The Absence of Eukaryotic Initiation Factor eIF(iso)4E Affects the Systemic Spread of a *Tobacco etch virus* Isolate in *Arabidopsis thaliana*

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Translation initiation factor eIF4E exerts an important role during infection of viral species in the family *Potyviridae*. Particularly, a eIF(iso)4E family member is required for *Arabidopsis thaliana* susceptibility to *Turnip mosaic virus*, *Lettuce mosaic virus*, and *Tobacco etch virus* (TEV). In addition, a resistance mechanism named restriction of TEV movement (RTM) in *A. thaliana* controls the systemic spread of TEV in Col-0 ecotype. Here, we describe that TEV-TAMPS, a Mexican isolate, overcomes the RTM resistance mechanism reported for TEV-7DA infection of the Col-0 ecotype but depends on eIF(iso)4E for its systemic spread. To understand at which level eIF(iso)4E participates in *A. thaliana* TEV-TAMPS infection, the viral RNA replication and translation were measured. The absence or overexpression of eIF(iso)4E did not affect viral translation, and replication was still observed in the absence of eIF(iso)4E. However, the TEV-TAMPS systemic spread was completely abolished in the null mutant. The viral protein genome-linked (VPg) precursor NIa was found in co-immunoprecipitated complexes with both, eIF(iso)4E and eIF4E. However, the viral coat protein (CP) was only present in the eIF(iso)4E complexes. Since both the VPg and the CP proteins are needed for systemic spread, we propose a role of *A. thaliana* eIF(iso)4E in the movement of TEV-TAMPS within this host.

Successful colonization of a plant host by viruses is mediated through molecular interactions between the host and viral factors. Such interactions mediate translation and replication of the viral genome, invasion of the host by cell-to-cell movement through plasmodesmata, and long-distance movement through the vascular system (Carrington and Whitham 1998; Taliany et al. 2008).

During the past decade, a central role of the translation initiation factor eIF4E has become evident during infection of viral species in the family *Potyviridae*. Point mutations or polymor-

phisms within eIF4E family members have been identified as the products of recessive resistance genes and are considered as markers for viral resistance in a broad spectrum of hosts and potyviruses (Ashby et al. 2011; Gao et al. 2004; Kang et al. 2005; Nicaise et al. 2007; Robaglia and Caranta 2006). Such mutations usually do not affect the endogenous cellular role of eIF4E. This protein is involved in translation initiation of capped mRNAs by directly binding the Cap structure, m⁷GpppN (where N is any nucleotide), at the 5' end of the mRNA through π - π stacking interactions as well as to the eIF4G protein to form the eIF4F complex (Jackson et al. 2010). In plants, three members of the eIF4E family have been characterized: eIF4E, eIF(iso)4E, and nCBP (Browning 2004). eIF4E forms a complex with eIF4G named eIF4F, whereas eIF(iso)4E binds to eIF(iso)4G to form the eIF(iso)4F complex. The expression of each member of the eIF4E family is apparently under differential regulatory mechanisms depending upon specific tissue and developmental stages (Dinkova et al. 2000; Rodriguez et al. 1998; Ruud et al. 1998). Particularly, eIF4E and eIF(iso)4E have been more broadly studied and have shown both redundant and specific roles as part of the eIF4F and eIF(iso)4F complexes during in vitro and in vivo translation of cellular mRNAs (Dinkova et al. 2011; Gallie 2001; Martinez-Silva et al. 2012).

Natural and insertional mutants in the eIF4E family have been studied in *Arabidopsis thaliana* (Duprat et al. 2002; Hwang et al. 2009; Jenner et al. 2010; Sato et al. 2005), pepper and tomato (Kang et al. 2005; Ruffel et al. 2002, 2005), pea (Gao et al. 2004), lettuce (Nicaise et al. 2003), and common bean (Naderpour et al. 2010). The mechanisms by which eIF4E proteins are involved in potyvirus infection are not completely understood. However, it has been shown that they interact with the viral genome-linked protein (VPg) bound to the 5' end of the viral genome. It has been proposed that inhibition of cellular mRNA translation might be achieved by the competition between VPg and Cap for a particular eIF4E factor (Khan et al. 2008; Leonard et al. 2000). Mutant alleles of eIF4E showing impairment of the interaction with VPg are responsible for observed resistances to potyviral infections (Gao et al. 2004; Lellis et al. 2002). The central region of the VPg has been implicated in the interaction with eIF4E (Roudet-Tavert et al. 2007), and the VPg protein itself has been proposed as an avirulence factor in the potyvirus-plant interaction (Rajamaki and Valkonen 1999).

Other host resistance factors different from eIF4E have also been documented. Some of them act at different steps of the

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viral infection such as short- or long-distance movement. For example, the restriction of *Tobacco etch virus* (TEV) movement (RTM) described in *A. thaliana* controls the systemic spread of TEV, *Plum pox virus* (PPV), and *Lettuce mosaic virus* (LMV) (Decroocq et al. 2006, 2009; Whitham et al. 1999). TEV belongs to the family *Potyviridae* and infects a broad range of host plants. Its genome is composed of a positive single-stranded RNA of about 10 kb with a VPg covalently attached to the 5' end and a poly(A) tail at the 3' end. The viral genome contains a long open reading frame coding for about 10 mature viral proteins (Urcuqui-Inchima et al. 2001) and a small open reading frame called PIPO, which is inserted in the P3 sequence and produces an additional protein (P3N-PIPO) that acts as a cell-to-cell movement protein (Chung et al. 2008; Wei et al. 2010).

The first experiments describing the RTM mechanism were performed using an isolate named TEV-7DA. The RTM response requires at least three loci, *RTM1*, *RTM2*, and *RTM3*. *RTM1* encodes a protein belonging to the jacalin family, which is involved in defense against insects and fungi (Chisholm et al. 2000). The *RTM2* locus corresponds to a protein with a trans membrane domain similar to small heat-shock proteins. Its expression is not heat-inducible and, apparently, is not involved in a thermo-tolerance mechanism (Whitham et al. 2000). *RTM1* and *RTM2* associate with the phloem, perhaps within the sieve elements, to restrict TEV long-distance movement (Chisholm et al. 2001). A third RTM loci (*RTM3*) was characterized (Cosson et al. 2010). It encodes a protein be-

longing to an undefined family of 29 members with Meprin and TRAF homology domains. Recently, two additional RTM genes have been identified, indicating that the nonconventional RTM resistance is a complex biological process (Cosson et al. 2012). At present, it is not clear how the RTM proteins restrict long-distance movement of potyviruses and whether they interact with viral components. However, a possible regulation by plant hormones opens new venues of virus resistance regulation (Cosson et al. 2012). A connection between RTM and eIF4E family member-associated resistance has not been reported to date.

In *A. thaliana*, eIF(iso)4E but not eIF4E is required for successful viral colonization in the case of TEV (Lellis et al. 2002) and other potyviruses, such as *Turnip mosaic virus* (TuMV) and LMV (Duprat et al. 2002). At least three potential roles have been considered for the VPg-eIF(iso)4E interactions during TEV infection: i) a function in viral RNA translation based on the eIF(iso)4E role in translation initiation, ii) protection of the viral mRNA 5' end against enzymatic degradation through the eIF(iso)4E bound to VPg, and iii) appropriate intracellular localization and transport of the potyviral RNA as part of ribonucleoprotein complexes through interaction with cellular microtubules (Lellis et al. 2002; Okade et al. 2009).

Here, we assayed the infectivity of a Mexican isolate of TEV (TEV-TAMPS) in *A. thaliana* plants (Col-0 ecotype). Curiously, we found that this isolate was able to overcome the RTM resistance mechanism reported for the Col-0 ecotype as compared with TEV-7DA, which is susceptible to RTM. However, when using *eIF(iso)4E* knockout plants, no systemic spread of TEV-TAMPS could be observed. The finding that TEV-TAMPS was able to overcome RTM only in the presence of eIF(iso)4E prompted us to investigate whether eIF(iso)4E was involved in the systemic spread of this isolate or in either viral translation, replication, or both. After sequencing the TEV-TAMPS VPg and coat protein (CP), we found particular amino acid changes in variable regions of these proteins, as compared with other TEV isolates deposited in the GeneBank database, mostly from the Americas (Allison et al. 1985; Chu et al. 1995) but one from Asia. Translation of TEV-TAMPS was not importantly affected by the absence or overexpression of eIF(iso)4E. However, the presence of eIF(iso)4E was required for the TEV-TAMPS systemic spread in *A. thaliana*. Finally, although the VPg precursor NIa was found in vivo in coimmunoprecipitated complexes with both eIF(iso)4E and eIF4E, the viral CP was only present in the eIF(iso)4E complexes. Since both the VPg and the CP proteins are needed for the systemic viral movement, we propose that eIF(iso)4E is required for the systemic spread of TEV-TAMPS.

RESULTS

A Mexican TEV isolate is able to overcome the RTM resistance mechanism of an *A. thaliana* Col-0 ecotype.

The RTM resistance mechanism reported for *A. thaliana* Col-0 ecotype affects the systemic spread of TEV, LMV, and PPV (Chisholm et al. 2001; Cosson et al. 2010; Mahajan et al. 1998). The viral isolates tested in these reports moved cell-to-cell in inoculated leaves but did not move systemically. For the same viruses, the cap-binding protein eIF(iso)4E has been identified as a factor required for successful infection (Robaglia and Caranta 2006).

Here, we tested whether a Mexican TEV isolate (TEV-TAMPS) was able to infect an *A. thaliana* Col-0 ecotype, previously reported to have the RTM resistance mechanism, as compared with the C24 ecotype. Additionally, *eIF(iso)4E* knockout mutant plants in the Col-0 ecotype background were tested to find at which level this factor is required in the viral

Fig. 1. *Arabidopsis thaliana* infection with a Mexican *Tobacco etch virus* (TEV) isolate (TEV-TAMPS). **A**, The TEV-TAMPS RNA was detected by final point reverse transcription-polymerase chain reaction in local (L) and systemic (S) leaves of wild-type Col-0 ecotype (Col-0), *AtelF(iso)4E-1* knockout mutant ([*iso*]4E1), *AtelF(iso)4E-2* overexpressing mutant ([*iso*]4E2), and wild-type C24 ecotype (C24) after inoculation (0L) and at 4 or 15 days postinoculation (4L, 4S, and 15S). The viral RNA was present in systemic leaves of Col-0, [*iso*]4E2, and C24 wild-type plants at 4 and 15 days postinoculation but was undetectable in systemic tissues of the [*iso*]4E1 knockout mutant. Mock = leaves inoculated with virus-free inoculation buffer; *eEF1α* = the elongation factor 1α, which was used as an internal control; RT (-) = reaction without reverse transcriptase. **B**, The percentage of *A. thaliana* Col-0 plants systemically infected with TEV-TAMPS or TEV-7DA isolates. Viral presence was measured with enzyme-linked immunosorbent assay against TEV coat protein.

infection. The TEV-TAMPS viral RNA was detected by reverse transcription-polymerase chain reaction (RT-PCR) at 4 days after inoculation (dpi) on either local or systemic leaves of wild-type Col-0 and C24 plants (Fig. 1A). At 15 dpi, TEV-TAMPS was detected at similar levels in the systemic leaves of both ecotypes. Interestingly, the TEV-TAMPS RNA was not detected in the systemic leaves of *AteIF(iso)4E-1* mutant (*[iso]4E-1*), previously reported as resistant to potyvirus infection (Duprat et al. 2002).

The ability of TEV-TAMPS to systemically spread in Col-0 wild-type (WT) plants was compared with TEV-7DA at 15 dpi, using CP quantitation by enzyme-linked immunosorbent assay (ELISA) (Fig. 1B). This experiment was performed with 20 plants per isolate in three independent experiments. Each plant was tested in duplicate technical replicate. The viral CP was detected in systemic leaves of only two from the 20 TEV-7DA-inoculated plants, whereas 19 of 20 of the TEV-TAMPS-inoculated plants gave systemically positive signal. These observations indicated that TEV-TAMPS but not TEV-7DA was able to overcome the RTM resistance mechanism reported for *A. thaliana* Col-0 (Mahajan et al. 1998). Although the molecular basis of RTM has not yet been discovered and the particular viral targets for this host defense mechanism are not known, several evidences have shown that the viral NIa protein and the CP are necessary for systemic spread of the virus (Dolja et al. 1995; Urcuqui-Inchima et al. 2001). Hence, amino acid changes in these proteins could be associated with the RTM overcome in *A. thaliana*, as has been shown for the PPV CP amino terminal region (Decroocq et al. 2009).

Amino acid differences within VPg and CP of TEV-7DA and TEV-TAMPS isolates in the context of other TEV isolates.

To find out whether TEV-TAMPS carries particular mutations as compared with those reported for TEV-7DA, the regions corresponding to these two proteins were sequenced. The TEV-TAMPS VPg and CP (GenBank accessions JX512812_ *MX_TAMPS* and JX512813_ *MX_TAMPS*, respectively) were aligned with sequences from TEV isolates available at the National Center for Biotechnology Information (NCBI) database, including accessions reported as unable to spread systemically in the *A. thaliana* Col-0 ecotype, such as TEV-7DA (*US_7DA_DQ986288*), TEV-HAT (*US_HAT_M11458*), *US_M15239*, *US_NW_L38714*, and *VE_CT8_EU334787*, as well as isolates for which the ability of systemic movement in *A. thaliana* has not been described. Several amino acid substitutions were found, particularly in a variable core region and in a less variable one at the carboxy terminus of the VPg sequence (Supplementary Fig. S1). These substitutions lie outside the region reported to interact with eIF(iso)4E or the viral RNA. The alignment of CP sequences showed high variability, particularly at the TEV-TAMPS amino-terminal region near to the DAG motif needed for aphid transmission (Supplementary Fig. S2), as has been shown for other potyviruses (Rybacki and Shukla 1992; Silva-Rosales et al. 2000).

Using the above alignments, the evolutionary relatedness between TEV-TAMPS and other TEV isolates affected by the RTM mechanism was inferred from phylogenetic analysis using the Geneious alignment and tree builder, with the Jukes-Cantor genetic distance model and the neighbor-joining method. The phylogenetic tree based on the VPg sequence showed separated groups for isolates from two countries in North America (the United States and Mexico) and one in South America (Venezuela) (Fig. 2A). Viral isolates affected by the RTM resistance mechanism (*US_7DA_DQ986288* and *US_HAT_M11458*) grouped together. TEV-TAMPS (*MX_TAMPS_JX512812*), overcoming the RTM resistance mechanism, stood

alone on a separate branch of the other Mexican isolates, whose ability to spread systemically in the *A. thaliana* Col-0 ecotype is not known. The same applies for the Venezuelan isolates that form another group.

Fig. 2. Phylogenetic relationship of Tobacco etch virus (TEV) isolates based on the amino acid sequences of **A**, viral genome-linked protein (VPg) and **B**, coat protein (CP). The TEV-TAMPS VPg branch (*MX_TAMPS_JX512812*) was distant from the group of TEV-7DA, and TEV-HAT isolates reported as susceptible to the *Arabidopsis thaliana* Col-0 restriction of Tobacco etch virus (TEV) movement mechanism (*US_7DA_DQ986288* and *US_HAT_M11458*) and, curiously, from other Mexican isolates grouped together as well. According to the CP sequence, a single branch was formed between TEV-TAMPS (*MX_TAMPS_JX512813*) and *US_NW_L38714*, although the length of each line indicates a considerable distance between these isolates. In this case, a separate branch was formed by *US_7DA_DQ986288*, *US_HAT_M11458*, and *US_M15239*.

To see whether this phylogeny would also apply for the CP sequence, a similar analysis was done for this cistron. The analysis showed again that TEV-TAMPS clusters separately to the TEV isolates susceptible to RTM (Fig. 2B). In this case, a single group formed by TEV-TAMPS and the *US_NW_L38714* was observed, although the distance between these isolates is con-

siderable. For this phylogenetic tree, different Mexican isolates were not included, due to the unavailability of corresponding CP sequences.

eIF(iso)4E is not required for TEV-TAMPS RNA replication or stability in *A. thaliana* Col-0.

The interaction between eIF(iso)4E and the viral VPg has been proposed by several authors to participate in RNA stability, translation, or movement (Lellis et al. 2002; Okade et al. 2009). Additionally, eIF(iso)4E sequestration by VPg might impair translation of host mRNAs and promote viral RNA translation (Eskelin et al. 2011; Khan et al. 2008). To further understand the potential role of eIF(iso)4E in TEV-TAMPS infection of *A. thaliana*, the replication and presence of viral RNA was evaluated in local and systemic leaves of plants inoculated with semipurified viral particles obtained as described below. In addition to the knockout line (*iso*4E-1, an eIF(iso)4E-overexpressing line on Col-0 ecotype background, (*iso*4E-2, was included in the analysis. Accumulation of the virus positive and negative RNA strands was quantified by real time quantitative (q)RT-PCR in local leaves at 0 and 4 dpi as well as in systemic leaves at 4 and 15 dpi (Fig. 3A and B). The positive RNA level in local leaves at 0 dpi (discussed below) was employed as the reference for each line used in the experiment. It must be noted, here, that the inoculum, despite intense washing, importantly contributed to the number of virus-positive strands detected in the inoculated leaves, particularly at 0 dpi (Fig. 3A), but not to the negative strands (Fig. 3B).

The results indicated that TEV-TAMPS accumulation in local leaves at 4 dpi was impaired in the (*iso*4E-1 knockout mutant and enhanced in the (*iso*4E-2-overexpressing mutant as compared with the Col-0 WT plants (Fig. 3A and B). Accumulation of the viral strands in systemic leaves at 4 and 15 dpi was not detected in the (*iso*4E-1 mutant plants, in contrast to WT and the (*iso*4E-2-overexpressing mutant. Again, and taking together the data of positive and negative viral strands, viral accumulation in systemic leaves was enhanced in (*iso*4E-2 (Fig. 3A and B). These observations indicate that eIF(iso)4E is not strictly required for the replication of TEV-TAMPS RNA in local leaves, as it is in the case of TuMV and LMV inoculations (Duprat et al. 2002), but it is indispensable for the TEV-TAMPS systemic dispersion.

A. thaliana inoculation with TEV-TAMPS was not associated with visible symptoms of viral infection. Therefore, to test whether the systemic spread is able to produce viral proteins, a Western blot with commercially available TEV CP antiserum was performed on local and systemic leaves (Fig. 3C). The observed results were consistent with the behavior of positive and negative RNA strands (Fig 3A and B), and the CP level in systemic tissues was similar in the cases of Col-0 WT and (*iso*4E-2 plants. CP was not detected in systemic leaves of the (*iso*4E-1 knockout. These data further confirm that eIF(iso)4E is required for the TEV-TAMPS movement.

Arabidopsis thaliana eIF(iso)4E is not required for TEV-TAMPS RNA translation.

We evaluated the translational efficiency of TEV-TAMPS in the presence or lack of eIF(iso)4E to determine whether this particular factor is able to enhance translation of the viral RNA in planta. For this assay, inoculated *A. thaliana* leaves, at 4dpi, from (*iso*4E-1, (*iso*4E-2, and WT plants were used. The distribution of viral RNA was measured along sucrose density gradients (polyribosomal fractionation), using qRT-PCR of a region corresponding to the CP RNA. The mRNAs efficiently initiated for translation are found on polyribosomes (high sucrose concentration), whereas a decrease in translation initiation shows a shift towards nonpolyribosome (fractions with

Fig. 3. Effect of absence or overexpression of eIF(iso)4E on TEV-TAMPS RNA accumulation. **A**, The TEV-TAMPS positive or **B**, the negative RNA strand was quantified by real time quantitative reverse transcription-polymerase chain reaction in inoculated *Arabidopsis thaliana* plants from ecotype Col-0 wild-type (white bars), (*iso*4E-1 knockout (black bars), and (*iso*4E-2 overexpressing (gray bars) plants. For quantification, total RNA was extracted from local leaves (0L, 0 days postinoculation [dpi]; 4L, 4 dpi) and systemic leaves (4S, 4 dpi; 15S, 15 dpi). The *eEF1α* mRNA was used as internal control for normalization and the 0L value was considered as basal line in order to represent the rest of samples as fold of change with respect to the inoculated viral RNA. Asterisks (*) indicate the difference was statistically significant at $P < 0.05$. **C**, Immunoblots of total protein extracts from local or systemic leaves of TEV-TAMPS inoculated plants, as described in the upper part of the figure, were performed with anti-coat protein (CP) serum to detect *Tobacco etch virus* (TEV) or with anti-eEF1Bβ serum as loading control.

lower sucrose concentration). The profiles during fractionation were similar for WT, *(iso)4E-1*, and *(iso)4E-2* plants (Fig. 4A), indicating that there were no meaningful changes on global translation levels by the absence or overexpression of eIF(iso)4E. The viral RNA was found in free RNA particles (fractions 2 to 4) and polyribosomal fractions with a peak of abundance between fractions 10 and 14 (Fig. 4B). This indicates that only a fraction of the total positive RNA strand is subjected to active translation in the absence, presence, or overexpression of eIF(iso)4E. The distribution of viral RNA appeared mostly unaltered in *(iso)4E-1* and *(iso)4E-2* lines as compared with WT. The distribution of a cellular mRNA used as control, the eukaryotic elongation factor 1 α (*eEF1 α*) mRNA, was found mostly in heavy polysomes (Fig. 4), indicating that it is efficiently translated in the presence of the viral RNA, regardless of the eIF(iso)4E levels. The presence of viral RNA in the fractions corresponding to polysomes was dependent on ribosome association as shown by puromycin polysome-disrupting treatments (Supplementary Fig. S3). These results do not support the hypothesis that TEV-TAMPS VPg interaction with eIF(iso)4E impairs translation of endogenous transcripts or enhances its own translation, at least in the *A. thaliana* host. Moreover, it was confirmed that eIF(iso)4E is not required for TEV-TAMPS translation.

TEV-TAMPS CP and VPg are found in complexes with *A. thaliana* eIF(iso)4E in planta.

The eIF(iso)4E factor has been previously associated with potyvirus translation and replication (Cotton et al. 2009; Khan

et al. 2006, 2008). However, in this work we obtained evidence that, in *A. thaliana*, the eIF(iso)4E absence or overexpression does not affect translation (association to polysomes). Moreover, eIF(iso)4E absence does not impede virus replication in local leaves. Nevertheless, eIF(iso)4E is required for the viral movement, either cell to cell or at long distance, and its systemic spread. Therefore, we asked whether eIF(iso)4E is particularly associated to viral proteins involved in the systemic spread. This interaction was tested by coimmunoprecipitation assays using protein extracts from *(iso)4E-1*, *(iso)4E-2*, and WT local leaves at 4 dpi. Three immunoprecipitations were performed using the antibody for *A. thaliana* eIF(iso)4E (IP-[iso]4E) or eIF4E (IP-4E) and the antibody against TEV CP. The immunoprecipitated protein complexes were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), were blotted, and were detected with antibodies against eIF(iso)4E, eIF4E, CP, and NIa (Fig. 5).

The Western blot against eIF(iso)4E and eIF4E in IP-(iso)4E and IP-4E demonstrated that the antibodies are able to specifically distinguish each isoform without cross-reactivity and, hence, each immunoprecipitate is expected to contain protein complexes specific for each isoform. eIF(iso)4E was not detected in *(iso)4E-1* plants, and none of the other Western assays showed a detectable product for the IP-(iso)4E of the knockout (Fig. 5 second panel). Detection with either α -CP or α -NIa in the same immunoprecipitate rendered a positive signal for WT and *(iso)4E-2* but not in the *(iso)4E-1* knockout, indicating specific coimmunoprecipitation of both viral proteins in complex with eIF(iso)4E. To corroborate the specificity of this

Fig. 4. Quantitation of TEV-TAMPS RNA in polysomal fractions from *Arabidopsis thaliana* wild-type Col-0 (black bars), *(iso)4E-1* knockout (white bars), and *(iso)4E-2* overexpressing (gray bars) plants. **A**, The polysomal fractionation was performed on continuous sucrose gradients (15 to 60%), and profiles were detected at 260 nm. **B**, The RNA isolated from each fraction (pair numbers) was subjected to quantitative reverse transcription-polymerase chain reaction for the viral RNA (CP), and cellular *eEF1 α* RNA. The percentage of RNA corresponding to each sucrose fraction is shown. The distribution of viral RNA along the profile was not affected in the *(iso)4E-1* knockout or *(iso)4E-2* overexpressing mutants, similar to the behavior of the cellular mRNA in mono (M) and polysomal (P) pools.

interaction, immunoprecipitation assays using α -CP or α -eIF4E were performed with protein extracts from 4-dpi TEV-TAMPS local leaves. The CP was detected in immunoprecipitation assays of all plants, since the extract was obtained from local leaves. However, eIF(iso)4E and NIa were immunodetected only in the immunoprecipitation assays from WT and (iso)4E-2 plants (Fig. 5, third panel). On the other hand, immunodetection performed with the same antibodies of IP-4E complexes returned positive for eIF4E and NIa but not CP and eIF(iso)4E (Fig. 5, fourth panel). These data suggest the presence of at least two different viral protein complexes, one involving CP, NIa, and eIF(iso)4E, the other one including NIa and eIF4E. The antibody against NIa includes the portion corresponding to VPg, and the band detected in immunoprecipitates was about 26 kDa, indicating that the protein found in these complexes is actually VPg. Therefore, TEV-TAMPS VPg is able to interact in planta with either eIF(iso)4E or eIF4E from *A. thaliana*, similar to reports of previous in vitro and in vivo studies with VPg from other potyviruses (Grzela et al. 2006).

DISCUSSION

Members of the translation initiation factor eIF4E family constitute a major group of host-recessive resistance genes for potyviruses (Nicaise et al. 2007; Piron et al. 2010; Robaglia and Caranta 2006; Ruffel et al. 2002). Several genetic and biochemical approaches have demonstrated that the VPg is the predominant factor mediating the eIF4E resistance and susceptibility mechanism. Based on the eIF4E function in eukaryotic translation and on the observed VPg interference of its binding to a Cap (7 mGTP) analogue or during in vitro mRNA translation (Grzela et al. 2006; Khan et al. 2008), it has been proposed that the resistance mechanism mediated through eIF4E family members is at the level of viral translation enhancement,

cellular translation inhibition, or both. However, other evidences argue against this proposal. One of these is the presence of an internal translation initiation mechanism promoted by specific regions found in the 5' untranslated region of several potyviral RNAs able to efficiently support in vitro translation by directly binding other initiation factors, such as eIF4G or eIF(iso)4G, without the presence of eIF4E or eIF(iso)4E (Gallie 2001; Ray et al. 2006). The other is that inhibiting cellular translation through eIF4E sequestration by VPg, as has been demonstrated by in vitro and, recently, in vivo experiments (Eskelin et al. 2011; Miyoshi et al. 2008), would not fully explain the specificity of the virus requiring a particular eIF4E isoform depending upon the host.

In this work, we aimed to dissect the level of eIF(iso)4E participation in the infection of *A. thaliana* with a Mexican isolate of the TEV named TEV-TAMPS. When analyzing inoculations of TEV-TAMPS in *A. thaliana* Col-0 WT and *eIF(iso)4E* knockout plants, we noticed that this isolate reproducibly overcame the RTM mechanism only in the WT plants. TEV-TAMPS mutations in VPg and CP, both proteins involved in the systemic movement of potyviruses, might be responsible for overcoming resistance mechanisms such as RTM and allowing the infection of *A. thaliana*. Recent publications support this idea, showing that amino acid substitutions, particularly at the N-terminus of CP, are determinants for the ability of LMV and PPV isolates to break the RTM resistance mechanism in *A. thaliana* (Decroocq et al. 2009). Furthermore, mutations in the VPg protein from TuMV have been shown to be sufficient to break the resistance imposed by *A. thaliana* *eIF(iso)4E* or *eIF(iso)4G* knockout mutants by a mechanism independent of its interaction with these factors (Gallois et al. 2010).

The null mutation for eIF(iso)4E in *A. thaliana* (Duprat et al. 2002) was previously reported as resistant to both TuMV and LMV, even at local tissues. The observation that TEV-TAMPS is able to overcome the RTM mechanism only in the

Fig. 5. Interaction of *Arabidopsis thaliana* Col-0 eIF(iso)4E and eIF4E with TEV-TAMPS proteins. Immunoprecipitation assays were performed using total protein extracts obtained from local leaves of *A. thaliana* wild-type Col-0, (iso)4E-1 knockout, and (iso)4E-2 overexpressing plants at 4 days postinoculation. Immunoprecipitates (IP) with α -CP-TEV, α -(iso)4E, and α -4E, as well as 10 μ g of total protein extract (Input) were resolved with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were immunoblotted against α -CP-TEV, α -NIa-TEV (which also recognizes viral genome-linked protein [VPg]), α -(iso)4E and α -4E. IP with α -(iso)4E (IP-[iso]4E) contained complexes formed by *A. thaliana* eIF(iso)4E and the coat protein (CP) (30 kDa) and VPg (26 kDa) viral proteins but not the *A. thaliana* eIF4E protein. Reciprocally, the IP with α -CP (IP-CP) contained the same proteins. The IP with α -4E (IP-4E) did not show the presence of viral CP or *Arabidopsis* eIF(iso)4E but displayed a positive signal for the viral VPg (in α -NIa).

presence of eIF(iso)4E but is detected in local tissues regardless the eIF(iso)4E presence allowed us to study in planta the viral translation, replication, and systemic spread. Our results indicated that abolishment of eIF(iso)4E did not impede TEV-TAMPS RNA accumulation in local leaves, although overexpression of this protein positively influenced, to some extent, viral replication in both local and systemic leaves (Fig. 3). Such observation is consistent with previous findings describing the presence of eIF(iso)4E and other translational factors, such as the PolyA binding protein 2 (PABP2) and the translation elongation factor 1A (eEF1A) in large vesicles associated with viral replication (Cotton et al. 2009; Hafren et al. 2010). However, TEV-TAMPS replication was not strictly dependent on the eIF(iso)4E presence, since the knockout mutant presented newly synthesized negative RNA strands in local leaves (Fig. 3B). In addition, this mutant did not show impairments in global cellular translation or in the viral RNA translation (Fig. 4), ruling out the requirement of eIF(iso)4E for these steps in the viral infective cycle, at least for the TEV-TAMPS–*Arabidopsis* system. Other work makes reference to two different sites within eIF4E that independently bind VPg and the Cap analogue m⁷GDP with similar affinity in vitro. The VPg-binding site would be different but structurally related to the CAP-binding site and reduces the mRNA Cap affinity (Michon et al. 2006). Similar interactions could occur with the TEV-TAMPS VPg and eIF(iso)4E of *A. thaliana* Col-0 but probably without a reduction in the mRNA Cap affinity, as there was not an interference with host mRNA translation.

Using co-immunoprecipitation assays, we were able to detect the TEV-TAMPS VPg in either eIF(iso) 4E or eIF4E complexes. However, we found the viral CP only in the eIF(iso)4E complexes (Fig. 5). The presence of CP only in the eIF(iso)4E-dependent protein complex has not been previously reported and could correlate with the requirement of this specific isoform for the viral movement in *A. thaliana*. Whether the interaction between eIF(iso)4E and CP is direct or is supported by other proteins within the complex remains to be solved. However, it is unlikely that VPg would recruit CP in the complex, since we found VPg but not CP in the eIF4E-bound complexes. Taking into account that both CP and VPg are proteins required for viral movement, we propose that particular complexes formed by eIF(iso)4E and these proteins are involved in the systemic spread of TEV-TAMPS. In the absence of eIF(iso)4E, such complexes could not be formed, hence, the viral movement is restricted.

Previous reports have described the interaction between eIF(iso)4E and TuMV VPg or its precursors VPg-Pro (NIa) and 6K-VPg-Pro (6K-NIa), suggesting a role in the disruption of cellular functions or in viral translation or replication (Beauchemin et al. 2007; Cotton et al. 2009). *A. thaliana* is a TuMV-susceptible plant showing severe symptoms upon infection with this potyvirus. The same plant species could be infected by TEV-TAMPS isolates without developing any obvious symptoms. For both TuMV and TEV *A. thaliana* susceptibility, the requirement of eIF(iso)4E has been demonstrated (Duprat et al. 2002; Lellis et al. 2002). However, whereas the presence of TuMV RNA could not be detected in the inoculated leaves of an *A. thaliana* eIF(iso)4E knockout (Duprat et al. 2002), viral products from TEV-TAMPS could be observed in local leaves when compared with WT plants, even though the inoculated virus might account for the majority of the detected RNA. Therefore, replication of TuMV strands may be strictly dependent on eIF(iso)4E, whereas replication of TEV, although negatively affected, still occurs in the absence of eIF(iso)4E.

The analysis of CP and VPg sequences reveals that specific amino acid changes may be responsible for the ability of TEV-TAMPS to overcome the RTM resistance mechanism. It will

be interesting to get a further insight in the phylogeny of these proteins, when more sequences for both proteins are available from the same isolates in different geographic regions. Noticeably, TEV-TAMPS VPg interacts with both eIF(iso)4E and eIF4E in planta. However, each isoform is apparently involved in different complexes with the viral proteins, leading us to conclude that they probably exert different roles within the viral life cycle.

MATERIALS AND METHODS

Plant and virus materials.

Arabidopsis thaliana Columbia 0 (Col-0) ecotype was used as WT as well as the genetic background for the *eIF(iso)4E* knockout *AteIF(iso)4E-1* (Duprat et al. 2002) and the *eIF(iso)4E*-overexpressing mutant *AteIF(iso)4E-2* (Martinez-Silva et al. 2012) lines. For some experiments, the ecotype Columbia 24 (C24) reported as susceptible to the TEV systemic movement was used (Mahajan et al. 1998). Individual plants were grown in pots with Metromix 200 substrate and were kept at 23°C, under 100 µmol m⁻² s⁻¹ light intensity, with an 8-h light and 16-h dark photoperiod, for 6 weeks before inoculation.

TEV-TAMPS was used for inoculation of all plants. TEV-TAMPS from the federal Mexican state of Tamaulipas was propagated in *Nicotiana tabacum* cv. Xanthi plants (6 weeks old) and were maintained under standard greenhouse conditions. The TEV-7DA isolate used in this work was expressed from a p35TEV plasmid previously reported (Bedoya and Daros 2010) and was propagated in *N. tabacum* cv. Xanthi plants (6 weeks old) under greenhouse conditions. TEV-TAMPS and TEV-7DA viral particles were partially purified from *N. tabacum* symptomatic leaves, according to a procedure previously described (Gopalan 2007). The amount of viral particles was determined using ELISA quantitation with α-CP-TEV (Agdia Inc., Elkhart, IN, U.S.A.). Six-week-old rosette leaves from *A. thaliana* plants were mechanically inoculated using a cotton swab tip with estimated similar amounts of viral particles.

Total RNA isolation and TEV-TAMPS detection by final point RT-PCR.

Total RNA from *A. thaliana* and *N. tabacum* plants was extracted using the Trizol method (Invitrogen Corp.; San Diego, CA, U.S.A.) and following the manufacturer's indications. The concentration was quantified by measuring absorbance at 260 nm, and its quality was verified by electrophoresis on a 1% agarose gel. The reverse transcription reaction was performed with M-MLV reverse transcriptase (Invitrogen Corp.) according to the manufacturer's instructions, using 1 µg of total RNA. Oligo dT was used as primer in the reverse transcriptase reaction. PCR assays were performed with recombinant *Taq* polymerase (Invitrogen Corp.) following the manufacturer's instructions, using the appropriate primers as described in Supplementary Table S1. The amplified products were resolved on a 1% agarose gel electrophoresis.

Polysomal RNA purification and analysis.

The separation of polysomal complexes was performed using 2 g of TEV-inoculated leaves, according to a previously described protocol (Davies and Abe 1995), adapted to *A. thaliana* (Martinez-Silva et al. 2012). After concentrating through a 60% sucrose cushion, ribosome-bound RNA was loaded on 15 to 60% sucrose gradients and was centrifuged for 2 h at 4°C in a SW-55 rotor at 250,000 × g. Nine fractions were separated with an Auto Densi-flow system (Labconco, Kansas City, MO, U.S.A.) coupled to a 260 nm absorbance detector Econo UV Monitor EM-1 (Bio-Rad, Hercules, CA, U.S.A.) and a plotter LKB BROMMA 2210 (Pharmacia LKB, Uppsala, Sweden) to

obtain the polysomal profiles. The RNA was extracted from each fraction using phenol-chloroform. The treatment with puromycin was done by adding GTP buffer (20 mM Tris-HCl, pH 8, 400 mM KCl, 3 mM MgCl₂, and 0.5 mM puromycin) to the pellet obtained after the 60% sucrose cushion centrifugation and incubating for 45 min at 37°C. Afterwards, the extract was run through a 15 to 60% sucrose gradient.

qRT-PCR.

RNA (2 µg) were treated with RQ1 DNase (Promega Corp., Madison, WI, U.S.A.) and were reverse-transcribed using M-MLV-RT (Invitrogen). The qPCR was performed with qTEV primers designed with the aid of the "Primer Tools" within the Geneious program). *eEF-1α* was used as an internal normalizing control gene. For the negative viral-strand quantitation, the 5' forward oligonucleotide TEV3 was used in the reverse transcriptase reaction. The PCR amplification was performed in a 7500 DNA analyzer (Applied Biosystems, Foster City, CA, U.S.A.), using SYBR Green PCR master mix (Applied Biosystems) for signal detection. The relative viral expression was calculated after normalization with the reference gene (*eEF-1α*), using the $2^{-\Delta\Delta C_t}$ method and was represented as fold of change with respect to the inoculated RNA (either positive or negative strand) collected from the local leaves 2 h after inoculation. To avoid with the quantification of inoculated viral particles that did not enter the plant cells, the local leaves were extensively washed with virus-free solution and, afterwards, were subjected to RNA isolation or were further grown under standard conditions to collect local leaves at 4 dpi and systemic leaves at 4 or 15 dpi. Tukey honestly significant difference analysis ($P < 0.05$) was performed on the data to estimate statistical differences between the three *A. thaliana* lines (WT, [iso]4E-1, and [iso]4E-2). The quantification of viral RNA distributed along polysomal fractions, obtained by sucrose gradients, was performed as described previously (Dinkova et al. 2005; Martínez-Silva et al. 2012), and the percentage of the RNA in nonpolysomal and polysomal fractions was determined.

Immunoblot and ELISA assays.

Total proteins were extracted by grinding 500 mg of leaf tissue and homogenizing it in 500 µl of extraction buffer (200 mM Tris-HCl, pH 8.5, 50 mM KCl, 25 mM MgCl₂, 2 mM EGTA, 2% PTE (polyoxyethylene [10] tridecyl ether), 1% Igepal, and 5 µg of cycloheximide per milliliter, 1 mM dithiothreitol (DTT), and 1 mM orthovanadate). After centrifugation (12,000 × g, 5 min), the supernatant was recovered, and the concentration was determined by the Bradford assay (Bio-Rad), using bovine serum albumin as standard. After SDS-PAGE, 5 µg of total proteins were used directly for immunoblot analysis. The α-eIF4E and α-eIF(iso)4E antibodies supplied by K. S. Browning from the Department of Chemistry and Biochemistry, University of Texas at Austin, were used at 1:10,000 dilution. The α-CP-TEV (Agdia Inc.) was used at 1:10,000 dilution, and the α-NIA antibody (from W. Dougherty) was used at 1:15,000 dilution. The antigen-antibody complexes were detected using a horseradish peroxidase-coupled goat α-rabbit immunoglobulin G (IgG) according to the manufacturer's recommendations. Immunoreactive bands were detected with Immobilon Western Chemiluminescent HRP substrate (Millipore, Bedford, MA, U.S.A.).

For ELISA experiments, 100 µg of total proteins obtained from uninfected or infected (15 dpi) systemic leaves of *A. thaliana* Col-0 with TEV-TAMPS or TEV-7DA were incubated, with α-CP-TEV (Agdia) as capture antibody at 1:500 dilution. As secondary antibody, an alkaline phosphatase-coupled goat α-rabbit IgG was used at a 1:1,000 dilution. The activity of alkaline phosphatase was indirectly estimated at 450 nm using

NBT (nitro-blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt) in carbonate buffer (0.1 M NaHCO₃, 1 mM MgCl₂, pH 9.8) as substrate.

Immunoprecipitation assays.

To evaluate interactions between eIF(iso)4E and viral proteins, 3 g of local or systemic leaves were ground. The slurry was transferred to 15-ml tubes with 5 ml of extraction buffer (200 mM Tris-HCl, pH 8.5, 50 mM KCl, 25 mM MgCl₂, 2 mM EGTA, heparin at 100 µg/ml, 2% PTE, 1% Igepal [previously Nonidet P40], and cycloheximide at 5 µg/ml, 1 mM DTT, and 1 mM orthovanadate). The extracts were vigorously mixed and were centrifuged for 15 min at 10,000 × g at 4°C. The upper clear supernatant was used to perform immunoprecipitation assays according to a previous report (Peritz et al. 2006), using Protein Agarose A (Millipore). The α-eIF(iso)4E, α-CP-TEV, and α-eIF4E antibodies were used at 1:1,000 dilutions. The total protein extracts (Input) and the immunoprecipitates were analyzed by immunoblots.

TEV-TAMPS VPg and CP sequencing and phylogenetic analysis.

The TEV-TAMPS isolate was obtained from *Capsicum annum* cultivars in the county of Tampico Madero in the federal state of Tamaulipas, Mexico. The nucleotide sequences of VPg and CP were obtained after RT-PCR amplification, using specific oligonucleotides and cloning into pGEM-T Easy vector (Promega Corp.). Both sequences were cured using the BioEdit Sequence Alignment Editor, version 7.0.9.0 for Windows, and were tested against GenBank sequences using the Blast software freely accessible on the NCBI website. The sequences were manually assembled and deposited in the GenBank database with the following accession numbers: TEV-TAMPS VPg, *JX512812_MX_TAMPS*; TEV-TAMPS CP, *JX512813_MX_TAMPS*.

Available VPg and CP sequences from other TEV isolates were obtained from GenBank. Unfortunately, CP sequences were not available for all accessions used in the VPg analysis and vice versa. Nucleotide sequences for both VPg and CP cistrons were aligned using Geneious Pro 4. 8.3 created by Biomatters (available from the Biomatters Geneious Pro website) with the Clustal W algorithm (Larkin et al. 2007) and cost matrix with gap extension cost of 100. The aligned nucleotide sequences were further translated *in silico*, with the same program, and were used with the Geneious tree builder with the Jukes-Cantor genetic distance model and the neighbor-joining method. A bootstrapping with a random seed of 500,000 and 1,000 replicates and no outgrouping was used. The consensus tree was selected with a threshold of 50.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Biomatters Geneious Pro website: www.geneious.com
 NCBI Blast software: blast.ncbi.nlm.nih.gov/
 NCBI GenBank: www.ncbi.nih.gov