The Plant Journal (2006) **48**, 452–462 doi: 10.1111/j.1365-313X.2006.02885.x

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

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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

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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 Bailey-Serres, 2002). 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 syntenic 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

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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 $= 3 \times 10^{-57}$). Notably, the list of annotated genes from BAC F15J15 displayed eIF4E at position 18040.

Based on the alignment of the eIF4E 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 *eIF(iso)4E* and a band of 1900 bp corresponding to *eIF4E* (*Cm-eIF4E*). Specific primers were designed based on the *Cm-eIF4E* 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-eIF4E* within this region. After sequencing PCR products for *Cm-eIF4E* alleles from the parental lines PI 161373, 'Piel de sapo' and 'Védrantais', two cleaved amplified polymorphism markers were developed for the two mapping populations used in the high-resolution mapping, the F_2 (PI 161375 × 'Piel de sapo') and the BC_1 ((Védrantais × PI 161375) × PI 161375) (Morales *et al.*, 2005). Marker M-Cm-eIF4E was tested in the recombinant individuals of both mapping populations and a perfect co-segregation with *nsv* was found in more than 3000 F_2 and BC_1 individuals.

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

nsv-linked markers	Predicted gene function	BLAST Hit	*E*-value[a]
38B12U	Hypothetical protein	At4g18980	0.56
38B12sp6	Putative protein	At4g18220	0.38
41I23sp6[b]	Unknown protein	At4g18260	8×10^{-14}
5A6U	Translation initiation factor eIF4E	At4g18040	0.14
7K20U[b]	Putative protein	At4g18810	8×10^{-12}
7K20sp6[b]	LRR protein	At4g20140	7×10^{-04}
34H17U	Disease resistance protein	At4g19510	1.4
34H17sp6	Putative protein	At4g19650	0.28
5B3U	Family 17-hydrolase glycosyl	At4g18340	0.39
9N8U	Putative protein	At4g17910	0.15
9N8sp6	Lil3 protein	At4g17600	1.0
1L3[b]	6-phosohate threolase synthase	At4g17770	2×10^{-06}
1R3[b]	L32 Ribosomal protein	At4g18100	3×10^{-47}

[a]Obtained performing the BLASTX and TBLASTX search.
[b]Markers with significant hits in the Arabidopsis region are in bold.

A single amino acid change in Cm-eIF4E correlates with nsv *resistance*

Full-length *Cm-eIF4E* cDNAs were cloned and sequenced from melon cultivar Védrantais (Ved) (*Cm-eIF4E-Ved*, GenBank accession no. DQ393831) and PI 161375 (PI) (*Cm-eIF4E-PI*, 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-eIF4E* encodes a protein of 235 amino acids. Cm-eIF4E amino acid sequence similarity was highest (91%) with the pea homologue, Ps-eIF4E (GenBank accession no. AY423375). Amino acid similarities

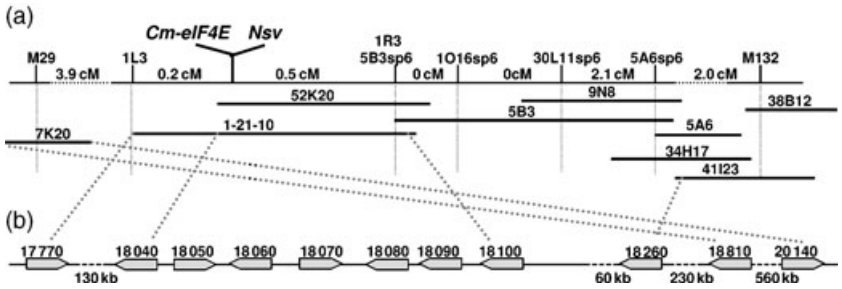

Figure 1. Genetic dissection of the *nsv* locus in melon and the orthologous region in Arabidopsis.
(a) Genetic and physical map of the *nsv* locus in melon. The genetic map is based on the analysis of more than 3000 *Nsv*-segregating plants. The *Nsv* locus physical map is based on the screening of 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.

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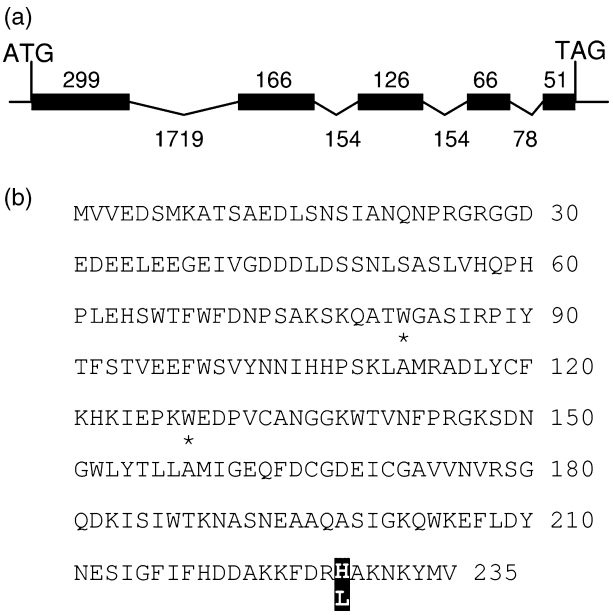

Figure 2. The *Nsv* coding sequence and primary structure of the Nsv protein.
(a) Schematic diagram of the *Nsv* coding sequence. Exons are indicated by black rectangles. Introns are indicated by broken lines. The numbers indicate the size of introns and exons in bp.
(b) Predicted Nsv protein sequence. The protein sequence shown is derived from a susceptible genotype. The black box indicates the polymorphic amino acid between susceptible and resistance genotypes. Conserved Tryptophan residues involved in the m^7G binding to the cap pocket are indicated by asterisks (Marcotrigiano *et al.*, 1997)

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).

Table 2 MNSV-Mα5 resistance in melon germplasm is associated with the identity of the amino acid at position 228 in Cm-eIF4E

Melon cultivars	Inoculation with MNSV-264	Inoculation with MNSV-Mα5	SNP	aa at position 228
PI 161375	S[a]	R[a]	T	Leu
Gulfstream	S	R	T	Leu
Planters Jumbo	S	R	T	Leu
Eros	S	R	T	Leu
Pepe	S	R	T	Leu
Quito	S	R	T	Leu
C-178	S	R	T	Leu
C-105	S	S	A	His
Doublon	S	S	A	His
MR-1	S	S	A	His
Seminole	S	S	A	His
Vedrantais	S	S	A	His
WMR-29	S	S	A	His

[a]S, susceptible to MNSV; R, resistant to MNSV.

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). The constructs derived from the NRB strain (MNSV-Mα5) and the RB strain (MNSV-264) of the virus are referred to as pBMα5 and pB264, respectively. The *Cm-eIF4E* constructs derived from resistant (PI) and susceptible (Ved) genotypes are referred to as pB4E-PI and pB4E-Ved, respectively (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 pBMα5 was co-bombarded with either pB4E-Ved and pB4E-PI constructs into the leaves of resistant and

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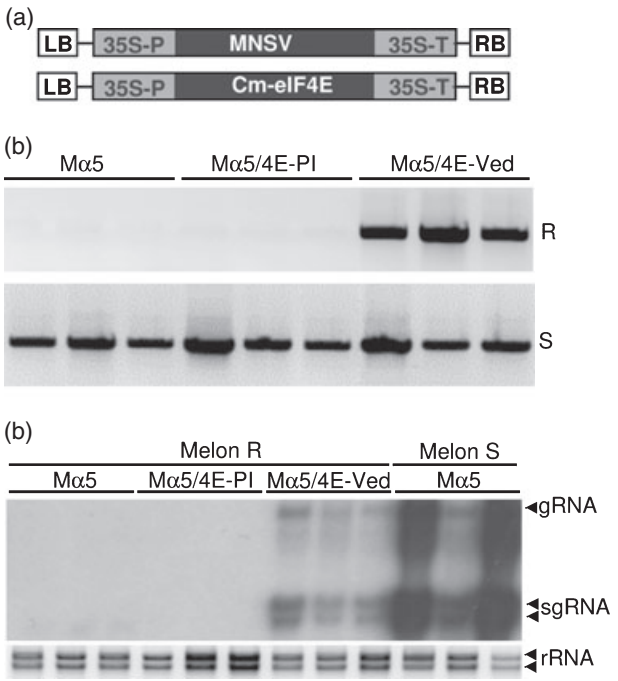

Figure 3. Biolistic transient expression assay of *Cm-eIF4E-Ved* in melon.
(a) Schematic structure of *Melon necrotic spot virus* (MNSV) and *Cm-eIF4E* constructs used in the transient expression assay. cDNAs were cloned into the binary vector pBIN61 between the left (LB) and right (RB) borders of the Agrobacterium Ti plasmid. The 35S promoter and terminator are indicated as 35S-P and 35S-T, respectively.
(b) RT-PCR detection of MNSV-Mα5. The pBMα5 (Mα5) construct was bombarded separately and in combination with either pB4E-PI (4E-PI) or pB-4E-Ved (4E-Ved) into the leaves of resistant (R) and susceptible (S) melon. Virus accumulation was assessed using RT-PCR at 2 days post-bombardment.
(c) Northern-blot analysis of MNSV-Mα5 RNA accumulation in bombarded leaves. The transient expression assay was carried out as described in (b). Virus accumulation was assessed by northern-blot analysis, using a [P^{32}]-labelled probe complementary to the MNSV-Mα5 coat protein. Each lane of the gel was loaded with 2 μg of total RNA. gRNA and sgRNA indicate MNSV genomic and subgenomic RNAs, respectively. rRNA was used as an equal loading control.

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-Mα5 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 pBMα5 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 eIF4E 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-Mα5 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 eIF4E amino acid mutation conserved in both species.

Using primers specific for *Nt-eIF4E* (GenBank accession no. AY702653), we RT-PCR amplified the *eIF4E N. benthamiana* homologue, *Nb-eIF4E* (GenBank accession no. DQ393833). Figure 4 shows the sequence alignment of eIF4E 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 transient expression. The system is based on the co-expression of *eIF4E* 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-eIF4E*. We predicted from this expression assay that the overexpression of an *eIF4E* allele is likely to replace the endogenous eIF4E protein. Virus accumulation was assessed by a northern blot at 4 days post-agroinfiltration.

MNSV-Mα5 was unable to accumulate in *N. benthamiana* leaves in combination with any of the *eIF4E* 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-Mα5 in *N. benthamiana*. *N. benthamiana* may lack another factor required for MNSV-Mα5 infection. As it was expected, MNSV-264 accumulated in *N. benthamiana* leaves independently of the co-expressed *eIF4E* alleles. However, the level of virus accumulation was dependent on the identity of the agroinfiltrated *eIF4E* allele. There was eight-fold MNSV-264 RNA accumulation when the virus was co-agroinfiltrated

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Figure 4. Comparison of the eIF4E amino acid in various plants species. Alignment of predicted eIF4E amino acid sequences from *Nicotiana benthamiana* (*Nb-eIF4E*), *Nicotiana tabacum* (*Nt-eIF4E*), *Cucumis melo* cultivar PI (*Cm-eIF4E-PI*) and cultivar Ved (*Cm-eIF4E-Ved*), pepper (*Capsicum annuum*, *Ca-eIF4E*, AY122052), tomato (*Lycopersicon esculentum*, *Le-eIF4E*, AY723734), pea (*Pisum sativum*, *Ps-eIF4E*, AY423375), *Arabidopsis thaliana* (*At-eIF4E*, AY093750) and lettuce (*Lactuca sativa*, *Ls-eIF4E*, AF530162). Black boxes indicate amino acid differences observed between *Melon necrotic spot virus* (MNSV)-resistant and MNSV-susceptible genotypes. Amino acids implicated in the binding of the mRNA cap structure are marked by arrowheads and amino acids implicated in the recognition of eIF4G and 4E binding proteins are marked by grey boxes (Marcotrigiano *et al.*, 1997).

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Ca-eIF4E     MATAEMEKTTTFDEAEKVKLN------ANEADDEVEEGEIVEETDDTTSYLS----KEIA 50
Le.eIF4E     MAAAEMERTMSFDAAEKLKAADGG---GGEVDDELEEGEIVEESNDTASYLG----KEIT 53
Nt-eIF4E     -MVDEVEKPVSLEESK------------TNTREVEEEGEIVGESDDTMSSLGN---PSMA 44
Nb-eIF4E     -MVDEVEKPASLEESK------------TNTREVEEEGEIVGESDDTMSSLGN---PSKA 44
Ls-eIF4E     -MKSEEQKLIDVNKHRGVRSD-------GEEDEQLEEGEIVGGDADTLSSSSSSRPGTAI 52
Cm-eIF4E-Ved MVVEDSMKATSAEDLSNSIANQNPRGRGGDEDEELEEGEIVG-DDDLDSSNLSA--SLVH 57
Cm-eIF4E-PI  MVVEDSMKATSAEDLSNSIANQNPRGRGGDEDEELEEGEIVG-DDDLDSSNLSA--SLVH 57
Ps-eIF4E     MVVEETPKSIITDDQ-----ITTNPNRVIEDDNNLEEGEILD-EDDS--SATSK--PVVH 50
At-eIF4E     MAVEDTPKSVVTEEAK-PNSIENPIDRYHEEGDDAEEGEIAGGEGDGNVDESSK--SGVP 57
                 :  :    :            .   :  : *****    *      .

                                                  ▼▼ ▼
Ca-eIF4E     TKHPLEHSWTFWFDNPVAKSKQAAWGSSLRNVYTFSTVEDFWGAYNNIHHPSKLVVGADL 110
Le.eIF4E     VKHPLEHSWTFWFDNPTTKSRQTAWGSSLRNVYTFSTVENFWGAYNNIHHPSKLIMGADF 113
Nt-eIF4E     MKHALEHSWTFWFDNPSGKSKQAAWGSSIRPIYTFSTVEDFWSVYNNIHHPSKLAVGADF 104
Nb-eIF4E     MKHPLEHSWTFWFDNPSGKSKQAAWGSSIRPIYTFSTVEDFWSVYNNIHHPSKLAVGADF 104
Ls-eIF4E     AQHPLEHSWTFWFDTPSAKSKQVAWGSSMRPIYTFSSVEEFWSLYNNIHRPSKLAQGADF 112
Cm-eIF4E-Ved QPHPLEHSWTFWFDNPSAKSKQATWGASIRPIYTFSTVEEFWSVYNNIHHPSKLAMRADL 117
Cm-eIF4E-PI  QPHPLEHSWTFWFDNPSAKSKQATWGASIRPIYTFSTVEEFWSVYNNIHHPSKLAMRADL 117
Ps-eIF4E     QPHLLENSWTFWFDTPAAKSKQPDWG-SMRPIYTFSTVEEFWSIYNNIHHPGKLAVGADF 109
At-eIF4E     ESHPLEHSWTFWFDNPAVKSKQTSWGSSLRPVFTFSTVEEFWSLYNNMKHPSKLAHGADF 117
               * **:*******.*  **:*  **:*:* ::***:**:**. ***:::*.**   **:

                                    ▼       ▼▼ ▼
Ca-eIF4E     HCFKHKIEPKWEDPVCANGGTWKMSFSKGKSDTSWLYTLLAMIGHQFDHEDEICGAVVSV 170
Le.eIF4E     HCFKHKIEPKWEDPVCANGGTWKMSFSKGKSDTSWLYTLLAMIGHQFDHGDEICGAVVSV 173
Nt-eIF4E     HCFKNKIEPKWEDPVCANGGKWTMSFSRGKSDTCWLYTLLAMIGEQFDCGDEICGAVINV 164
Nb-eIF4E     HCFKNKIEPKWEDPVCANGGKWTMSFSRGKSDTCWLYTLLAMIGEQFDCGDEICGAVINV 164
Ls-eIF4E     YCFKNKIEPKWEDPVCANGGKWTMTFTKAKSDTCWLYTLLAMIGEQFDHGDDICGAVVNV 172
Cm-eIF4E-Ved YCFKHKIEPKWEDPVCANGGKWTVNFPRGKSDNGWLYTLLAMIGEQFDCGDEICGAVVNV 177
Cm-eIF4E-PI  YCFKHKIEPKWEDPVCANGGKWTVNFPRGKSDNGWLYTLLAMIGEQFDCGDEICGAVVNV 177
Guisante     YCFKHKIEPKWEDPICANGGKWTANYPKGKSDTSWLYTLLAMIGEQFDHGDEICGAVVNV 169
At-eIF4E     YCFKHIIEPKWEDPICANGGKWTMTFPKEKSDKSWLYTLLALIGEQFDHGDEICGAVVNI 177
             :***: ********:*****.*. .:.: ***. *******:**.***  *:*****:.:

Ca-eIF4E     RGKGEKISLWTKNAANETAQVSIGKQWKQFLDYSDSVGFIFHDDAKRLDRNAKNRYTV 228
Le.eIF4E     RAKGEKIALWTKNAANETAQVSIGKQWKQFLDYSDSVGFIFHDDAKRLDRNAKNRYTV 231
Nt-eIF4E     RVRQEKIALWTRNAANETAQVSIGKQWKEFLDYNDSVGFIFHDDAKKLDRAAKNRYSV 222
Nb-eIF4E     RVRQEKIALWTRNAANETAQVSIGKQWKEFLDYNDSIGFIFHDDAKKLDRAAKNRYSA 222
Ls-eIF4E     RARQEKIALWTKNAANESAQLSIGKQWKEFIDYNDTIGFIFHEDAKTLDRSAKNKYTV 230
Cm-eIF4E-Ved RSGQDKISIWTKNASNEAAQASIGKQWKEFLDYNESIGFIFHDDAKKFDRHAKNKYMV 235
Cm-eIF4E-PI  RSGQDKISIWTKNASNEAAQASIGKQWKEFLDYNESIGFIFHDDAKKFDRLAKNKYMV 235
Ps-eIF4E     RGRAEKISIWTKNASNEAAQVSIGKQWKEFLDYNETMGFIFHDDARKLDRNAKNKYVV 227
At-eIF4E     RGKQERISIWTKNASNEAAQVSIGKQWKEFLDYNNSIGFIIHEDAKKLDRNAKNAYTA 235
             *   ::*::**:**:**:** *******:*:**.:::***:*:**: :** *** * .
```

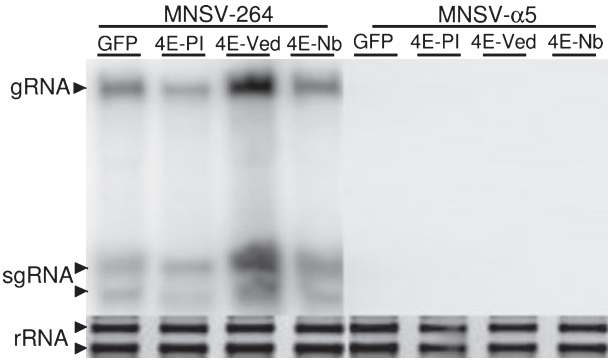

Figure 5. Agrobacterium transient expression assay of *Melon necrotic spot virus* (MNSV) and *eIF4E* alleles in *N. benthamiana*. pBMα5 and pB264 constructs were agroinfiltrated into *Nicotiana benthamiana* leaves either in combination with *eIF4E* alleles or with GFP as a negative control. Virus accumulation was assessed by northern-blot analysis 4 days post-agroinfiltration. Genomic (gRNA) and subgenomic RNAs (sgRNA) were detected after hybridization with a [P^{32}]-labelled probe complementary to the MNSV coat protein gene. Each lane of the gel was loaded with 2 μg of total RNA. rRNA was used as equal loading control (bottom panel). The virus accumulation was quantified using the Packard Instant Imager (Packard Bioscience Co., Meriden, CT, USA).

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 m^7GpppA instead of m^7GTP, 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 m^7G-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

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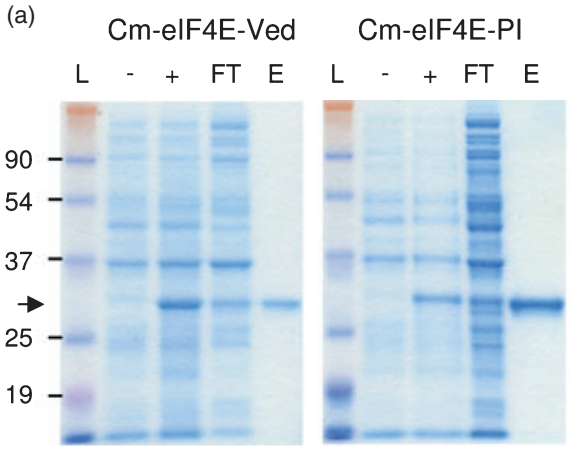
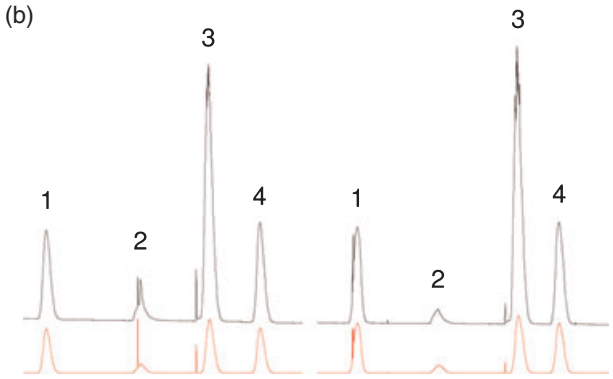
(a)



(b)



(c)

Cm-eIF4E	Ved	PI
Injected	200 µg	200 µg
Eluted	109 µg	120 µg

Figure 6. Cm-eIF4E-PI and Cm-eIF4E-Ved show a similar cap-binding affinity.
(a) *Cm-eIF4E-PI* and *Cm-eIF4E-Ved* full-length cDNAs were expressed in *Escherichia coli*. The crude protein extracts before (–) and after (+) Isopropyl-β-D-thiogalactopyranoside (IPTG) treatment showed a ~27-kDa-induced product (arrow head) consistent with the recombinant eIF4Es. The soluble fractions were prepared and passed through an m^7G-sepharose column (FT, flow through). After washing the column, the bound eIF4E proteins were eluted (e) with an m^7GDP-cap analogue. L, ladder in kDa.
(b) Comparative cap-binding assay: 200 µg of purified Cm-eIF4E-Ved (left) and Cm-eIF4E-PI (right) were sequentially loaded (peak 2) and eluted with m^7GDP (peak 3) on the same m^7G-sepharose column, both giving a comparable signal. No protein release was detected when the same quantity of cap analogue was injected either before (peak 1) or after (peak 4) the binding assay. Upper chromatogram, 215 nm; lower chromatogram, 253 nm.
(c) The eluted proteins were assayed by the Bradford method, confirming that both eIF4E proteins show similar cap-binding activities.

quantitative assay using the m^7G-affinity column. The Ved and PI Cm-eIF4E purified fractions were dialyzed to eliminate the m^7GDP-cap analogue from the buffer, and defined quantitities 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 eIF4E 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-eIF4E 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-eIF4E* 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-eIF4E* 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-eIF4E to bind m^7GTP *in vitro*. In the case of potyviridae resistance mediated by eIF4E, non-conservative amino acid substitutions implicated in resistance occur in two regions of the eIF4E 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 eIF4E variants encoded by alleles

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conferring potyvirus resistance showed reduced *in vitro* m^7GTP 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 eIF4G 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 eIF4F 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 unkown. 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 F_2 population from the cross

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PI 161375 (*nsv*/*nsv*) × 'Piel de Sapo' (*Nsv*/*Nsv*). MNSV-264 and MNSV-Mα5 are RB and NRB virus strains, respectively (Diaz *et al.*, 2004). Wild-type virus infections were obtained using the infectious clones pTOPO-264 and pTOPO-Mα5L2, as described in Diaz *et al.* (2004).

Cloning the Cm-eIF4E *marker and full-length* Cm-eIF4E *cDNAs*

A fragment of the melon *eIF4E* gene comprising a region of exon 1, intron 1 and a region of exon 2 was cloned using the degenerate primers eIF4E-dF (5′-TGGACITTYTGGTTYGAYAA-3′) and eIF4E-dR (5′-GGRTCYTCCCAYTTIGGYTC-3′). *Cm-eIF4E* was mapped as marker M-Cm-eIF4E in the F_2 and BC_1 mapping populations after digestion of the PCR product obtained with the specific primers meIF4E-F (5′-CCGATCTATACCTTCTCTACC-3′) and meIF4E-R (5′-TACAAATCTGCCCTCATCGCC-3′) with the restriction enzymes *Rsa*I and *Mbo*II, respectively. To map the SNP at the codon position 228, melon DNA was PCR amplified with the primers meIF4E-F2 (5′-GTTTCTGATACgATGTTGTTTCCCTG-3′) and meIF4E-R2 (5′-GCCGAGATGCAGCAGgATGCTTTGCAC-3′) and polymorphism was detected by digestion with the restriction enzyme *Nla*III. 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 following primers: 5′-GAGGGCGGTGCCATTCTTCTTCGG-3′ and 5′-CTCAGATTCTTGTTCTGTACTTGAC-3′.

Constructs used for transient expression

Full-length cDNAs from MNSV-Mα5 and MNSV-264 cDNAs (Diaz *et al.*, 2004) were cloned into the *Xba*I–*Sma*I 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 pBMα5 and pB264, respectively. The forward primers used containing the *Xba*I site were: Mα5-F (5′-GCTCTAGAGGATATCTCTAGCCGGATCCCCG-3′) and 264-F (5′-GCTCTAGAGGGATTACTCTAGCCGGATCCCCG-3′). The reverse primers were: Mα5-R (5′-GAAGGCCTAGGGCGGGATGGGCCGAAAACCCATCT-3′) and 264-R (5′-GAAGGCCTAGGGCGGGGGGCTCCGATAGAACCCCT-3′). *Cm-eIF4E* open reading frame sequences were amplified from cDNAs of PI and Ved melon genotypes using primers FullcDNA-F (5′–GCTCTAGATAACTCTCCATTCCACAAAG-3′) and FullcDNA-R (5′-CCTCCCGGGACGCCACGCATTAGAAACCGTACAG-3′), and cloned into pBIN61 giving rise to pB4E-PI and pB4E-Ved. The *N. benthamiana eIF4E* construct is referred to as pB4E-Nb. All constructs were checked by sequencing.

Biolistic 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′α5-R (5′-GGAACAAACTTGGAGAGTATACAAAGAG-3′) was used to synthesize the viral first strand and Seq1-F (5′-CCCATCAAAACACGCAAACTGTATTGTC-3′) and Seq1-R (5′-ACACTGAAACCCGAATTGTCTCCAGTG-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 used in hybridization was amplified using the primers Seq3-F (5′-CTTCCATTAAAACACAATACGTTGGCATC-3′) and Seq3′α5-R (5′-GGAACAAACTTGGAGAGTATACAAAGAG-3′).

Agroinfiltration transient expression assay

The constructs in pBIN61 were transformed into the Agrobacterium strain C58C1 carrying the virulence helper plasmid pCH32 (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 μ ml^{-1} carbenicilin. At an OD_{600} of 0.6, the culture was heated for 2 min at 42°C and protein expression was induced with 1 mM isopropyl-β-D-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 m^7GTP-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 μM m^7GDP (Sigma, St Louis, MO, USA). The chromatographies were run on an AKTA Explorer apparatus (Pharmacia, Munich, Germany) and OD_{253} was used to follow the m^7GDP nucleotide and OD_{215} was used for the proteins.

Acknowledgements

We thank Andy Maule and Olivier LeGall for their critical comments on the manuscript. G. Orjeda was supported by Le Conseil Régional-IDF, C. Nieto and M. Morales were supported by short-stay Marie Curie fellowships. C. Nieto was also the recipient of a ''Formación de Personal Investigador'' fellowship of the Ministerio de Educación y Ciencia (Spain). This work was supported by grants AGL2003-02739, 2FD97-0286 and HF2003-176.

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