belongs to the family Tombusviridae (genus Carmovirus) and is an uncapped and non-polyadenylated RNA virus. In melon, nsv-mediated resistance is a natural source of recessive resistance against all strains of MNSV except MNSV-264. Analyses of chimeras between non-resistance-breaking and resistance-breaking strains have shown that the avirulence determinant maps to the 3′-untranslated region (3′-UTR) of the viral genome. Using a combination of positional cloning and microsynteny analysis between Arabidopsis thaliana and melon, we genetically and physically delimited the nsv locus to a single bacterial artificial chromosome clone and identified the melon eukaryotic translation initiation factor 4E (Cm-eIF4E) as a candidate gene. Complementation analysis using a biolistic transient expression assay, confirmed Cm-eIF4E as the product of nsv. A single amino acid change at position 228 of the protein led to the resistance to MNSV. Protein expression and cap-binding analysis showed that Cm-eIF4E encoded by a resistant plant was not affected in it’s cap-binding activity. The Agrobacterium-mediated transient expression of the susceptibility allele of Cm-eIF4E in Nicotiana benthamiana enhanced MNSV-264 accumulation. Based on these results, a model to explain melon resistance to MNSV is proposed. These data, and data from other authors, suggest that translation initiation factors of the eIF4E family are universal determinants of plant susceptibility to RNA viruses.

Keywords: Carmovirus, avirulence determinant, map-based cloning, eIF4E, microsynteny, cap-binding.

Introduction

Plant viruses are obligate parasites that multiply within their hosts by establishing specific interactions between viral factors and macromolecules, structures and processes of the plant, which determine the plant susceptibility to viral infection (Carrington and Whitham, 1998; Maule et al., 2002). Either a mutation or the loss of one such susceptibility factor may result in resistance to the virus. In this case, resistance is expected to be genetically recessive (Fraser, 1990, 1999). The vast majority of the defined recessive resistances operate against viruses belonging to the Potyviridae.
family (Diaz-Pendon et al., 2004), and the characterization of some natural recessive resistance genes (Gao et al., 2004; Kang et al., 2005a; Kanyuka et al., 2005; Nicaise et al., 2003; Ruffel et al., 2002, 2005; Stein et al., 2005) and mutagenesis of model hosts (Duprat et al., 2002; Lellis et al., 2002) have implicated translation initiation factors of the 4E family [eIF4E and eIF(iso)4E] as susceptibility factors required for virus multiplication (reviewed in Robaglia and Caranta, 2006). In addition, recessive ethyl methane sulfonate-induced mutations controlling Arabidopsis resistance to Cucumber mosaic virus (CMV, genus Cucumovirus) and Turnip crinkle virus (TCV, genus Carmovirus) have also been shown to correspond to eIF4E and eIF4G, respectively (Yoshii et al., 2004). Recently, the role of eIF(iso)4G as a natural resistance gene in rice against Rice yellow mottle virus (RYMV, genus Sobemovirus) has been characterized (Albar et al., 2006).

Factor eIF4E has an essential role in the initiation step of protein synthesis. Efficient translation of mRNAs is thought to occur in a closed-loop format, in which the 5′- and 3′-termini are brought together through interactions with translation initiation factors (reviewed in Kawaguchi and Rochon, 2006). Interestingly, only a small proportion of positive-strand RNA plant viruses have genomic and subgenomic mRNAs structured like host mRNAs, with a 5′-cap and a poly(A) tail, and often lack either one or both of these features (Fauquet et al., 2005). For example, viruses of the family Potyviridae have a poly(A) tail at the 3′-end of the genomic RNA, but instead of a 5′-cap structure they contain a 5′-terminal genome-linked protein (VPG) covalently attached to the 5′-end of the viral RNA.

Regions of the potyviral VPG have been mapped as avirulence determinants for several eIF4E-mediated resistances (Borgstrom and Johansen, 2001; Kang et al., 2005b; Moury et al., 2004). It has also been shown for several potyviruses that the VPG can bind to either eIF4E or to its isoform eIF(iso)4E in yeast two-hybrid and in vitro binding assays (Kang et al., 2005a; Leonard et al., 2000; Schaad et al., 2000; Wittmann et al., 1997), and at least in one of these cases this interaction correlates with virus infectivity (Leonard et al., 2000). In addition, mutations in eIF4E always resulted in amino acid changes near the cap-binding region of the protein for all natural recessive resistance genes that have been characterized to date (reviewed in Robaglia and Caranta, 2006). All these data suggest that a physical interaction between eIF4E and the potyviral VPG is necessary for viral infection, with VPG perhaps acting as a cap mimic that serves for the recruitment of the translation initiation complex to the potyviral RNA, thus facilitating cap-independent translation (Schaad et al., 2000). However, other possibilities have also been proposed, such as a role for eIF4E in the cellular localization of viral complexes (Gao et al., 2004).

The melon resistance to Melon necrotic spot virus (MNSV) is among the few identified monogenic recessive resistances against a non-potyvirus. MNSV belongs to the genus Carmovirus (Fauquet et al., 2005) and is endemic in cucurbit crops worldwide. MNSV has a single-stranded positive sense RNA genome of 4.3 kb, which contains at least five open reading frames (Diaz et al., 2004; Riviere and Rochon, 1990). The genomic and subgenomic MNSV RNAs lack both a 5′-cap structure and a 3′-poly(A) tail. There are at least two known sources of resistance to MNSV in melon: the cultivar ‘Gulfstream’ and the Korean accession PI 161375, both controlled by the recessive gene nsv (Coudriet et al., 1981). The nsv gene is effective against all known strains of the virus except for the recently described MNSV-264 strain (Diaz et al., 2004). Analyses of protoplasts of susceptible and resistant melon cultivars inoculated with MNSV have shown that the resistance trait conferred by this gene is expressed at the single-cell level (Diaz et al., 2004). Interestingly, studies performed with chimeras between MNSV-264 and a non-resistance breaking strain have shown that the viral avirulence determinant corresponding to nsv is located at the 3′-untranslated region (3′-UTR) of the virus genome (Diaz et al., 2004).

Recently, we obtained a high-resolution genetic map in the nsv locus and identified a single bacterial artificial chromosome (BAC) clone 1-21-10 containing the resistance gene (Morales et al., 2005). In this paper, we describe the characterization of the nsv locus using a combination of positional cloning and microsynteny analysis of Arabidopsis and melon. We demonstrate that the nsv gene encodes the melon eIF4E (Cm-eIF4E). We also show that a single amino acid change at position 228 of the protein led to the resistance to MNSV. Moreover, protein expression and cap-binding analysis indicated that Cm-eIF4E encoded by a resistant plant was not affected in its cap-binding activity. All together, we demonstrated that a recessive resistance mediated by eIF4E is active against a non-potyviridae, uncapped and non-polyadenylated virus. This resistance also involves the non-coding 3′-end region of the viral RNA as an avirulence determinant.

Results

Markers tightly linked to the nsv locus in melon match sequences on Arabidopsis thaliana chromosome 4

The nsv gene was previously mapped to linkage group 11. A single ~100-kb BAC clone (1-21-10) from the susceptible genotype that physically contained the resistance gene was also identified using two mapping populations with more than 3000 segregating individuals (Morales et al., 2005). To speed up the isolation of the nsv locus, we used a combination of positional cloning and synteny analysis between melon and Arabidopsis. Based on previous work showing that sequence co-linearity between these two species may be limited to small intervals (van Leeuwen et al., 2003), we hypothesized that markers that are tightly linked to nsv in
melon should match a single genomic region in Arabidopsis.

Markers linked to nsv derived from BAC-end sequences and spanning an 8.7-cM genetic interval were compared using BLAST analysis allowing the identification of a region in Arabidopsis chromosome 4 as the most probable nsv syntenic region (Table 1). Among the nsv most tightly linked markers there were two, 1R3 and 1L3, which mapped within an 182-kb Arabidopsis genomic region located between genes At4g17770 and At4g18100 (Figure 1). Additional melon BAC-ends also showed homologies with Arabidopsis genes in this region, but with lower E-values. A search was performed for genes annotated on the Arabidopsis BACs corresponding to the gene region depicted in Figure 1 in order to find a possible candidate gene for nsv. The homology with a higher E-value was found between BAC-end 1R3 (from BAC 1-21-10) and Arabidopsis BAC F15J15 (E-value = 3 × 10⁻⁵⁷). Notably, the list of annotated genes from BAC F15J15 displayed elf4E at position 18040.

Based on the alignment of the elf4E proteins from pepper (AY122052), tomato (AF259801) and Arabidopsis (AY093750), a pair of degenerate primers was designed from two conserved motifs. After the amplification of melon genomic DNA two PCR products were obtained, a band of 520 base pair (bp) corresponding to the isoform elf(iso)4E and a band of 1900 bp corresponding to elf4E (Cm-elf4E). Specific primers were designed based on the Cm-elf4E partial sequence, and when tested in the BAC contig (Figure 1) we obtained positive amplifications in BACs 52K20 and 1-21-10, confirming the presence of Cm-elf4E within this region. After sequencing PCR products for Cm-elf4E alleles from the parental lines PI 161373, ‘Piel de sapo’ and ‘Védraintais’, two cleaved amplified polymorphism markers were developed for the two mapping populations used in the high-resolution mapping, the F₂ (PI 161375 × ‘Piel de sapo’) and the BC₁ ((Védraintais × PI 161375) × PI 161375) (Morales et al., 2005). Marker M-Cm-elf4E was tested in the recombinant individuals of both mapping populations and a perfect co-segregation with nsv was found in more than 3000 F₂ and BC₁ individuals.

A single amino acid change in Cm-elf4E correlates with nsv resistance

Full-length Cm-elf4E cDNAs were cloned and sequenced from melon cultivar Védraintais (Ved) (Cm-elf4E-Ved, GenBank accession no. DQ393831) and PI 161375 (P₁) (Cm-elf4E-P₁, GenBank accession no. DQ393830), which were homozygous for the dominant and the recessive allele at the nsv locus, respectively. The cDNAs obtained were 1153 bp in length with a 5’-UTR of 122 bp, a coding region of 708 bp and a 3’-UTR of 323 bp. Cm-elf4E encodes a protein of 235 amino acids. Cm-elf4E amino acid sequence similarity was highest (91%) with the pea homologue, Ps-elf4E (GenBank accession no. AY423375). Amino acid similarities

Table 1 BAC-end sequences tightly linked to the nsv locus in melon match sequences on Arabidopsis thaliana chromosome 4

<table>
<thead>
<tr>
<th>nsv-linked markers</th>
<th>Predicted gene function</th>
<th>BLAST Hit</th>
<th>E-value</th>
</tr>
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<tr>
<td>38B12U</td>
<td>Hypothetical protein</td>
<td>At4g18880</td>
<td>0.56</td>
</tr>
<tr>
<td>38B12sp6</td>
<td>Putative protein</td>
<td>At4g18220</td>
<td>0.38</td>
</tr>
<tr>
<td>41I23sp6</td>
<td>Unknown protein</td>
<td>At4g18260</td>
<td>8 × 10⁻¹⁴</td>
</tr>
<tr>
<td>5A6U</td>
<td>Translation initiation factor elf4E</td>
<td>At4g18040</td>
<td>0.14</td>
</tr>
<tr>
<td>7K20U</td>
<td>Putative protein</td>
<td>At4g18810</td>
<td>8 × 10⁻¹²</td>
</tr>
<tr>
<td>7K20sp6</td>
<td>LRR protein</td>
<td>At4g20140</td>
<td>7 × 10⁻⁰⁴</td>
</tr>
<tr>
<td>34H17U</td>
<td>Disease resistance protein</td>
<td>At4g19510</td>
<td>1.4</td>
</tr>
<tr>
<td>34H17sp6</td>
<td>Putative protein</td>
<td>At4g19650</td>
<td>0.28</td>
</tr>
<tr>
<td>5B3U</td>
<td>Family 17-hydrolase glycosyl</td>
<td>At4g18340</td>
<td>0.39</td>
</tr>
<tr>
<td>9N8U</td>
<td>Putative protein</td>
<td>At4g17910</td>
<td>0.15</td>
</tr>
<tr>
<td>9N8sp6</td>
<td>Li3 protein</td>
<td>At4g17600</td>
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</tr>
<tr>
<td>1L3b</td>
<td>6-phosphate threolase synthase</td>
<td>At4g17770</td>
<td>2 × 10⁻⁰⁶</td>
</tr>
<tr>
<td>1R3b</td>
<td>L32 Ribosomal protein</td>
<td>At4g18100</td>
<td>3 × 10⁻⁴⁷</td>
</tr>
</tbody>
</table>

*aObtained performing the blastx and tblastx search.

*bMarkers with significant hits in the Arabidopsis region are in bold.

Figure 1. Genetic dissection of the nsv locus in melon and the orthologous region in Arabidopsis.

(a) Genetic and physical map of the nsv locus in melon. The genetic map is based on the analysis of more than 3000 Nsv-segregating plants. The Nsv locus physical map is based on the screening of two bacterial artificial chromosome libraries from melon of Nsv/Nsv and nsv/nsv genotypes. Only the minimal tiling path is shown. Markers derived from BAC clones were anchored to the genetic map (Morales et al., 2005).

(b) The Nsv orthologous region in Arabidopsis. Numbers above the genes indicate the Arabidopsis Genome Initiative (AGI) code number. The broken lines indicate physical distances and are not to scale. The dotted lines indicate Nsv-linked markers showing a significant homology with Arabidopsis genes.
with the Arabidopsis factors At-eIF4E (EMBL accession no. Y10548) and At-eIF(iso)4E (EMBL accession no. Y10547) were 88% and 68%, respectively. As eIF4E belongs to a small multigenic family (Rodriguez et al., 1998), it was necessary to demonstrate that the cloned Cm-eIF4E cDNAs corresponded to the M-Cm-eIF4E marker co-segregating with the nsv locus.

Cm-eIF4E genomic DNA was cloned and sequenced. The gene contained four introns and five exons (Figure 2). A sequence alignment between marker M-Cm-eIF4E and Cm-eIF4E genomic sequence showed that the former overlaps exon 1, intron 1 and a part of exon 2 of Cm-eIF4E (data not shown).

Sequence comparison of Cm-eIF4E proteins from the resistant (PI) and the susceptible (Ved) cultivars revealed a single amino acid substitution at position 228. The susceptible genotype carries a Histidine and the resistant genotype carries a Leucine (Figure 2). A single nucleotide polymorphism (SNP) led to this amino acid change in the protein. A molecular marker was derived from the SNP and mapped relative to the nsv locus in a mapping population of more than 3000 segregating plants. No recombination events were identified between MNSV resistance and the SNP. We also analysed the conservation of the SNP in 13 MNSV-resistant and -susceptible melon accessions. In this analysis we identified a perfect association between the SNP and the resistance to MNSV (Table 2).

Expression of Cm-eIF4E-Ved in resistant melon complements MNSV infection

To demonstrate that the nsv locus codes for an allele of Cm-eIF4E, we developed a transient expression assay based on microprojectile bombardment (McCabe et al., 1988). Because nsv-mediated resistance is recessive, we predicted that the co-bombardment of the susceptibility allele of Cm-eIF4E with the non-resistance breaking strain of MNSV in resistant plants would complement virus accumulation. The constructs used in this assay comprised full-length infectious clones for resistance breaking (RB) and non-resistance breaking (NRB) MNSV strains (Diaz et al., 2004) and Cm-eIF4E alleles under the control of the Cauliflower mosaic virus (CaMV) 35S promoter (Figure 3a).

MNSV constructs were separately coated onto gold particles and then bombarded into the leaves of susceptible and resistant melons. Virus accumulation was assessed using RT-PCR and northern-blot hybridization at 2 days post bombardment. As expected, both strains accumulated in susceptible melon leaves and only the RB strain accumulated in the leaves of resistant plants (data not shown).

For the complementation experiment, the construct pBM5 was co-bombarded with either pB4E-Ved and pB4E-PI constructs into the leaves of resistant and

<table>
<thead>
<tr>
<th>Melon cultivars</th>
<th>Inoculation with MNSV-264</th>
<th>Inoculation with MNSV-M5</th>
<th>aa at position 228</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI 161375</td>
<td>S*</td>
<td>R*</td>
<td>T</td>
</tr>
<tr>
<td>Gulfstream</td>
<td>S</td>
<td>R</td>
<td>T</td>
</tr>
<tr>
<td>Planters Jumbo</td>
<td>S</td>
<td>R</td>
<td>T</td>
</tr>
<tr>
<td>Eros</td>
<td>S</td>
<td>R</td>
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<tr>
<td>Pepe</td>
<td>S</td>
<td>R</td>
<td>T</td>
</tr>
<tr>
<td>Quito</td>
<td>S</td>
<td>R</td>
<td>T</td>
</tr>
<tr>
<td>C-178</td>
<td>S</td>
<td>R</td>
<td>T</td>
</tr>
<tr>
<td>C-105</td>
<td>S</td>
<td>S</td>
<td>A</td>
</tr>
<tr>
<td>Doublon</td>
<td>S</td>
<td>S</td>
<td>A</td>
</tr>
<tr>
<td>MR-1</td>
<td>S</td>
<td>S</td>
<td>A</td>
</tr>
<tr>
<td>Seminole</td>
<td>S</td>
<td>S</td>
<td>A</td>
</tr>
<tr>
<td>Vedrantais</td>
<td>S</td>
<td>S</td>
<td>A</td>
</tr>
<tr>
<td>WMR-29</td>
<td>S</td>
<td>S</td>
<td>A</td>
</tr>
</tbody>
</table>

*S, susceptible to MNSV; R, resistant to MNSV.
susceptible plants. As above, virus accumulation was assayed using RT-PCR and northern-blot hybridization at 2 days post bombardment (Figure 3b,c). In this analysis, MNSV-Mx5 accumulated in susceptible leaves independently of the Cm-eIF4E co-bombarded allele. In contrast, in leaves from resistant plants, virus accumulation was observed only when p8Mx5 was co-bombarded with pB4E-Ved, the construct carrying the allele derived from the susceptible genotype. As it was expected, all co-bombardments of pB264 with either pB4E-PI or pB4E-Ved in either resistant or susceptible leaves resulted in virus multiplication (data not shown). These results demonstrated that the nsv locus codes for an elf4E factor, and that expression of the Cm-eIF4E-Ved allele in resistant melon is both necessary and sufficient to restore susceptibility to the NRB strain of MNSV.

Expression of Cm-eIF4E-Ved in Nicotiana benthamiana enhances virus accumulation

Previously it was demonstrated that the ability of MNSV-264 to infect melon of the nsv genotype and N. benthamiana maps to the 3'-UTR of the viral genomic RNA. In these analyses, chimeras between RB and NRB viruses harbouring MNSV-264 3'-UTR were able to infect N. benthamiana and melon of the nsv genotype. In contrast, chimeras harbouring MNSV-Mx5 3'-UTR were unable to infect either N. benthamiana or melon of the nsv genotype (Diaz et al., 2004). Based on this, we hypothesized that the melon and N. benthamiana resistances to MNSV could be similarly controlled, perhaps being the result of an elf4E amino acid mutation conserved in both species.

Using primers specific for Nt-elf4E (GenBank accession no. AY702653), we RT-PCR amplified the elf4E N. benthamiana homologue, Nb-elf4E (GenBank accession no. DQ393833). Figure 4 shows the sequence alignment of elf4E proteins derived from N. benthamiana, melon and other plant species. Only the part of the protein around amino acid 228 responsible for virus resistance in melon is shown. Interestingly, N. benthamiana and melon of the nsv genotype carry Alanine and Leucine at position 228, respectively, which are both neutral and non-polar amino acids. At the corresponding position, susceptible melon carries a Histidine, a basic and polar amino acid. The other species shown in the alignment are non-hosts of MNSV and carry either Asparagine or Serine at the same position, both of which are neutral and polar amino acids.

To test whether the identity of amino acid 228 controls the virus accumulation in N. benthamiana, as it does in melon, we developed a complementation assay using Agrobacterium tumefaciens transient expression. The system is based on the co-expression of elf4E alleles and NRB and RB strains of MNSV in N. benthamiana leaves. The constructs used in this assay comprised pB4E-PI and pB4E-Ved, used in the bombardment expression assay, as well as pB4E-Nb expressing Nb-elf4E. We predicted from this expression assay that the overexpression of an elf4E allele is likely to replace the endogenous elf4E protein. Virus accumulation was assessed by a northern blot at 4 days post-agroinfiltration.

MNSV-Mx5 was unable to accumulate in N. benthamiana leaves in combination with any of the elf4E alleles (Figure 5). Our interpretation of these data was that the expression of Cm-eIF4E-Ved controlling the susceptibility in melon is not sufficient to confer susceptibility to MNSV-Mx5 in N. benthamiana. N. benthamiana may lack another factor required for MNSV-Mx5 infection. As it was expected, MNSV-264 accumulated in N. benthamiana leaves independently of the co-expressed elf4E alleles. However, the level of virus accumulation was dependent on the identity of the agroinfiltrated elf4E allele. There was eight-fold MNSV-264 RNA accumulation when the virus was co-agroinfiltrated
with the Cm-eIF4E-Ved allele. No difference was observed when the virus was co-agroinfiltrated with GFP either as control or with the other eIF4E alleles (Figure 5). We interpreted these data as indicating that MNSV-264 either prefers to use the Cm-eIF4E-Ved allele or is unable to use the other co-expressed allele, such as Cm-eIF4E-PI that differs only by one amino acid substitution.

Mutation His228Leu does not affect Cm-eIF4E cap-binding activity

The His228Leu mutation is localized in the C-terminal arm of the protein, far from the conserved cap-recognition pocket; however, when the crystal structure of human eIF4E was determined, and complexed with m7GpppA instead of m7GTP, it was shown that this region also has an important role for cap-binding and the functional regulation of the protein (Tomoo et al., 2002). A modelling of Cm-eIF4E based on the crystal structure of human eIF4E identified His228 on an accessible region of the protein (data not shown). To analyze the consequence of the His228Leu mutation of Cm-eIF4E for cap-binding activity, the Cm-eIF4E proteins from resistant and susceptible alleles were first stably expressed in Escherichia coli; the two proteins showed similar levels in crude extracts (Figure 6a). The Cm-eIF4Es from PI and Ved could both be purified on m7G-sepharose matrix, indicating their ability to bind to a cap analogue in vitro. The cap-binding ability of the two proteins was then compared in a
quantitative assay using the m^7G-affinity column. The Ved and PI Cm-ef4E purified fractions were dialyzed to eliminate the m^7GDP-cap analogue from the buffer, and defined quantities of the proteins were sequentially loaded on the column and eluted. The similarity of the chromatograms (Figure 6b) and the comparable quantities of eluted proteins (Figure 6c) obtained with both elF4E variants indicated no major differences regarding their affinity for the cap. Therefore the mutation His228Leu conferring the resistance to MNSV does not affect the Cm-elF4E cap-binding ability.

Discussion

We were interested in characterizing the melon nsv gene because it is among the few natural defined recessive resistance genes that are effective against a non-potyviridae (Diaz et al., 2004; Diaz-Pendon et al., 2004). Through a map-based cloning strategy and microsynteny analysis we were able to identify Cm-elF4E as a candidate gene for nsv, and transient complementation analysis provided the confirmation that nsv indeed codes for this translation factor.

The role that microsynteny with Arabidopsis had in this case for the identification of a candidate gene for nsv is remarkable. In a previous study, in melon linkage group 4, near an R-gene cluster, some degree of localized synteny and co-linearity was found between two Arabidopsis duplicated regions and melon (van Leeuwen et al., 2003); it was concluded that diverse mechanisms of gene reshuffling acted during evolution to separate the two species. Microsynteny has been reported between distant species like Arabidopsis and tomato near the ovate gene, the lateral suppressor region and the Diageotropica gene (Ku et al., 2000; Oh et al., 2002; Rossberg et al., 2001). In some of these examples, comparative mapping between tomato and Arabidopsis facilitated the positional cloning of the genes of interest (Liu et al., 2002; Oh et al., 2006). Microsynteny has also been reported between Arabidopsis and legume species (Mudge et al., 2005). Recent data obtained after sequencing BAC 1-21-10 suggests that there is some degree of gene conservation between the melon nsv region and Arabidopsis and Medicago truncatula (Garcia-Mas, unpublished results).

Cloning and sequencing Cm-elF4E cDNAs from susceptible and resistant melon genotypes showed little variation for this gene in melon. However, the importance of the single amino acid change (from His228 to Leu228) correlating with the resistance phenotype was confirmed by the complementation of virus multiplication in resistant genotypes with the corresponding opposite allele. Despite the dramatic change of phenotype that this amino acid change confers regarding virus multiplication, it does not seem to have any effect on the ability of Cm-elF4E to bind m^7GTP in vitro. In the case of potyviridae resistance mediated by elF4E, non-conservative amino acid substitutions implicated in resistance occur in two regions of the elF4E 3D structure located near the predicted cap-recognition pocket of the protein, in close proximity to highly conserved residues involved in cap binding (reviewed in Robaglia and Caranta, 2006). Only some of the elF4E variants encoded by alleles
confering potyvirus resistance showed reduced in vitro m7GTP affinity (Gao et al., 2004; Kang et al., 2005a,b).

Collectively, data on natural recessive resistance genes (Gao et al., 2004; Kang et al., 2005a; Kanyuka et al., 2005; Nicaise et al., 2003; Ruffel et al., 2002, 2005; Stein et al., 2005 and this report) and on Arabidopsis mutants (Duprat et al., 2002; Lellis et al., 2002; Yoshii et al., 2004) point towards a central function for the eIF4E family of translation factors on plant virus multiplication. In the host cell, eIF4E is a part of the eIF4F protein complex, which has an essential role in the initiation step of cap-dependent mRNA translation. In eukaryotes, most cellular RNAs contain both a 5'cap and a 3'poly(A) tail, and these terminal structures act synergistically to stimulate translation. This translational enhancement depends on a protein bridge formed between these two structures by eIF4E binding to the 5'cap, poly(A) binding protein (PABP) binding to the poly(A) tail, and eIF4F binding simultaneously to both of these proteins, thereby forming a closed loop. This closed loop is a requisite for efficient translation initiation of most mRNAs, as it seems to enhance the recruitment of the 43S ribosomal initiation complex to the 5'untranslated region (5'-UTR) of the cellular transcripts (reviewed in Kawaguchi and Bailey-Serres, 2002). Significantly, positive-sense single-stranded RNA viruses often lack the 5' cap, the poly(A) tail or both of these structures, yet they need to use the host translational machinery to translate their mRNAs. A diverse array of strategies have either been shown or proposed to be used by viral genomes to circumvent this problem (Dreher and Miller, 2006).

For members of the family Potyviridae, it is not yet clear how eIF4E functions in conferring host susceptibility. Experimental data on genetic and physical interactions between potyviral VPg and eIF4E suggest that VPg probably functions as a 5'-cap substitute recruiting the translation initiation complex through interaction with either eIF4E or eIF(iso)4E (Kang et al., 2005a; Schaad et al., 2000). However, arguments exist against this hypothesis (Dreher and Miller, 2006), and other possible roles for eIF4E during the potyviral life cycle have been proposed: the VPg-eIF4E interaction may inhibit the cap-dependent translation of hosts mRNAs, freeing ribosomes for viral RNA translation (Dreher and Miller, 2006), it may play a role during genome replication (Robaglia and Caranta, 2006) and/or it may be relevant to other steps of the viral infection cycle, such as facilitating the cell-to-cell movement of viral RNA (Gao et al., 2004).

Members of the plant virus family Tombusviridae, to which MNSV belongs, neither posses a 5'cap nor a poly(A) tail. For some of them the expression of viral RNAs has been studied in some detail, showing that they carry out cap-independent translation via structural RNA elements in their 3'-UTRs, and that they do not utilize internal ribosome entry (Koh et al., 2002; Meulewaeter et al., 2004; Mizumoto et al., 2003; Qu and Morris, 2000; Shen and Miller, 2004; Timmer et al., 1993). Similarly, mRNAs of Barley yellow dwarf virus (BYDV; family Luteoviridae), another uncapped and non-polyadenilated plant virus, are able to form a closed loop by direct base-pairing of a stem loop in the 3'-UTR with a stem loop in the 5'-UTR, and this base-pairing allows a sequence in the 3'-UTR to confer translation initiation at the 5' proximal AUG (Guo et al., 2001). Moreover, the cap-independent translation of Satellite tobacco necrosis virus (STNV) is facilitated by an interaction between the 5'-UTR and a ~100-nucleotide translational enhancer (TED) located at the 3'-UTR. Translation of STNV RNA without a functional TED can be restored in vitro by the addition of a 5' cap, and the STNV TED is a potent inhibitor of in vitro translation when added in trans. Additionally, STNV TED RNA is able to bind to eIF4E and eIF(iso)4F complexes and their subunits (including eIF4E and eIF(iso)4E), and all these data suggest that the STNV TED RNA is a functional mimic of a 5'-cap group (Gazo et al., 2004). Interestingly, we have been able to show that the genetic determinant of the MNSV ability to overcome nsv (avr determinant) is located at the 3'-UTR of the viral genome (Diaz et al., 2004) in a region of less than 100 nucleotides, which has the potential to form a structurally conserved stem loop (Truniger, Nieto and Aranda, unpublished). In addition, we have been able to identify in the MNSV 3'-UTR, outside of the avr determinant, nucleotide stretches with complementarity to nucleotides of a conserved loop in the MNSV 5'-UTR (Truniger, Nieto and Aranda, unpublished). Therefore, it is possible that MNSV RNA translation, as for that of other tombusviruses, occurs through a 5'–3' RNA–RNA interaction that effectively circularizes the message. It can be speculated that the MNSV avr determinant in the 3'-UTR interacts with eIF4E acting as a 5'-cap substitute, bringing the eIF4F complex to the 5'-UTR through the 5'–3' RNA–RNA interaction. In this model, interaction between the MNSV 3'-UTR and Cm-eIF4E would control the initiation of translation of the MNSV genomic RNA and, hence, the plant susceptibility to this virus. However, further experiments are required to validate this model. The way in which MNSV-264, the resistance-breaking isolate, infects the plant in the absence of the Cm-eIF4E allele, which confers susceptibility to other isolates, is also unknown. It may be that either MNSV-264 is able to use all known Cm-eIF4E alleles or, alternatively, that MNSV-264, with a differentiated 3'-UTR (Diaz et al., 2004) uses another host factor to achieve the same function. Again, further experiments are needed to clarify these aspects.

Experimental procedures

Plant and virus materials

Nsv-segregating populations were derived from a backcross between the susceptible Charentais-type line ‘Vedrantais’ and the resistant accession PI 161375 (Vedrantais (Nsv/Nsv) × PI 161375 (nsv/nsv)) × PI 161375 (nsv/nsv) and an F2 population from the cross...
Cloning the Cm-eIF4E marker and full-length Cm-eIF4E cDNAs

A fragment of the melon elf4E gene comprising a region of exon 1, intron 1 and a region of exon 2 was cloned using the degenerate primers elf4E-df (5'-TGGACCTTGTGGTGGAYAA-3') and elf4E-dr (5'-GGRTCTYCCAYTGGYTC-3'). Cm-eIF4E was mapped as marker M-Cm-eIF4E in the F2 and BC1 mapping populations after digestion of the PCR product obtained with the specific primers meIF4E-F (5'-CCGATCTATACCTTCTCTCC-3') and meIF4E-R (5'-TACAAATCTGCCCTACTGCC-3') with the restriction enzymes RsaI and MboI, respectively. To map the SNP on the codon position 228, melon DNA was PCR amplified with the primers elf4E-F2 (5'-GGTCTGATACgATGTTGTTTCCCTG-3') and elf4E-R2 (5'-GCCGAGTGACGCAagATGTTGCAC-3') and polymorphism was detected by digestion with the restriction enzyme NlaIII. The 5' and 3' ends of the nsv cDNA were determined by rapid amplification of cDNA ends (RACE)-PCR, as described in Clepet et al. (2004). The primers used in the RACE-PCR are derived from the sequence of the molecular marker M-Cm-eIF4E. Full-length Cm-eIF4E cDNAs from the different melon genotypes were obtained in conventional RT-PCR experiments (Sambrook and Russel, 2001) using the two molecular marker M-Cm-eIF4E. Full-length Cm-eIF4E cDNAs from the different melon genotypes were obtained in conventional RT-PCR experiments (Sambrook and Russel, 2001). These two constructs are referred to as pBM5 and pBM6, respectively. The forward primers used containing the XbaI site were: M5-F (5'-GCTCTAGAGGA-CTTCCATTAAAACACAATACGTTGGCATC-3') and M5-R (5'-GAAGGCCTAGGGCGGGGGTGACCATCCTGAG-3') and TCTCAGATTCTTTGCTGATC-3'. Constructed used for transient expression

Full-length cDNAs from MNSV-Mx5 and MNSV-264 cDNAs (Diaz et al., 2004) were cloned into the XbaI-Smal sites of the binary vector pBIN61 and expressed under the control of the CaMV 35S promoter (Bendahmane et al., 2000). These two constructs are referred to as pBM5 and pBM6, respectively. The forward primers used containing the XbaI site were: M5-F (5'-GCTCTAGAGGA-CTTCCATTAAAACACAATACGTTGGCATC-3') and M5-R (5'-GAAGGCCTAGGGCGGGGGTGACCATCCTGAG-3').

Cm-eIF4E open reading frame sequences were amplified from cDNAs of PI and Vd melon genotypes using primers FullICDNA-F (5'-GCTCTAGAATCTTCCATTCAAAAAG-3') and FullICDNA-R (5'-CCCTCCGGAGCCCGAGTATAGAAACCCTGAC-3'), and cloned into pBIN61 giving rise to pB4E-PI and pB4E-Vd. The N. benthamiana elf4E construct is referred to as pB4E-Nb. All constructs were checked by sequencing.

Biological transient expression assay

Plasmid DNAs were isolated using the alkaline lysis method and purified on cesium chloride gradients (Sambrook and Russel, 2001). Plasmid DNA (20 μg) from viral and Cm-eIF4E expression vectors were mixed in a ratio of 1:3 before being coated to 1.0-μm Gold particles (Bio-RAD, Hercules, CA, USA) as described previously (McCabe et al., 1988). Detached leaves from 6-week-old plants were bombarded with the gold particles coated with plasmid DNAs, using the Biolistic PDS-1000/He System (Bio-RAD). The leaves were incubated in moistened Petri dishes at 25°C for 48 h. RNA extraction (TRizol Reagent; Invitrogen, Carlsbad, CA, USA) was performed and then analysed for virus accumulation using RT-PCR. The primer Seq3'5'-R (5'-GGACCAATGTTGAGAGTATAACAGAG-3') was used to synthesize the viral first strand and Seq1'F (5'-CCCATC-AAAAACCCGAAACTGTTGTC-3') and Seq1'R (5'-ACA-CTGAAAGCGGATTGTTGCCATG-3') primers were used in PCR experiments. Northern-blot analysis was also performed to detect the presence of viral genomic (gRNA) and subgenomic (sgRNA) RNAs. A probe complementary to the MNSV coat protein was hybridized using the primers Seq3'F (5'-CTTCCATTAAACACAAATCAGTTGGCATAC-3') and Seq3'5'-R (5'-GGACCAATGTTGAGAGTATAACAGAG-3').

Agroinfiltration transient expression assay

The constructs in pBIN61 were transformed into the Agrobacterium strain C58C1 carrying the virulence helper plasmid pHCh2 (Hamilton et al., 1996). Agroinfiltration into N. benthamiana leaves was carried out as described previously (Bendahmane et al., 1999). After 4 days post-agroinfiltration, total RNA was extracted and virus accumulation was quantified using Northern-blot analysis.

Protein expression and cap binding

Cm-eIF4E cDNAs were cloned into pET-15b vector (Novagen, Darmstadt, Germany) and expressed under the control of the T7 promoter and lac operator. E. coli BL21 cells were transformed and grown at 37°C in Luria Broth medium supplemented with 50 μg ml⁻¹ carbenicilin. At an OD₆₀₀ of 0.6, the culture was heated for 2 min at 42°C and protein expression was induced with 1 mM isopropyl-β-thiogalactopyranoside (iPTG) at 20°C for 2 h 30 min. Cells were harvested by centrifugation, washed with chilled STE buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA, 100 mM NaCl) and disrupted by sonication. After centrifugation at 22 000 g for 1 h, the soluble fraction was straight loaded on a m7GTP-Sepharose 4B affinity column (Amersham Biosciences, Little Chalfont, Bucks, UK), using the running buffer and conditions reported previously (Webb et al., 1984). Elutions were performed with 100 mM m7GDP (Sigma, St Louis, MO, USA). The chromatographies were run on an AKTA Explorer apparatus (Pharmacia, Munich, Germany) and OD₂₅₃ was used to follow the m7G nucleotide and OD₂₁₅ was used for the proteins.

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