Melon RNA interference (RNAi) lines silenced for *Cm-eIF4E* show broad virus resistance

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SUMMARY

Efficient and sustainable control of plant viruses may be achieved using genetically resistant crop varieties, although resistance genes are not always available for each pathogen; in this regard, the identification of new genes that are able to confer broad-spectrum and durable resistance is highly desirable. Recently, the cloning and characterization of recessive resistance genes from different plant species has pointed towards eukaryotic translation initiation factors (eIF) of the 4E family as factors required for the multiplication of many different viruses. Thus, we hypothesized that eIF4E may control the susceptibility of melon (*Cucumis melo* L.) to a broad range of viruses. To test this hypothesis, *Cm-eIF4E* knockdown melon plants were generated by the transformation of explants with a construct that was designed to induce the silencing of this gene, and the plants from T2 generations were genetically and phenotypically characterized. In transformed plants, *Cm-eIF4E* was specifically silenced, as identified by the decreased accumulation of *Cm-eIF4E* mRNA and the appearance of small interfering RNAs derived from the transgene, whereas the *Cm-eIF(iso)4E* mRNA levels remained unaffected. We challenged these transgenic melon plants with eight agronomically important melon-infecting viruses, and identified that they were resistant to *Cucumber vein yellowing virus* (CVYV), *Melon necrotic spot virus* (MNSV), *Moroccan watermelon mosaic virus* (MWMV) and *Zucchini yellow mosaic virus* (ZYMV), indicating that *Cm-eIF4E* controls melon susceptibility to these four viruses. Therefore, *Cm-eIF4E* is an efficient target for the identification of new resistance alleles able to confer broad-spectrum virus resistance in melon.

INTRODUCTION

Viral diseases of cucurbit crops are major limiting factors for their production and may cause massive damage, particularly to melon and squash (Lovisolo, 1980), resulting in huge economic

losses (Gaba *et al.*, 2004). Major viruses that infect melon include: *Cucurbit aphid-borne yellows virus* (CABYV, genus *Polyomavirus*), *Watermelon mosaic virus* (WMV, genus *Potyvirus*), *Zucchini yellow mosaic virus* (ZYMV, genus *Potyvirus*), *Papaya ringspot virus* (PRSV, genus *Potyvirus*), *Moroccan watermelon mosaic virus* (MWMV, genus *Potyvirus*) and *Cucumber mosaic virus* (CMV, genus *Cucumovirus*), which are transmitted by aphids; *Cucurbit yellow stunting disorder virus* (CYSDV, genus *Crinivirus*), *Beet pseudoyellows virus* (BPYV, genus *Closterovirus*) and *Cucumber vein yellowing virus* (CVYV, genus *Ipomovirus*), which are transmitted by whiteflies (Abou-Jawdah *et al.*, 2000; Bananej and Vahdat, 2008; Gil-Salas *et al.*, 2009; Kassem *et al.*, 2007; Lecoq *et al.*, 2003, 2011; Papayiannis *et al.*, 2005; Sevik and Arli-Sokmen, 2003; Yakoubi *et al.*, 2007); and *Melon necrotic spot virus* (MNSV, genus *Carmovirus*), which is transmitted by the soil fungus *Olpidium bornovianus* (Bananej and Vahdat, 2008; Cuadrado *et al.*, 1993).

One of the most efficient and sustainable strategies for controlling plant virus infections is the use of genetically resistant plants. Around one-half of the approximately 200 known resistance genes that target plant viruses are recessively inherited (Díaz-Pendón *et al.*, 2004; Kang *et al.*, 2005b). A large number of recessive resistance genes have been cloned from crop species and shown to encode eukaryotic translation initiation factors (eIF) of the eIF4E or eIF4G family (Robaglia and Caranta, 2006; Truniger and Aranda, 2009). Thus, recessive resistance is often achieved through the absence of appropriate host factors that are essential for the virus to complete its biological cycle (hereafter referred to as susceptibility factors) (Truniger and Aranda, 2009), although this does not always seem to be the case (Gonzalez-Ibeas *et al.*, 2012). In plant cells, two eIF4F translation initiation complexes exist, eIF4F and eIF(iso)4F, which are formed by the cap-binding proteins, eIF4E and eIF(iso)4E, that are bound to the scaffold proteins eIF4G and eIF(iso)4G, respectively (Browning, 2004). Both factors have been shown to play similar roles in mRNA translation *in vitro* (Browning, 1996) and to have redundant functions *in vivo* (Duprat *et al.*, 2002; Ruffel *et al.*, 2006; Sato *et al.*, 2005; Yoshii *et al.*, 2004), but additional unique roles have also been described (Gallie and Browning, 2001). In dicotyledons, more than one gene can code for proteins of the eIF4E or eIF(iso)4E subfamily. For example, in *Arabidopsis*, there are three genes that code for eIF4E

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proteins and, in tomato, two, and, in both species, only one gene encodes for eIF(iso)4E (Piron *et al.*, 2010; Yoshii *et al.*, 2004). In melon, only one gene encoding for eIF4E and one encoding for eIF(iso)4E have been identified (M. A. Aranda *et al.*, unpublished data; Clepet *et al.*, 2011; Gonzalez-Ibeas *et al.*, 2007). Resistances that are mediated by eIF4E or its isoform to several viruses belonging to the family *Potyviridae* have been described in different plant species (Hwang *et al.*, 2009; Naderpour *et al.*, 2010, reviewed in Truniger and Aranda, 2009). Outside of the family *Potyviridae*, two cases of eIF4E-mediated resistance have been described, one in *Arabidopsis* to CMV (Yoshii *et al.*, 2004) and one in melon to MNSV (Nieto *et al.*, 2006; Truniger *et al.*, 2008). The finding that different viruses have common susceptibility factors suggests that, as a biotechnological approach, transgenic resistance to a wide range of viruses may be achieved by a reduction in the expression levels of these factors through RNA silencing (Hwang *et al.*, 2009, reviewed in Reddy *et al.*, 2009).

The identification of eIF4E as a common susceptibility factor for different viruses led us to search for new *Cm-eIF4E* alleles in the natural diversity of the melon (*Cucumis melo* L.) species by EcoTILLING, which is a variant of the TILLING strategy (Nieto *et al.*, 2007). Unfortunately, no *Cm-eIF4E* variants were identified, with the exception of one known single amino acid change that results in resistance to MNSV, but not to other viruses (Nieto *et al.*, 2006). Before using other time-consuming strategies to generate novel *Cm-eIF4E* variants (e.g. by TILLING) (Comai and Henikoff, 2006), we decided to test whether *Cm-eIF4E* controls the susceptibilities of plants to melon-infecting viruses other than MNSV. For this assessment, we used an RNA silencing strategy. Here, we describe the generation of transgenic melon plants expressing a transcript that forms a RNA hairpin structure that is able to induce the specific silencing of *Cm-eIF4E*. We demonstrated that the transgenic plants show down-regulated *Cm-eIF4E* expression, whereas the expression of its isoform *Cm-eIF(iso)4E* is not affected. We challenged these transgenic melon plants with agronomically important melon-infecting viruses, and identified resistances to CVYV, MNSV, MWMV and ZYMV, indicating that *Cm-eIF4E* is a susceptibility factor that is required for these infections to take place.

RESULTS

Generation of transgenic melon plants

The aim of this study was to reduce the expression of the translation initiation factor *Cm-eIF4E* in melon by post-transcriptional gene silencing. This had to be performed carefully to avoid affecting indirectly the expression of its isoform, *Cm-eIF(iso)4E*, because large regions of both genes share up to 75% of their nucleotide sequences. Thus, the fragment of *Cm-eIF4E* that showed the

lowest sequence identity relative to its isoform was chosen for the construction of the silencing vector. This fragment consisted of 175 nucleotides and is in the first 5' quarter of the *Cm-eIF4E* transcript, beginning exactly after the start codon (Fig. S1a, see Supporting Information). It was inserted as an inverted repeat into the pHANNIBAL vector (Wesley *et al.*, 2001) on both sides of the *pdk* intron, so that expression results in an intron-hairpin RNA (ihpRNA) able to induce RNA silencing of *Cm-eIF4E* (Fig. S1b). Melon hypocotyl explants were transformed with this transgene using *Agrobacterium tumefaciens*. Transformed buds were selected with kanamycin. Over 30 putative independently transformed kanamycin-resistant T0 plants were further analysed by polymerase chain reaction (PCR) to screen for the integrity of the inserted transgene and by flow cytometry to determine their ploidy levels. Most kanamycin-resistant/PCR-positive plants that were selected after transformation were tetraploid or chimeric. Eight diploid transgenic plants were self-pollinated. These plants were often affected in growth and fertility; thus, we obtained abundant T2 seeds only from one transformed T0 line. These mainly segregating T2 generations were used for all the work presented in this article, as they are ideal to compare directly nontransformed and transformed plants. The segregation ratio for kanamycin resistance in the T1 generation of this transgenic line was 3 : 1 (resistant : susceptible), strongly suggesting the presence of a single transgene insertion.

Specific silencing of *Cm-eIF4E* in transgenic melon plants

The effectiveness and specificity of the silencing induced by the ihpRNA, as expressed from the 35S promoter (Fig. S1b), was determined by the analysis of the accumulation levels of *Cm-eIF4E* and *Cm-eIF(iso)4E* mRNA using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The results showed a significant decrease in the accumulation of *Cm-eIF4E* mRNA in transgenic T2 plants compared with nontransgenic T2 and wild-type (WT) control plants (Fig. 1a, left), suggesting that the silencing was efficient. Importantly, the levels of *Cm-eIF(iso)4E* mRNA remained unchanged, reaching very similar levels in the transgenic, nontransgenic and WT plants (Fig. 1a, right). Thus, the chosen sequence seemed to be able to induce the specific silencing of *Cm-eIF4E*, but not of its isoform. To assess whether the presence of reduced *Cm-eIF4E* mRNA levels in the transformed plants was truly the consequence of RNA silencing, its correlation with the production of transgene-derived small interfering RNAs (siRNAs) was analysed by Northern blot. These experiments showed the accumulation of *Cm-eIF4E*-derived siRNAs in all transgenic plants, whereas none could be detected in the nontransgenic and WT plants (Fig. 1b), demonstrating that the low *Cm-eIF4E* mRNA levels were the result of transgene-induced RNA interference (RNAi).

Fig. 1 *Cm-elf4E* silencing in melon is specific and correlates with the appearance of *Cm-elf4E*-derived small interfering RNAs (siRNAs). (a) *Cm-elf4E* and *Cm-elf(iso)4E* mRNAs were quantified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Average *Cm-elf4E* (left) and *Cm-elf(iso)4E* (right) expression values of 16 noninoculated transformed (T) and 10 noninoculated nontransformed (NT) plants. NT corresponds to segregating T2 plants that did not carry the transgene. WT corresponds to wild-type BGV130, used as reference in all RT-qPCR quantifications. (b) Transgene-derived siRNAs were detected by Northern blot. Digoxigenin-labelled RNA and cRNA, complementary to both directions of the 175-nucleotide *Cm-elf4E* fragment of the transgenic construct, were used as probes. The presence of the transgene was identified by PCR.

Transgenic plants are resistant to MNSV

We have demonstrated previously that *Cm-elf4E* is essential for the translation of MNSV RNAs and hence for its multiplication in melon (Nieto *et al.*, 2006; Truniger *et al.*, 2008). Thus, MNSV was used as a control for the susceptibility assays. Cotyledons of plants from a segregating T2 generation, together with WT plants as controls, were inoculated with MNSV. At 5 days post-inoculation (dpi), inoculated cotyledons were sampled, and the following analyses were performed using the extracted DNA or RNA: (i) the determination of the presence of the transgene by PCR; (ii) the quantification of the viral and *Cm-elf4E* mRNAs by RT-qPCR; and (iii) the identification of transgene-derived siRNAs by Northern blot.

The results showed that viral multiplication was limited to almost undetectable levels in all of the plants that contained the transgene [PCR(+)] plants, Fig. 2a, plants 1–10], suggesting that the down-regulation of *Cm-elf4E* expression resulted in MNSV resistance. However, sister nontransgenic plants [PCR(–)] plants, Fig. 2a, plants 11–13] that expressed large amounts of *Cm-elf4E* mRNA were susceptible to MNSV and showed similar values to WT plants. In addition, Northern blot analysis detected *Cm-elf4E*-derived siRNAs in all of the PCR(+) but not PCR(–) plants, including WT and mock plants. Therefore, a perfect correlation of resistance to the *Cm-elf4E*-dependent virus MNSV, with plants containing the transgene [PCR(+)] showing reduced *Cm-elf4E* expression and presenting siRNAs derived from the transgene,

Fig. 2 *Cm-elf4E* silencing in melon results in resistance to Melon necrotic spot virus (MNSV). (a) Analyses of plants of a segregating T2 generation inoculated with MNSV. The relative amounts of MNSV RNA and *Cm-elf4E* mRNA were quantified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Transgene-derived siRNAs were detected by Northern blot. The presence of the transgene was identified by PCR. (b) Inoculated cotyledons at 5 and 12 days post-inoculation (dpi). WT (wild type), T (transformed).

was found, suggesting that the virus resistance phenotype of this transgenic line was the result of *Cm-elf4E* silencing. Interestingly, although, at 5 dpi, the inoculated cotyledons of the transgenic melons showed no symptoms, at 12 dpi, very small lesions could be observed (Fig. 2b), but this infection did not progress to systemic infection.

Multiplication of different viruses in *Cm-elf4E*-silenced plants

We tested the susceptibilities of the *Cm-elf4E*-silenced plants to seven other agronomically important melon-infecting viruses, including PRSV, WMV, CMV, ZYMV, CVYV, MWMV and CABYV; the first six were mechanically inoculated, whereas CABYV was agroinoculated (Prüfer *et al.*, 1995). Cotyledons of T2 plants were

Fig. 3 *Cm-elf4E* silencing in melon results in resistance to *Zucchini yellow mosaic virus* (ZYMV). (a) Analyses of plants from a segregating T2 generation inoculated with ZYMV. The relative amounts of ZYMV RNA and *Cm-elf4E* mRNA were quantified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Transgene-derived siRNAs were detected by Northern blot. The presence of the transgene was detected by PCR. (b) Disease symptoms on the leaves of wild-type (WT) melon plants at 21 and 30 days post-inoculation (dpi) and the symptomless leaves of transgenic (T) melon plants at 30 dpi.

inoculated with these viruses, and samples were obtained at the indicated dpi for further analyses. All of the plants were susceptible to PRSV, WMV, CMV and CABYV, whether or not they were transformed, and showed similar virus accumulation levels to those of WT plants (as detected by RT-qPCR or Northern blot; see Fig. S2 in Supporting Information for examples). However, no virus accumulation was observed for ZYMV, CVYV or MWMV, as illustrated in the corresponding figures. All transgenic PCR(+) plants were symptomless after inoculation with ZYMV (Fig. 3b) and showed no virus RNA multiplication and low levels of *Cm-elf4E* mRNA accumulation, whereas transgene-derived siRNAs were detected (Fig. 3a, plants 1–11). In contrast, the PCR(–) plants showed symptoms and high virus and *Cm-elf4E* mRNA accumulation values (Fig. 3a, plants 12–14), whereas no transgene-derived siRNAs were detected. For T2 plants inoculated with CVYV, similar results were observed: only the PCR(+) plants were asymp-

Fig. 4 *Cm-elf4E* silencing in melon results in resistance to *Cucumber vein yellowing virus* (CVYV). (a) Analysis of homozygous plants from a T2 generation inoculated with CVYV. The relative levels of CVYV RNA and *Cm-elf4E* mRNA were quantified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Transgene-derived siRNAs were detected by Northern blot. The presence of the transgene was determined by PCR. (b) Disease symptoms on the leaves of wild-type (WT) melon plants at 21 and 30 days post-inoculation (dpi) and the symptomless leaves of transgenic (T) melon plants at 30 dpi.

tomatic (Fig. 4b) and showed no accumulation of virus RNA and low levels of *Cm-elf4E* mRNA, correlating with the presence of transgene-derived siRNAs (Fig. 4a). Similar results were obtained for inoculations with MWMV: all PCR(−) plants showed strong symptoms of viral infection at 4 dpi (Fig. 5, plants 1, 2, 6 and 7) and died a few days later, whereas the corresponding PCR(+) plants showed no symptoms (Fig. 5, plants 3–5, 8 and 9). Resistant plants remained asymptomatic after 30 dpi (Fig. 5). Because, for these three viruses, all resistant plants were PCR(+) and therefore transformed, and all susceptible plants were PCR(−) and therefore not transformed, we may conclude that *Cm-elf4E* is a susceptibility factor for MWMV, ZYMV and CVYV. Average *Cm-elf4E* mRNA levels were similar in mock-inoculated (healthy) transgenic plants (Fig. 1; average value of relative quantification, 0.19), in transgenic plants showing resistance to CVYV, ZYMV or MWMV (Figs 3–5; average value of relative quantification, 0.16) and in

Fig. 5 *Cm-elf4E* silencing in melon results in resistance to Moroccan watermelon mosaic virus (MWMV). Representative plants of a segregating T2 generation inoculated with MWMV at 5 days post-inoculation (dpi). The presence of the transgene was determined by polymerase chain reaction (PCR). Susceptible plants died a few days later, whereas resistant plants remained symptomless, as shown at 30 dpi. WT, wild-type.

	14m1	14m2	14m3	14m4	14m5	14m6	14m7	14m8	14m9	WT	mock
P C R	-	-	+	+	+	-	-	+	+	-	-
M W M V	S	S	R	R	R	S	S	R	R	S	-

transgenic plants showing susceptibility to PRSV, WMV, CABYV or CMV (Fig. S2; average value of relative quantification, 0.16).

DISCUSSION

In this study, melon transgenic plants expressing an ihpRNA that was able to induce the specific silencing of *Cm-elf4E* were generated and subjected to inoculations with eight agronomically important melon-infecting viruses (Bananej and Vahdat, 2008; Desbiez *et al.*, 2009; Kassem *et al.*, 2007). We found that the expression of reduced levels of *Cm-elf4E* and increased levels of transgene-derived siRNAs in these transgenic plants caused them to be resistant to CVYV, MNSV, MWMV and ZYMV, suggesting that *Cm-elf4E* is a susceptibility factor for these four viruses.

We were concerned that the silencing of *Cm-elf4E* could also result in the indirect silencing of *Cm-elf(iso)4E* because these two genes share 75% of their nucleotide sequences. Nonspecific silencing, which is based on partial sequence homology, has been described previously (Jackson and Linsley, 2004; Scacheri *et al.*, 2004). Recently, it has been shown that the silencing of *HC-Pro* from ZYMV in cucumber and melon resulted in resistance to ZYMV, but also to PRSV and WMV (Leibman *et al.*, 2011). The *HC-Pro* genes of these two viruses have sequence identities of 63% and 67% with that of ZYMV, respectively. We found that our transgenic plants were specifically silenced for *Cm-elf4E*, and that the levels of *Cm-elf(iso)4E* mRNA were not affected. This is possibly the result of having selected the fragment with the lowest sequence homology between the *Cm-elf4E* and *Cm-elf(iso)4E* genes for the silencing construct. In several cases, the reduced or absent expression of one gene from the 4E subfamily increased the expression of an isoform, suggesting that a homeostatic control mechanism exists that serves to maintain the essential cellular functions of *elf4E*. For example, the tomato *elf4e1* TILLING mutant, which did not affect growth, overexpressed *elf4E2* (Piron *et al.*, 2010). In addition, in *Arabidopsis* mutants and transgenic tobacco plants, *elf(iso)4E* depletion resulted in a compensatory increase in *elf4E* levels (Combe *et al.*, 2005; Duprat *et al.*, 2002;

Yoshii *et al.*, 2004). However, the down-regulation of *elf4E* in tobacco did not trigger a reciprocal increase in *elf(iso)4E* levels (Combe *et al.*, 2005). We also did not observe a compensating increase in the expression of *Cm-elf(iso)4E* in our *Cm-elf4E*-silenced transgenic melon plants, suggesting that, in melon, if partial compensation occurs, it is not regulated at the level of transcription or mRNA stability.

Our group has demonstrated previously that *Cm-elf4E* is essential for the translation and therefore multiplication of MNSV in melon (Nieto *et al.*, 2006; Truniger *et al.*, 2008). We have shown that *elf4E*-mediated resistance acts at the single-cell level on translation, and thus no MNSV multiplication is observed in resistant melon protoplasts (Diaz *et al.*, 2004). We first took advantage of this trait to utilize this virus as a control for the susceptibility assays to identify whether the levels of silencing that were obtained in the transgenic plants were sufficient to avoid viral multiplication. Our results showed that silencing decreased *Cm-elf4E* expression 6–10-fold and that the reduced *Cm-elf4E* levels correlated perfectly with resistance to MNSV. Thus, the virus resistance phenotype of the transgenic line was the result of *Cm-elf4E* silencing. This was confirmed by the 100% correlation of resistance to CVYV, ZYMV and MWMV with *Cm-elf4E* silencing. Importantly, the successful infections of PRSV, WMV, CMV or CABYV did not affect the levels of *Cm-elf4E* mRNAs in the RNAi lines, suggesting that the silencing suppressors of these four viruses did not affect *Cm-elf4E* silencing. A closer inspection of the RT-qPCR values that were obtained from the quantification of the virus RNAs of MNSV, CVYV and ZYMV in the transgenic plants compared with the mock-inoculated plants (Figs 2–4) revealed that the transgenic plants showed higher resistances to CVYV and ZYMV than to MNSV (average values of relative quantification: 5.0e-6, 2.5e-6 and 1.4e-2, respectively). Thus, the low levels of *Cm-elf4E* in transgenic plants seemed to still allow some residual multiplication of MNSV and, as a consequence, very small lesions on the inoculated cotyledons appeared after 12 dpi. This infection did not progress to systemic infection. However, no symptoms were observed in either the inoculated cotyledons or upper leaves

until 30 dpi in the transgenic plants inoculated with CVYV, ZYMV and MWMV. These results suggest that the multiplication of potyvirus RNAs requires higher concentrations of eIF4E in the host cells than that of the MNSV genome, whose translation is independent of cap and VPg.

PRSV, WMV, ZYMV, CVYV and MWMV belong to the family *Potyviridae*. For several viruses of this family, eIF4E has been described as a susceptibility factor. Here, we found that three of these viruses depend on eIF4E for their multiplication in melon plants. This agrees with the previous finding that potyviruses demonstrate notable isoform specificity, with eIF4E and eIF(iso)4E usually having distinct and nonoverlapping functions in their infection cycles (Truniger and Aranda, 2009). In *Arabidopsis*, for example, the loss of eIF4E confers resistance to *Clover yellow vein virus* (CIYVV) (Sato *et al.*, 2005), whereas the loss of eIF(iso)4E confers resistance to *Turnip mosaic virus* (TuMV), *Tobacco etch virus* (TEV) and *Lettuce mosaic virus* (LMV) (Duprat *et al.*, 2002; Lellis *et al.*, 2002). Although most potyviruses require one specific eIF4E isoform to multiply in a specific host, others are able to use both, as is the case for *Pepper veinal mottle virus* (PVMV) and *Chilli veinal mottle virus* (ChiVMV) in pepper (Hwang *et al.*, 2009; Ruffel *et al.*, 2006). Thus, in the case of PRSV and WMV, these viruses are either able to use both eIF4E and its isoform, or depend only on the eIF4E isoform, being eIF4E independent. The possibility that these viruses do not need any of the factors of the eIF4E family cannot be excluded. Our finding that ZYMV requires eIF4E for its multiplication in melon supports the work of Ling *et al.* (2009), who showed that a single nucleotide polymorphism in exon 1 of *eIF4E* in watermelon was closely associated with ZYMV resistance. Thus, it seems that eIF4E is a ZYMV susceptibility factor in both melon and watermelon. This is not always the case: for some potyviruses, it has been shown that the same virus requires different eIF4E isoforms to multiply in different hosts. For example, TEV and LMV require eIF(iso)4E to successfully infect *Arabidopsis* (Duprat *et al.*, 2002; Lellis *et al.*, 2002), whereas these same viruses require eIF4E in pepper and tomato (TEV) and in lettuce (LMV) (Kang *et al.*, 2005a; Nicaise *et al.*, 2003; Ruffel *et al.*, 2005). Although CMV is not a potyvirus, this host-dependent isoform specificity may explain why we found normal levels of CMV multiplication in transgenic melon plants, suggesting that its multiplication in melon is independent of eIF4E, although, in *Arabidopsis*, an eIF4E mutant, *cum1*, has been shown to be resistant to CMV (Yoshii *et al.*, 2004).

From a practical point of view, we have identified that *Cm-eIF4E* is required for the multiplication of three important emergent viruses, CVYV, ZYMV and MWMV, in melon. Because of its importance, CVYV has been included in the EPPO A2 Action List of pathogens that are recommended for regulation (Bananej and Vahdat, 2008; Lecoq *et al.*, 2007; Yakoubi *et al.*, 2007) (<http://www.eppo.org/QUARANTINE/listA2.htm>). However, MWMV has been reported previously in several countries (Lecoq *et al.*, 2008;

Yakoubi *et al.*, 2008) and has recently been identified as a virus of increasing incidence in south-eastern Spain (M. Juárez and M. A. Aranda, unpublished data). Finally, it has been established in several different countries that ZYMV is one of the most important cucurbit-infecting viruses after CABYV, WMV and CMV (Bananej and Vahdat, 2008; Desbiez *et al.*, 2009). These results suggest that eIF4E-mediated resistance in melon could serve as a strategy for the control of these important viral diseases, either modifying the transformation strategy (Rommens *et al.*, 2007) or searching for mutants in mutagenized melon populations using TILLING (Comai and Henikoff, 2006). In natural melon populations, no *Cm-eIF4E* variants were identified using Eco-TILLING (Nieto *et al.*, 2007). By TILLING melon populations, several induced mutations in *Cm-eIF4E* have been identified in two different melon cultivars, Piel de Sapo and Charentais, but their virus susceptibilities have not yet been tested (González *et al.*, 2011).

EXPERIMENTAL PROCEDURES

Construction of the binary vector for RNAi

To obtain specific silencing of *Cm-eIF4E*, a 175-nucleotide fragment from the *Cm-eIF4E* unigene (ICuGI accession no. MU21698) was chosen based on its low sequence identity with the *Cm-eIF(iso)4E* unigene (ICuGI accession no. MU5578) (ICuGI, <http://www.icugi.org/>), as identified by sequence alignment using CLUSTALX (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). This *Cm-eIF4E* fragment was PCR amplified using the melon expressed sequence tag (EST) clone MU21698 as the template and the primers CE-79 (5'-GGCCTCTAGACTCGAGTAGTTGAAGATTCGATG-3'), which contained *XhoI* (bold) and *XbaI* (italics) restriction sites, and CE-80 (5'-GGCCGAATTCGGATCCGAGGCTGATGCTAG-3'), which contained *EcoRI* (bold) and *BamHI* (italics) restriction sites, for the cloning in inverted directions into the same restriction sites of the pHANNIBAL plasmid. Primers CE-163 (5'-CCCCACCCACGAGG-3') and CE-164 (5'-CCGGCGGT AAGGATC-3'), corresponding to the *Cauliflower mosaic virus* (CaMV) 35S promoter and octopine synthase (OCS) terminator sequences, respectively, were used to verify the correct cloning by sequencing. The *NotI* fragment of this construct was further subcloned into the binary vector pART27 (CSIRO, Plant Industry, Clayton South, Victoria, Australia), and the resulting plasmid was verified by sequencing with the primers CE-163/4.

Agrobacterium tumefaciens-mediated transformation

The transformation protocol is available elsewhere (B. Gosalvez *et al.*, unpublished data). Briefly, transformation was performed using hypocotyl explants from the melon accession BGV-130. The explants were incubated with *A. tumefaciens* EHA105 culture. Incubated explants were cultured for 3 days before they were transferred to a selection medium containing 150 mg/L kanamycin (Duchefa, Haarlem, North Holland, Netherlands) for the selection of transformed cells. Once the elongated shoots produced roots, they were subcultured in the presence of 15 mg/L kanamycin to select out the transformed shoots that stably expressed the transgene. Once the root systems were well developed, the plants were transferred to soil and acclimatized in a confined glasshouse and multiplied by self-pollination.

Seeds from melons that resulted from self-pollination were peeled, sterilized and germinated in the dark for 48 h in a germination medium [Murashige and Skoog salts (Duchefa), 10 g/L sucrose, 8 g/L agar, pH 5.7] containing 50 mg/L kanamycin to select for the resistant transformed seeds.

Identification of transgenic plants by PCR

Genomic DNA was extracted from young leaves or the cotyledons of melon plants according to Dellaporta *et al.* (1983) and evaluated by PCR to detect the presence of the transgene. PCR was performed using 200 ng of genomic DNA in a final volume of 25 µL with 0.3 µM each of the primers CE-404 (5'-CGCACAATCCCACTATCCTTC-3', pHANNIBAL-specific sequence) and CE-409 (5'-CCACGTCCTCTAGGGTTTG-3', *Cm-elf4E*-specific sequence) according to the Go-Taq polymerase (Promega, Madison, WI, USA) protocol. DNA from the WT plants was used as a negative control. The amplification of a 157-nucleotide fragment indicated the presence of the transgene (PCR+).

Virus inoculations

Treatments were performed on 10–15 plants from a T2 generation (homozygous or heterozygous). Cotyledons of 6–7-day-old plants were mechanically inoculated with fresh inoculum (in 0.03 M potassium phosphate buffer, pH 8.0) from cucurbits that had been infected with MNSV (isolate MNSV-Mx5), PRSV (isolate PRSV-WZ43), WMV (isolate WMV-M116), CMV (isolate CMV-fny), ZYMV (isolate ZYMV-C71), CVYV (isolate CVYV-AILM) and MWMV (isolate MWMV-CE) following standard procedures (Hull, 2002). CABYV infection was achieved by the agroinoculation of cotyledons with an agroinfectious clone [pBINCAB (Prüfer *et al.*, 1995) in *A. tumefaciens* strain C58C1]. Plants were grown in a glasshouse (16 h, 26 ± 2 °C/8 h, 18 ± 2 °C day/night, respectively). After inoculation, they were monitored daily for the appearance of symptoms. For the plants that had been inoculated with MNSV and CABYV, the inoculated cotyledons were harvested at 5 dpi. For the other systemically infecting viruses, the third leaf was harvested at approximately 21 dpi. Genomic DNA (see above) and total RNA (see below) for further analyses were extracted from the harvested cotyledons or leaves. For MWMV, only DNA was extracted from the cotyledons because this virus killed the susceptible plants, and no systemically infected leaves were able to be harvested. Viral accumulation was detected by RT-PCR (see below), with the exception of the agroinoculated CABYV that was detected and quantified by Northern blot using a CABYV-specific probe (Kassem *et al.*, 2007).

Detection of siRNAs by Northern blot hybridization

Total RNA was isolated from melon plant tissues (leaves or cotyledons) using the TRI Reagent (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. Ten micrograms of RNA in 50% formamide were separated in a 20% polyacrylamide gel containing 7 M urea and 0.5 × Tris-Borate-EDTA (TBE) buffer, run in 0.5 × TBE buffer (180 V/90 min), transferred to a positively charged nylon membrane (Roche Applied Science, Indianapolis, IN, USA) in 0.5 × TBE buffer (80 mA/25 min) and fixed by UV exposure (1200 J × 100 min). The blotted membrane was hybridized with digoxigenin-labelled (DIG) RNA labelling mix, Roche

Applied Science) RNA and cRNA probes corresponding to the selected 175-nucleotide *Cm-elf4E* fragment at 42 °C overnight. After hybridization, the membrane was washed twice at 52 °C with 2 × standard saline citrate (SSC) and 2% sodium dodecylsulphate (SDS), once with 1 × SSC and 1% SDS, and once with 0.5 × SSC and 1% SDS for 10 min. The membrane was further developed according to the DIG labelling protocol (Roche Applied Science).

RT-qPCR analysis to determine mRNA expression levels in melon plants

Prior to RT, the total RNA was treated with DNase I (New England Biolabs, Ipswich, MA, USA) and subsequently purified by phenol–chloroform extraction. RT was performed following the protocol of the transcriptase (Roche Applied Science) using an oligo-dT(16) primer. Because neither MNSV nor CMV has poly(A) tails, the primers CE-294 or CE-169 (complementary to the 3' end of MNSV or CMV, respectively, see below) were added to the corresponding RT reactions. Relative qPCRs to quantify *Cm-elf4E* and *Cm-elf(iso)4E* mRNAs and virus concentrations were performed using the ABI 7500 System (Applied Biosystems International, Foster City, CA, USA), as described in Gonzalez-Ibeas *et al.* (2007), using SYBR green chemistry and cyclophilin expression (Melogen database, cCL3169) as internal reference. Data analyses were conducted using SDS-7500 software (version 1.4, Applied Biosystems International). The following specific primer pairs were designed using Primer Express Software 3.0 (Applied Biosystems International): *elf4E*-CE-147 (5'-TTCGGTTCC TTCCCTTCAT-3') and CE-148 (5'-CCGCCGATGTAGCTTTCATC-3'); *elf(iso)4E*-CE-149 (5'-GGTATTTTCAGCGCCCAAGA-3') and CE-150 (5'-CCAATTTATGGGACTGCGGAT-3'); MNSV-CE-293 (5'-GCCCCAGGGAAT CCTAGAAT-3') and CE-294 (5'-CCGCTGTACACAGTTCCTTA-3'); CMV-CE-168 (5'-ATGGGCAATGCGTTTCGTTA-3') and CE-169 (5'-CCGCTTACGATT CCAACTGT-3'); PRSV-CE-703 (5'-CGGAGAGACACAGTGGAAGAT-3') and CE-704 (5'-AACACACTAGCGCGAGTATTCAT-3'); WMV-CE-170 (5'-TGTTGCTTCATGGAAGATTGGT-3') and CE-171 (5'-AAAATTGTGCCATCA GGTGCTA-3'); CVYV-CE-638 (5'-CGCAGCGCGACAACCTTAAC-3') and CE-639 (5'-TTGTCTATGGCTTGGGATGGA-3'); ZYMV-CE-172 (5'-TGCCA CCACTAGCGAAGACA-3') and CE-173 (5'-CGGCCTACCCCTTACTGCATT-3'); and Cyclophilin-CE-155 (5'-CGATGTGGAATTGACGGAA-3') and CE-156 (5'-CGGTGCATAATGCTCGGA-3').

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NOTE ADDED IN PROOF

While this article was in proof a new publication appeared, that provides evidence that both *elf4E1* and *elf4E2* are involved in broad spectrum resistance in tomato against potyviruses.

Mazier, M., Flamaing, F., Nicolai, M., Sarnette, V. and Caranta, C. (2011) Knock-down of both *elf4E1* and *elf4E2* genes confers broad-spectrum resistance against potyviruses in tomato. *PLoSone*, **6**, e29595.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Construct for *Cm-elf4E* silencing. (a) Sequence alignments of *Cm-elf4E* (MU21698) and *Cm-elf(iso)4E* (MU5578) using CLUSTALX. The selected *Cm-elf4E* sequence stretch (175 nucleotides) is shown in bold and italics, and the primer sequences that were used for the amplification of the fragment are additionally underlined; stars mark identical nucleotides. (b) Schematic representation of the intron-hairpin-structured RNA (ihp4E) expressed by the transgenic construct. The 175-nucleotide inverted complementary sequence fragments from the *Cm-elf4E* gene (175-4E) form the stem, which is separated by the *pdv* intron.

Fig. S2 *Cm-elf4E* silencing in melon does not affect the susceptibility of melon to *Papaya ringspot virus* (PRSV), *Watermelon mosaic virus* (WMV), *Cucumber mosaic virus* (CMV) and *Cucurbit aphid-borne yellows virus* (CABYV). Results obtained in the analyses of plants from a segregating T2 generation inoculated with PRSV, WMV, CMV and CABYV; the relative amounts of virus RNA and *Cm-elf4E* mRNA were quantified by reverse transcription-polymerase chain reaction (RT-PCR) (with the exception of CABYV agroinoculated plants, for which virus accumulation was quantified by Northern blot), the transgene-derived small interfering RNAs (siRNAs) were detected by Northern blot, and the presence of the transgene was detected by PCR.

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