INTRODUCTION

The outcome of virus infections is ultimately controlled by molecular interactions between host and viral factors. Consequently, understanding of the complex interaction network linking these partners in infected plant cells is crucial to elucidate the coordinated roles of the viral and cellular factors involved in the infection process. Recent efforts have generated a first vision of the protein interaction network linking these partners in infected plant cells is crucial to elucidate the coordinated roles of the viral and cellular factors involved in the infection process. Recent efforts have generated a first vision of the protein interaction network in the case of members of the genus Potyvirus, the largest and most economically important genus of positive-stranded ssRNA plant viruses (Shukla et al., 1994). As an example, cellular proteins Hsc70-3 and PABP2 and the viral RNA-dependent RNA polymerase Nlb protein have been proposed to participate in the turnip mosaic virus (TuMV) replication complex (Dufresne et al., 2008). Similarly, although their precise contribution to the viral infection process remains to be established, several chloroplastic proteins (RuBisCO, 37 kDa, PSI-K, Rieske Fe/S, NtMind and Fd V) have recently been shown to interact with various potyviral proteins, including the capsid protein (CP), the cylindrical inclusion (CI) helicase, and the P1 and HCPro proteins (Cheng et al., 2008; Feki et al., 2005; Jiménez et al., 2006; Jin et al., 2007; McClintock et al., 1998; Shi et al., 2007). In several cases, reduction or abolition of the expression of these cellular proteins has been shown to affect virus accumulation, thus providing functional validation of a contribution of these cellular partners to virus invasion of the host (Duprat et al., 2002; Lellis et al., 2002).

A cell protein potentially involved in transcriptional control (VIP) and two host RNA helicase-like proteins (AtRH8 and PpDDXL) (Dunoyer et al., 2004; Huang et al., 2010) have been shown to interact with the potyviral VPg. However, one of the most extensively described plant–potyvirus interactions involves the eukaryotic translation initiation factor 4E (eIF4E) or its isoform eIF(iso)4E, and the potyviral VPg. For several potyviruses, this interaction has been shown to be absolutely required for virus infection, so that alleles of eIF4E unable to interact with the VPg of an infecting virus behave as recessive resistance genes (Kang et al., 2005; Yeam et al., 2007). In many crop species, including pepper, tomato, pea, lettuce, melon and barley, eIF4E and/or its isoform eIF(iso)4E are the only recessive resistance genes identified so far (Robaglia & Caranta, 2006). Moreover, eIF4G, another component of the eIF4F translation complex, or eIF4F itself, have been shown to interact directly or indirectly...
with the potyviral VPg (Khan et al., 2006; Michon et al., 2006), and both eIF4E and eIF4G are recruited in a coordinated manner by potyviruses in Arabidopsis thaliana (Nicaise et al., 2007). This suggests that these translation initiation factors are required for virus infection and resistance function, opening the route to the investigation and creation of new durable resistances (Piron et al., 2010). Furthermore, it was shown that mutations in the VPg of eIF4E-mediated resistance-breaking potyviruses restore binding with the product of resistance alleles, suggesting that eIF4E and VPg are involved in a co-evolutionary arms race between host plant and virus (Charron et al., 2008). To date, VPg is the main potyviral protein identified as a virulence determinant in eIF4E-mediated resistance breaking (Ayme et al., 2006). The eIF4E–VPg interaction has been characterized in detail through in vivo and in vitro experiments, even if its exact biological function still remains unclear. Biological bimolecular fluorescence complementation (BiFC), immunochemical (ELISA) and spectrophotometric (fluorescence measurements) approaches led, for example, to the description of a subcellular localization in planta (Beauchemin et al., 2007), to mapping of the central domain of VPg as the virus-interacting domain (Roudet-Tavert et al., 2007) and to definition of biochemical quantitative parameters for the eIF4E–VPg interaction (Khan et al., 2006; Michon et al., 2006).

A salient feature of members of the family Potyviridae is the accumulation in infected host cells of laminate cytoplasmic inclusion bodies ('pinwheels') (Edwardson, 1992), formed by the so-called CI protein. Like most other potyviral proteins, the CI is a multifunctional protein. Structural and genetic evidence has unambiguously shown its involvement in virus cell-to-cell movement (Carrington et al., 1998; Gómez de Cedrón et al., 2006; Roberts et al., 1998; Wei et al., 2010), whilst its RNA-binding, RNA helicase and ATPase activities established its role in virus replication (Fernández et al., 1995, 1997; Merits et al., 1998). Beside its self-interaction (López et al., 2001), the CI has been described in yeast two-hybrid (YTH) screens or overlay assays as binding to other viral proteins, including the HC-Pro protein (Guo et al., 2001) and the P1 and P3 proteins (Merits et al., 1998), confirming previous microscopy observations in the last two cases (Arbatova et al., 1998; Rodríguez-Cerezo et al., 1993). The CP of several potyviruses has been shown to be associated with the CI in pinwheels and in plasmodesmata (Rodríguez-Cerezo et al., 1997), whilst the CI of potato virus A (PVA) co-purified with isolated virus particles (Gabrenaitė-Verkhovskaya et al., 2008), further reinforcing the evidence for the existence of CI–CP interactions. A few cellular CI-interacting proteins have been identified (Bilgin et al., 2003; Jiménez et al., 2006), and the TuMV and soybean mosaic virus CIs have been shown to be possible viral targets recognized by dominant resistance genes (Jennér et al., 2000; Zhang et al., 2009). All of these results suggest that the potyviral CI protein is an important player in the plant–potyvirus interaction network.

Previous studies have shown that eIF4E controls lettuce susceptibility to the potyvirus lettuce mosaic virus (LMV) (Nicaise et al., 2003). Indeed, the widely used mo1 and mo1' natural resistance alleles of the mo1 gene correspond to allelic forms of eIF4E and, depending on the particular LMV isolate, confer either a true-resistance phenotype (absence of detectable virus accumulation in inoculated plants) or a tolerance phenotype (lack of symptoms and sporadic virus accumulation). As for other potyviruses, the VPg of LMV interacts with the lettuce eIF4E in vitro (Michon et al., 2006; Roudet-Tavert et al., 2007) and in vivo (S. German-Retana, unpublished results). However, in contrast to other systems, this interaction does not seem to be affected by the specific virus isolate–lettuce variety combination under investigation, so that the VPg of non-resistance-breaking isolates was shown to bind with essentially similar affinity to the susceptible eIF4E allele (eIF4E0) or to resistance alleles (eIF4E1 or eIF4E2) (T. Michon and others, unpublished results). Reverse-genetic studies have shown that resistance breaking is under complex control, with two LMV genomic regions able to independently confer the ability to overcome eIF4E-mediated resistance (Abdul-Razzak et al., 2009). Mutations in the VPg allow only the overcoming of mo1', but mutations in the C-terminal part of the CI helicase confer the ability to overcome both resistance alleles, demonstrating for the first time a role for CI in eIF4E-mediated resistance. In particular, a point mutation at position 621 of CI, changing a serine to a threonine, was shown to be sufficient to allow both mo1' and mo1' breaking (Abdul-Razzak et al., 2009).

In the present study, the possible binding of the CI helicase to the viral VPg and to its eIF4E cellular partner was investigated. For each protein, various allelic forms (resistance-breaking or non-resistance-breaking for viral proteins, susceptibility- or resistance-conferring for cellular proteins) were expressed and purified from Escherichia coli as recombinant fusion proteins and their interactions were analysed in vitro in ELISA-based assays. Furthermore, by using a BiFC approach, the interaction of the CI helicase with eIF4E was validated in vivo. These results demonstrate that the C-terminal end of the CI helicase, involved in resistance breaking, is able to interact with both the viral VPg and lettuce eIF4E.

RESULTS

The C-terminal portion of LMV CI binds in vitro to lettuce eIF4E

The in vitro binding of the C-terminal region of the LMV CI (aa 372–643 according to the LMV-0 CI amino acid sequence, GenBank accession no. X97704) to the lettuce eIF4E was first investigated. In order to evaluate possible binding under a plant–virus compatible interaction, the CI-Cter protein was derived from the common, non-resistance-breaking LMV-0 isolate (hereafter designated
CI-0), and eIF4E0, encoded by the lettuce mol0 susceptibility allele, was used. Both viral and cellular proteins were produced as recombinant fusion proteins in *E. coli* and purified to near homogeneity as described in Methods. The interaction between the two proteins was investigated using two-site ELISAs, in which the bait protein is first immobilized into the wells of ELISA plates and the binding of the prey interacting protein is revealed by using specific antibodies. After checking the specificity of the antibodies and detecting conjugates (see titration data of immobilized baits in Supplementary Fig. S1a, available in JGV Online), both orientations of the assay (immobilization of either CI-Cter or eIF4E in the wells) were used. Several control treatments were performed systematically, including omitting the immobilized bait, replacing it with purified *Aequorea victoria* GFP or omitting the prey protein in the assays. The results obtained are shown in Table 1. As indicated by *A*405 measurement, significant binding of the second partner (prey) was observed, irrespective of whether CI-0 or eIF4E0 was used as the immobilized bait. All control assays (Table 1), in which one or other of the partner proteins was omitted or replaced by GFP, gave *A*405 values that were either much lower than when testing the CI-Cter–eIF4E interaction or negligible, indicating that the detected interaction signal is specific. Further experiments demonstrated that the CI-Cter–eIF4E interaction signal is dose-dependent, as the *A*405 values for CI-Cter or eIF4E binding increased as a function of the concentration of the prey interacting protein, irrespective of which partner was used as the immobilized bait (Fig. 1).

These ELISA binding assays were then used to assess whether the detected interaction was affected by the identity of the allelic forms of the CI-Cter or of the eIF4E used and thus, ultimately, by the compatibility of the interaction between the LMV isolate providing the CI and the lettuce variety providing the eIF4E.

### Impact of allelic variations on the interaction between the C-terminal portion of LMV CI and the lettuce eIF4E

The viral CI-Cter proteins derived from either non-resistance-breaking (LMV-0) or resistance-breaking (LMV-E) isolates were compared for their ability to bind to each of the three allelic forms of eIF4E, from susceptible (eIF4E0) or resistant (eIF4E1 or eIF4E2) lettuce. The respective reactivities

<table>
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<tr>
<th>Immobilized bait</th>
<th>Interactant prey</th>
<th>Detection antibody</th>
<th><em>A</em>405</th>
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<tbody>
<tr>
<td>CI-0</td>
<td>CI-0</td>
<td>2A7</td>
<td>0.004 ± 0.000</td>
</tr>
<tr>
<td>CI-0</td>
<td>eIF4E0</td>
<td>2A7</td>
<td>0.304 ± 0.011</td>
</tr>
<tr>
<td>CI-0</td>
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<tr>
<td>CI-0</td>
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<td>38</td>
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</tr>
<tr>
<td>CI-0</td>
<td>eIF4E0</td>
<td>38</td>
<td>0.733 ± 0.051</td>
</tr>
<tr>
<td>CI-0</td>
<td>eIF4E0</td>
<td>38</td>
<td>0.159 ± 0.020</td>
</tr>
<tr>
<td>GFP</td>
<td>eIF4E0</td>
<td>38</td>
<td>0.120 ± 0.001</td>
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**Fig. 1.** Binding of the C-terminal part of the CI protein of LMV-0 (CI-0) to lettuce eIF4E0 in ELISA-based assays is dose-dependent. Wells were coated with either CI-0 (open bars) or eIF4E0 (filled bars), both purified proteins being used at a concentration of 4 μg ml−1. The purified prey proteins were used at variable concentrations. The *A*405 values are the means ± SD of three replicates from a typical experiment, after subtraction of the background value (obtained omitting immobilized bait).
obtained for the CI-0–eIF4E0 interaction was always lower
observed with CI-E (grey bars). In addition, the signal
readings tested than that seen with either eIF4E 1 or eIF4E 2.
Indeed, the CI-Cter from LMV-0 (CI-0) and the CI-Cter
forms bound to all three eIF4E allelic forms. However, despite the fact that similar amounts of CI-
and CI-E were used in all assays and that all isoforms showed comparable reactivity towards the detecting antibodies (see above), the A405 values measured with CI-0 [Fig. 2(a), white bars] were systematically lower than those observed with CI-E (grey bars). In addition, the signal obtained for the CI-0–eIF4E0 interaction was always lower than that obtained when either eIF4E1 or eIF4E2 was used. Both effects were reproducible and observed irrespective of which protein was used as the immobilized bait. On the other hand, no clear or reproducible differences were observed for the binding of different eIF4E allelic forms and CI-E.

To further evaluate the differences in binding observed for CI-0 between the various forms of eIF4E, measurements were performed in the presence of varying concentrations of the various eIF4E forms. Fig. 2(b) shows that the binding for eIF4E0 is significantly lower at all concentrations tested than that seen with either eIF4E1 or eIF4E2. This result was reproducible, and quantitative analysis of the ELISA data could be performed, allowing estimation of the dissociation constant of the CI-0–eIF4E0 binding at around 0.2 μM, while those for eIF4E1 and eIF4E2 were found reproducibly to be significantly lower (Kd=0.01–0.1 μM), confirming the results obtained using fixed concentrations of the three proteins. Similar experiments performed in parallel using CI-E did not show any significant differences in its binding with the various eIF4E forms, with essentially superimposable curves (see Supplementary Fig. S2a, b, available in JGV Online), with an evaluated Kd=0.024–0.08 μM for the three forms of eIF4E. Again, these results are in accordance with the data shown in Fig. 2(a) and obtained with fixed protein concentrations, although a slightly higher signal could sometimes be observed when eIF4E2 was used as the bait [see Fig. 2(a), right panel]. It therefore appears that the CI from the non-resistance-breaking LMV-0 isolate shows a lower affinity for the eIF4E factor from a susceptible lettuce cultivar and, more generally, that it binds less efficiently to all forms of eIF4E than the CI from the resistance-breaking LMV-E isolate.

Infection phenotype-altering mutations at position 621 of the CI C-terminal portion do not affect in vitro eIF4E-binding properties significantly

We showed previously by site-directed mutagenesis of the CI-Cter protein that a mutation at position 621 of the CI was sufficient to affect, in a reciprocal manner, the infection phenotype of the parental viruses LMV-0 and LMV-E. The S621T point mutation in LMV-0 is sufficient to confer resistance-breaking properties to LMV-0 and, conversely, the T621S mutation in LMV-E abolished its ability to overcome the mol1 or mol2 resistance genes. We therefore investigated whether these mutations affect the eIF4E-binding properties of the LMV-0 or LMV-E CI-Cter proteins.

The mutant forms CI-0-S621T and CI-E-T621S were expressed in E. coli and purified using the same purification
procedure as their wild-type counterparts. After verifying that all proteins reacted similarly with the detection antibodies (not shown), the ability of each mutant to bind eIF4E\textsuperscript{0} was then compared with that of its wild-type counterpart. Both mutated forms were able to interact with eIF4E\textsuperscript{0} and gave an interaction signal that was not significantly different from that observed with the cognate wild-type protein, indicating that point mutations at position 621 do not significantly affect the in vitro eIF4E-binding properties of either of the CI-Cter forms studied (Supplementary Fig. S3a, available in JGV Online). Similar binding experiments were conducted in parallel using the eIF4E\textsubscript{1} and eIF4E\textsubscript{2} forms (see Supplementary Fig. S3b) and demonstrated that, similarly to the situation with eIF4E\textsuperscript{0}, binding to the two resistance allelic forms of eIF4E is not affected by mutations at position 621 of CI.

The C-terminal portion of the LMV CI interacts in planta with the lettuce eIF4E

To test whether lettuce eIF4E and the LMV CI-Cter are able to associate in vivo, BiFC assays (Bracha-Drori et al., 2004) were performed following transient expression of eIF4E\textsuperscript{0} and CI-0 in Nicotiana benthamiana leaves using agroinfiltration. In this system, the N- and C-terminal fragments of YFP were both fused to the N and C termini of the coding sequences of lettuce eIF4E or LMV CI-Cter and all potentially compatible combinations were evaluated. A cytoplasmic fluorescence signal was observed reproducibly for the eIF4E-YN + YC-CI-Cter and eIF4E-YC + YN-CI-Cter combination (Fig. 3a–d), whereas no signal (or, in some cases, a very slight background signal clearly distinguishable from the one observed in Fig. 3a–d) was detected following individual expression of eIF4E-YN, eIF4E-YC, YC-CI-Cter or YN-CI-Cter (Fig. 3e, f; Supplementary Fig. S4c, f, available in JGV Online). In addition to those negative controls, a BiFC experiment was performed where the following combinations were checked: eIF4E-YN–eIF4E-YC and YC-CI-Cter–YN-CI-Cter. No fluorescence signal was recovered in these control experiments, except sometimes the background shown in Supplementary Fig. S4. Taken together, these results show that the LMV CI-Cter fragment is able to interact physically with lettuce eIF4E in the cytoplasm of tobacco cells.

The LMV C-terminal part of the CI helicase binds to the viral VPg

The VPg proteins from LMV-0 and LMV-E were expressed in E. coli and purified to near homogeneity as polyhistidine-tagged proteins. Potential interactions between the LMV CI-Cter and VPg were then evaluated in ELISA-based assays using the same experimental set-up used above to investigate CI-Cter–eIF4E interactions. As reported previously when analysing eIF4E–VPg interactions (Roudet-Tavert et al., 2007), erratic and irreproducible results were obtained when the VPg was used as the immobilized bait and this test format was therefore not investigated further (results not shown). However, when the CI-Cter protein from LMV-0 was used as bait, significant and reproducible VPg-binding signals were obtained, as illustrated in Table 2.

The detected CI-Cter–VPg interaction was specific, as no signal was obtained when either of the protein partners was omitted or replaced by GFP (used as a control) (Table 2). In homologous assays in which the binding of the VPg of a given isolate was assayed with the CI-Cter from the same isolate, the interaction was always observed. However, in parallel assays using titrated amounts of the two proteins (see Supplementary Fig. S1a), the CI-E–VPg-E interaction

![Fig. 3. Detection of in planta interactions between the LMV-0 CI-Cter and lettuce eIF4E\textsuperscript{0} in a BiFC assay. The various constructions were transiently expressed in N. benthamiana leaves following agroinfiltration of bacteria containing the relevant constructs in pairwise combinations as follows: (a, b) eIF4E-YN + YC-CI-Cter; (c, d) eIF4E-YC + YN-CI-Cter. A range of negative controls were performed, including the individual expression of all constructs used. As examples, (e) and (f) show respectively leaves in which eIF4E-YN or YN-CI-Cter were expressed alone. Images for fluorescence emitted by YFP (left) and for the transmitted light mode (right) are shown. Reconstructed YFP signals were observed in the epidermal cells using confocal microscopy at 2 days post-infiltration. Bars, 31 μm (a); 54.86 μm (b); 31.16 μm (c); 54.57 μm (d); 28 μm (e); 31.46 μm (f).](image-url)
Table 2. Binding of the CI-Cter of LMV-0 (CI-0) to the viral VPg (VPg-0)

Purified bait protein was immobilized in the wells of the ELISA plate using a concentration of 4 µg purified protein ml⁻¹ in coating buffer; − indicates that the coating step was performed in the absence of bait protein. Purified prey protein was used at a concentration of 8 µg ml⁻¹; − indicates that the binding step was performed in the absence of prey protein. All detection antibodies were used at the concentration indicated in Methods. The \( A_{405} \) values given are means ± SD of three replicates obtained in a typical experiment. Purified GFP was used as a negative interaction control.

<table>
<thead>
<tr>
<th>Immobilized bait</th>
<th>Interactant prey</th>
<th>Detection antibody</th>
<th>( A_{405} )</th>
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<tr>
<td>−</td>
<td>VPg-0</td>
<td>1H5</td>
<td>0.02 ± 0.004</td>
</tr>
<tr>
<td>CI-0</td>
<td>VPg-0</td>
<td>1H5</td>
<td>0.613 ± 0.017</td>
</tr>
<tr>
<td>CI-0</td>
<td>−</td>
<td>1H5</td>
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<td>GFP</td>
<td>VPg-0</td>
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</tr>
<tr>
<td>CI-0</td>
<td>GFP</td>
<td>Anti-GFP</td>
<td>0.045 ± 0.015</td>
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</table>

Experiments were performed to evaluate whether this difference in binding might be caused by differences in the CI-Cter or in the VPg-binding properties. The results obtained indicate that the VPg proteins from LMV-0 and LMV-E differed in their ability to bind to the CI-Cter, irrespective of its origin, whereas the CI-Cter proteins of LMV-0 and LMV-E do not differ substantially in their VPg-binding properties (Fig. 4a, b). Essentially similar binding curves were obtained with CI-0 and CI-E, with the LMV-0 VPg always showing a weaker signal than that of LMV-E. It therefore appears that the lower interaction signal observed for the CI-0–VPg-0 combination compared with the CI-E–VPg-E one was due to the lower binding of the VPg-0 and not to differences in the binding properties of the two assayed CI-Cter proteins.

DISCUSSION

We report here for the first time the interaction of a potyviral CI helicase with eIF4E, a translation initiation factor involved in natural recessive resistance against potyviruses. In the LMV–lettuce pathosystem, eIF4E was identified as the product of the durable mo1 recessive LMV-resistance gene (Nicaise et al., 2003), and its interaction with the viral VPg has been demonstrated (Michon et al., 2006; Roudet-Tavert et al., 2007). The results reported here using two-site ELISA binding and BiFC assays provide evidence that, in addition to the VPg, the lettuce eIF4E interacts physically with the C-terminal domain of the LMV CI (aa 372–643 of CI). This result confirms those obtained using YTH assays (J. Walter, unpublished data) and is consistent with the involvement of the LMV CI-Cter domain in mo1 resistance breaking (Abdul-Razzak et al., 2009).

Analysis of the eIF4E–CI interaction using various forms of the two proteins indicated that, whatever the combination

Fig. 4. Comparison of the binding in ELISA-based assays of the VPg of LMV-0 (VPg-0) or of LMV-E (VPg-E) to the CI-Cter of LMV-0 (CI-0) or of LMV-E (CI-E). Purified bait proteins CI-0 (a) or CI-E (b) were used at a concentration of 4 µg ml⁻¹. Purified VPg preys (∗, VPg-0; ■, VPg-E) were used at the concentrations indicated. The \( A_{405} \) values given are the means ± SD of three replicates from a typical experiment.
considered (CI from virulent or avirulent virus isolates; eIF4E from susceptible or resistant lettuce), a significant interaction was always observed. In particular, the CI from the non-resistance-breaking isolate (CI-0) showed significant interaction with the eIF4E from resistant cultivars (eIF4E\textsubscript{1} or eIF4E\textsubscript{2}). This means that the QGA deletion at positions 108–110 of eIF4E\textsubscript{1} or the alanine to proline substitution at position 70 of eIF4E\textsubscript{2}, which confer resistance to LMV, do not impede the interaction with CI-Cter. The resistance phenotypes of cultivars expressing the eIF4E\textsubscript{1} or eIF4E\textsubscript{2} forms are different (Abdul-Razzak \textit{et al.}, 2009), but no appreciable difference between eIF4E\textsubscript{1} and eIF4E\textsubscript{2} in CI-binding behaviour was observed under the conditions used. Furthermore, although introduction of mutation S621T into the CI-0 Cter conferred virulence to LMV-0 (and the reciprocal T621S substitution abolished LMV-E virulence), the binding behaviour of these mutated forms was shown to be similar to that of their wild-type counterparts. Similarly to the situation with the eIF4E–VPg interaction, it therefore seems that there is no strict correlation between the eIF4E–CI–Cter interaction and LMV–lettuce compatibility. The interactions involved therefore seem more complex than those reported in other plant–potyvirus pathosystems, where the sole VPg–eIF4E interaction is responsible for the eIF4E-mediated resistance or its overcoming (Kang \textit{et al.}, 2005; Yeam \textit{et al.}, 2007). Besides LMV, the only exception so far to this rule concerns clover yellow vein virus, for which the breaking of eIF4E-mediated resistance in pea involves the P1 protein (Nakahara \textit{et al.}, 2010).

Differences were observed in the strength of the eIF4E–CI–Cter interaction between forms of the two proteins. In particular, the CI of LMV-0 consistently showed a stronger interaction with the resistance-conferring alleles of eIF4E. A possibility is that, in resistant hosts, a reinforced interaction between eIF4E and CI might limit eIF4E availability, thus contributing to the resistance phenotype. This hypothesis seems excluded, however, as CI-E binds more efficiently than CI-0 to all eIF4E forms.

LMV VPg interacts with both lettuce eIF4E and the viral HC-Pro (Roudet-Tavert \textit{et al.}, 2007). The results presented here show that the C-ter part of the LMV CI is also able to interact \textit{in vitro} with the VPg. Such a CI–VPg interaction has only so far been reported in the case of papaya ringspot virus (Shen \textit{et al.}, 2010). However, it has been hypothesized that CI could associate with other viral proteins, such as HC-Pro, CP and VPg, in tip structures observed at one end of potyvirus particles. The VPg and HC-Pro of PVA and potato virus Y (PVY) have been observed in these tip structures (Torrance \textit{et al.}, 2006), whilst the CIs of tobacco vein mottling virus and PVA have been shown to be associated with virus particles (Gabrenaite-Verkhovskaya \textit{et al.}, 2008; Langenberg, 1993). The recent demonstration of the binding of eIF4E and eIF(iso)4E to the PVA, PVY and tobacco etch virus HC-Pro further extends this potential interaction network (Ala-Poikela \textit{et al.}, 2011).

The VPg–CI interaction was weaker for LMV-0, and analysis of heterologous interactions between LMV-0 and LMV-E proteins showed the binding differences to be linked to the VPg and not to the CI. As for the analysis of the eIF4E–CI interaction, careful calibration of the purified proteins used and verification that the antibodies do not react differentially against the various allelic forms (see Supplementary Fig. S1b) seem to exclude the possibility that the differences observed could be artefactual. However, the higher binding observed with the LMV-E VPg could be due either to its higher CI-binding efficiency or to a higher ability to form dimers (Chroboczek \textit{et al.}, 2012) under the conditions used.

Globally, the eIF4E-interaction network involving CI and VPg appears to be stronger for LMV-E, with higher interaction signals for both CI–eIF4E and CI–VPg. This could suggest the need for a minimal interaction threshold for infection success in resistant lettuces. All experiments reported here have been performed with a C-terminal fragment of the CI that was implicated in resistance breaking by reverse-genetics experiments (Abdul-Razzak \textit{et al.}, 2009) and which, in contrast to the full CI, can be expressed and purified efficiently. We cannot rule out, however, the possibility that the binding properties analysed here may be modulated by other parts at the CI. More precise measurement of the interaction parameters linking eIF4E, VPg and complete or truncated forms of the CI are needed in order to reinforce or disprove the hypothesis of a minimal interaction threshold outlined above.

Each of the three protein partners analysed in the present work (VPg, CI and eIF4E) can bind the two others. The VPg of potyviruses contributes to both genome replication and translation, which are mutually exclusive processes. In this context, mutually exclusive interactions between VPg and CI (replication) or cellular eIF4E (translation) might conceivably be associated with these two processes. In such a scenario, eIF4E-mediated resistance would be hypothesized to interfere with replication and/or translation. Whilst initial efforts to analyse potential ternary interactions using ELISA-based assays have been inconclusive, a thermodynamic analysis is in progress to determine whether the network involves exclusively binary interactions, or whether a ternary complex involving all three partners can be formed.

eIF4E-based resistance has also been postulated to act at the level of virus cell-to-cell movement. In pea and pepper, eIF4E assists potyvirus cell-to-cell movement (Arroyo \textit{et al.}, 1996; Gao \textit{et al.}, 2004), whilst in \textit{mo12} lettuce, grafting experiments showed the systemic movement of LMV to be severely impaired and cell-to-cell movement to be delayed (German-Retana \textit{et al.}, 2003). Potyvirus cell-to-cell movement involves several viral proteins, including CI, for which ultrastructural and genetic data suggest strongly that direct interaction with plasmodesmata and ribonucleoprotein complexes facilitates cell-to-cell movement (Carrington \textit{et al.}, 1998; Gómez de Cedrón \textit{et al.}, 2006; Roberts \textit{et al.}, 1998; Wei \textit{et al.}, 2010).
The eIF4E–CI interaction reported here could therefore contribute somehow to the cell-to-cell movement process, thus potentially explaining the resistance phenotypes sometimes associated with eIF4E-mediated resistance.

Taken together, data from this study and previous genetic, biochemical and ultrastructural studies suggest that the C-terminal domain of the LMV CI protein could be involved in a large interaction network of viral and cellular proteins, including plant translation initiation factors, viral VPg, Hc-Pro, and possibly P1 and P3. More effort, however, is still needed to understand this network and the underlying function(s) that are associated with the viral and host protein interactions involved.

METHODS

Expression and purification of lettuce eIF4E and LMV-VPg in E. coli. Each allelic form of eIF4E, isolated from mo1 and mo1 resistant lettuce (eIF4E1 and eIF4E2) and from mo1 susceptible lettuce (eIF4E0), was expressed with a polyhistidine tag and purified from E. coli, as described previously (German-Retana et al., 2008). The coding sequences of the VPg from LMV-0 (VPg-0) and LMV-E (VPg-E) were cloned into the pTrcHis-C expression vector (Invitrogen) in frame with an N-terminal histidine tag and purified from E. coli, as described previously (Michon et al., 2006). When required, an extra polishing step was added: samples eluted from the nickel-affinity resin were loaded onto a Superose 12 size exclusion column (GE Healthcare) to remove any contaminant protein species. Recombinant proteins were analysed by SDS-PAGE (15 % polyacrylamide) and fractions containing pure VPg were pooled and concentrated before use.

Expression and purification of LMV CI-Cter proteins in E. coli. The cDNAs encoding the C terminus of the CI proteins (nt 5192–6007 on the LMV-0 genome, GenBank accession no. X977704) derived from LMV-0, LMV-E and the mutants LMV-0S621T and LMV-E-S621T (Abdul-Razzak et al., 2009) were amplified using the primers 5'-CACCATGGGGACGGAAGAAATACC-3' and 0.6007m (5'-TCATTGTGATATAATGACATCGATGC-3') and cloned into the pENTER/D-TOPO vector (Invitrogen), then transferred into the pDEST-17 vector (Invitrogen) to allow production of N-terminal fusions with a 6×His tag. Protein expression was performed in E. coli BL21-AI cells grown at 37 °C in LB medium containing 100 μg ampicillin ml⁻¹. When the exponential phase was reached, protein expression was induced with 0.2 % arabinose for 4 h at 37 °C. All subsequent steps were performed at 4 °C. Cells from 1 l cultures were pelleted and resuspended in 50 ml TEX buffer (50 mM Tris/HCl, 2 mM EDTA, 1 mM DTT, 0.1 % Triton X-100, pH 8). One tablet of cocktail inhibitors (Roche) and 1 mg lysozyme ml⁻¹ were added. After 30 min incubation with gentle shaking, the sample was sonicated on ice for 5 min (0.4 s pulsations). Inclusion bodies were spun down at 10 000 g for 30 min. The supernatant was discarded and 10 ml SB buffer (8 M urea, 20 mM MOPS, 1 mM DTT, pH 7.2) was added to the pellet, which was solubilized with gentle shaking overnight. The insoluble material was removed by centrifugation (10 000 g for 30 min), and 2 ml Ni-NTA beads (Qiagen) equilibrated in WB buffer (SB buffer supplemented with 10 mM imidazole) were incubated with the protein extract for 5 min. The beads were packed in a column and washed with 25 ml WB buffer. Proteins were eluted with 3 × ml EB buffer (20 mM MOPS, 1 mM DTT, 500 mM imidazole, pH 7.2). The purity of the His-tagged CI proteins was checked by SDS-PAGE and Western blotting using the 2A7 anti-CI mAb (see below).

Development of a specific mAb directed against the LMV CI-Cter protein. BALB/c mice were immunized with 50 μg recombinant LMV-0 CI-Cter purified as described above. Following cell fusion, screening of the hybridoma cell lines was performed by direct ELISA; purified CI-Cter protein (4 μg ml⁻¹) or lysate of CI-Cter-expressing bacteria (25 μl in 25 μl ELISA carbonate coating buffer) was coated overnight at 4 °C into ELISA plates. Detection was performed using hybridoma culture supernatants (25 μl in 25 μl PBS buffer supplemented with 0.2 % Tween 20 and 0.2 % FBS), followed by alkaline phosphatase-conjugated goat anti-mouse immunoglobulins (Sigma). The 2A7-8 hybridoma cell line was finally selected, and the 2A7 mAb was purified and validated further by Western blotting and ELISAs.

ELISA-derived binding assays. All ELISA-derived interaction assays were performed as described previously (Roudet-Tavert et al., 2007) except that, in experiments using CI-Cter as a prey, the PBS contained 100 mM instead of 150 mM NaCl. In some experiments, purified mGFP expressed as a polyhistidine fusion and purified according to Dielen et al. (2011) was used as a control. Protein complexes were detected using specific polyclonal antibodies directed against lettuce eIF4E (pAb38), specific mAbs directed against LMV-VPg (mAb 1H5) or against LMV CI (mAb 2A7, used at 5 μg ml⁻¹), followed by anti-mouse or anti-rabbit alkaline phosphatase conjugates (Sigma), diluted 1/3000. For several sets of measurements, it was possible to distinguish the dissociation constant from the experimental data according to Michon et al. (2006).

BIFC assays. The lettuce eIF4E0 and LMV-0 CI C-termin binding sequences cloned in pENTR/D-TOPO (Invitrogen) were transferred into each of the four pBiFP vectors, pBiFP1, pBiFP2, pBiFP3 and pBiFP4 (Azimzadeh et al., 2008), in which the inserted coding sequences are cloned in fusion with the N- or C-terminal parts of YFP, as either N- or C-terminal fusions. All constructs were confirmed by sequencing. For fluorescence complementation tests, all eight compatible combinations between protein pairs (i.e. providing both parts of the YFP) were assayed by transient expression following agroinfiltration of N. benthamiana leaves (Nicaise et al., 2007). YFP fluorescence was detected 2 days after infiltration using a Leica TCS SP2 confocal microscope and the LCS Lite Leica software.

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