Short Communication

Single amino acid changes in the turnip mosaic virus viral genome-linked protein (VPg) confer virulence towards Arabidopsis thaliana mutants knocked out for eukaryotic initiation factors eIF(iso)4E and eIF(iso)4G

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Previous resistance analyses of Arabidopsis thaliana mutants knocked out for eukaryotic translation initiation factors showed that disruption of the At-eIF(iso)4E or both the At-eIF(iso)4G1 and At-eIF(iso)4G2 genes resulted in resistance against turnip mosaic virus (TuMV). This study selected TuMV virulent variants that overcame this resistance and showed that two independent mutations in the region coding for the viral genome-linked protein (VPg) were sufficient to restore TuMV virulence in At-eIF(iso)4E and At-eIF(iso)4G1xAt-eIF(iso)4G2 knockout plants. As a VPg-eIF(iso)4E interaction has been shown previously to be critical for TuMV infection, a systematic analysis of the interactions between A. thaliana eIF4Es and VPgs of virulent and avirulent TuMVs was performed. The results suggest that virulent TuMV variants may use an eIF4F-independent pathway.

The eukaryotic translation initiation factors eIF4E and eIF4G play a key role during virus infection in plants (Robaglia & Caranta, 2006). During mRNA translation, eIF4E provides the cap-binding function and is associated with eIF4G, the scaffold for the other components of the translation initiation complex, to form the eIF4F complex (Marcotrigiano et al., 1999). In plants, a second eIF4F complex called eIF(iso)4F is present, and results from the assembly of eIF(iso)4E and eIF(iso)4G isoforms (Browning, 2004). These two complexes perform essentially the same task in translation, but have different affinities for certain classes of mRNA substrates and are probably involved in different cellular events (Gallie & Browning, 2001). Plant genes encoding proteins from the eIF4F and eIF(iso)4F complexes belong to small gene families. In Arabidopsis thaliana, three genes encode the eIF4E subfamily proteins (eIF4E1, eIF4E2 and eIF4E3), one encodes eIF(iso)4E and one a non-canonical eIF4E-like protein, known as novel cap-binding protein (nCBP). A single gene encodes eIF4G, whilst two genes encode the eIF(iso)4G subfamily proteins [eIF(iso)4G1 and eIF(iso)4G2] (Robaglia & Caranta, 2006). Viruses that depend on these factors to perform their infectious cycle include members of the genus Potyvirus, the largest and the most diverse genus of plant viruses (Adams et al., 2005; Shukla et al., 1994). Potyviruses are characterized by a positive-sense, single-stranded RNA genome of about 10 kb that encodes at least ten multifunctional proteins. The genome is 3’-polyadenylated and covalently linked at its 5’ end to a virus-encoded protein (VPg) (Murphy et al., 1991; Revers et al., 1999).

Potyviruses may selectively use either the eIF4E or eIF(iso)4E isoform in A. thaliana to achieve a successful infection (Sato et al., 2005). We have demonstrated that potyviruses, including turnip mosaic virus (TuMV), selectively use members of the eIF4F4 family in a fashion that parallels the selective recruitment of eIF4E isoforms (Nicaise et al., 2007; Sato et al., 2005). Indeed, disruption of the eIF(iso)4E gene in A. thaliana (Duprat et al., 2002)
simultaneous disruption of both the $At-eIF(iso)4G1$ and $At-eIF(iso)4G2$ genes results in resistance to TuMV (Nicaise et al., 2007). Here, we investigated the occurrence of virulent TuMV isolates able to accumulate in $At-eIF(iso)4E$ knockout (KO) A. thaliana mutants, and characterized the viral amino acid residues crucial for TuMV gain of virulence in $At-eIF(iso)4E$ or $At-eIF(iso)4G1 \times At-eIF(iso)4G2$ plants.

In initial experiments, systemic accumulation of the avirulent TuMV isolate CDN1 (Jenner et al., 2000; Lehmann et al., 1997) in $At-eIF(iso)4E$ KO plants was occasionally observed at low frequency ($\leq 5\%$) during several independent inoculation experiments. Serial propagation of these viral isolates resulted in the selection of TuMV CDN1 variants able to systemically infect 100% of inoculated $At-eIF(iso)4E$ plants, as revealed by symptom development and RT-PCR assays (data not shown). In this way, three variants were obtained.

In most plant–potyvirus pathosystems, amino acid changes in the Vpg have been shown to be responsible for the ability of the virus to overcome eIF4E-mediated resistance (Ayme et al., 2006; Charron et al., 2008; Kang et al., 2005); therefore, the Vpg regions of the three virulent TuMV variant progeny were sequenced and compared with the Vpg of the avirulent TuMV CDN1 isolate. Vpg sequence comparisons revealed a single amino acid change in each variant compared with TuMV CDN1: one at position 116 shared by two variants and a second at position 163 in the third variant (Fig. 1). The first mutation in the central region of the Vpg corresponds to a glutamic acid (E116), which is replaced by a glutamine (Q), whilst at position 163 in the C-terminal region of the Vpg, an asparagine (N) is replaced by a tyrosine (Y). Sequence comparisons of the Vpg region from a representative collection of 15 potyvirus species showed that E116 and N163 did not correspond to highly conserved amino acids, although asparagine N163 is embedded within eight highly conserved amino acids ($^{160}\text{ERE}^{162}$ and $^{164}\text{ELRQT}^{168}$, data not shown).

In order to confirm that each single amino acid change in the Vpg cistron of TuMV determined its virulence on the $At-eIF(iso)4E$ mutant, PCR-based site-directed mutagenesis by overlap extension was used (see Supplementary Table S1, available in JGV Online, for primer sequences) to introduce the E116Q or the N163Y substitution into the polyprotein encoded by the full-length infectious cDNA clone of the UK1 isolate, named p35Tu (Sánchez et al., 1998, 2003; Tan et al., 2005). Brassica rapa plants were inoculated with the p35Tu, p35Tu-E116Q and p35Tu-N163Y clones, respectively, by particle bombardment, as described previously (German-Retana et al., 2008). After 8 days, all bombarded plants showed initial vein-clearing symptoms followed by mosaic symptoms characteristic of TuMV infection. Following biolistic inoculation of $B. \text{rapa}$, TuMV isolates were further mechanically inoculated onto $A. \text{thaliana}$ ecotype Columbia, $At-eIF(iso)4E$ and $At-eIF(iso)4G1 \times At-eIF(iso)4G2$ double mutant. As expected from previous experiments, Columbia was susceptible to TuMV UK1, whereas plants from the $At-eIF(iso)4E$ and $At-eIF(iso)4G1 \times At-eIF(iso)4G2$ mutants were resistant to TuMV UK1 infection (Fig. 2a, d). In

![Fig. 1. Amino acid sequence alignment of the VPgs of the TuMV UK1 and CDN1 isolates and the derived variants. Numbers represent amino acid residue positions of Vpg. Dashes indicate identical amino acids. The E116Q and N163Y mutation positions are indicated in bold and underlined in both the UK1 and CDN1 strain background. The GenBank accession numbers for TuMV UK1 and CDN1 are AF169561 and AB093610, respectively.](http://vir.sgmjournals.org)
contrast, all plants from the At-elF(iso)4E and At-elF(iso)4G1 × At-elF(iso)4G2 mutants were susceptible to the TuMV-E116Q and TuMV-N163Y variants (Fig. 2b, c, e, f). Visual symptoms resulting from the infection by both TuMV UK1 and the two variants appeared on the At-elF(iso)4E mutant at the same time as on the wild-type Columbia. The symptoms were similar in all cases: chlorotic and mottled leaf colouring was followed by severe stunted growth, distortion of leaf blades and delayed bolting (Fig. 2b, c, e, f). However, symptoms induced by the TuMV-E116Q and TuMV-N163Y variants on the At-elF(iso)4G1 × At-elF(iso)4G2 double mutant were observed with a delay of 4–6 days compared with those induced on At-elF(iso)4E mutant plants. In all cases, ELISA assays and
RT-PCR detection of the viral progeny were performed, and the identity and stability of each mutant were assessed by sequence analysis of the VPg cistron (Fig. 1). These results confirmed that a single point mutation in the VPg cistron of TuMV allowed the virus to overcome either the lack of eIF(iso)4E or the lack of both eIF(iso)4G1 and eIF(iso)4G2 for successful infection. Taken together, these data suggested that these virulent TuMV variants do not require the eIF(iso)4F complex as a whole to carry out infection.

Recently, eIF4E has been shown to interact physically with the potyviral VPg, as demonstrated using various protein–protein interaction assays, such as ELISA, yeast two-hybrid assays, bimolecular fluorescence complementation and surface plasmon resonance (Beauchemin et al., 2007; Charron et al., 2008; Léonard et al., 2000; Okade et al., 2009). Furthermore, this physical interaction was shown to correlate with successful viral infection of the plants (Beauchemin et al., 2007; Charron et al., 2008; Kang et al., 2005; Léonard et al., 2000; Yeam et al., 2007). Therefore, one would expect that the mutations E116Q and N163Y would confer on VPg the ability to interact with eIF4F (described by Nicaise et al., 2000; Yeam et al., 2007). Table 1 summarizes the behaviour of TuMV UK1, TuMV CDN1 and the derived virulent variants in terms of virus accumulation and symptom induction. In addition to the KO mutants for the three other eIF4E genes (At-eIF4E1, At-eIF4E2 and At-eIF4E3), the mutant At-nCBP was also tested, as it is depleted in expression of a protein shown to function as a cap-binding protein and to interact with eIF(iso)4G to form a complex that supports the protein synthesis initiation of a capped mRNA (Ruud et al., 1998). All mutants were found to be susceptible to the TuMV-E116Q and TuMV-N163Y variants derived from the UK1 and CDN1 strains (Table 1).

As a second step, a yeast two-hybrid system was used to monitor protein–protein interaction patterns between all A. thaliana eIF4E proteins and the different CDN1 and UK1 TuMV VPgs (wild-type and E116Q and N163Y forms) (see Supplementary Table S2 for primer sequences). An interaction between At-eIF(iso)4E and TuMV VPg was shown, whilst no interaction could be detected with the other four proteins, even at low stringency (Fig. 2g). Interactions with eIF4E and nCBP proteins were then tested using the E116Q and N163Y TuMV VPg variants, with the same results as with wild-type TuMV VPg (Fig. 2g and data not shown). We concluded from these two-hybrid assays that neither the E116Q nor the N163Y mutation interferes with the binding of VPg to At-eIF(iso)4E, nor did either induce any new interaction with another eIF4E isoform.

Overall, the results presented here show that a single amino change either in the central part or the C-terminal domain of the VPg from TuMV isolates UK1 and CDN1 permits breaking of the complete resistance of an A. thaliana mutant bearing a transposon-induced null allele for the At-eIF(iso)4E gene. Whilst the first mutation, E116Q, is

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**Table 1.** Susceptibility of eIF4E and eIF4G mutants to TuMV UK1 and CDN1 isolates, and their respective derived variants

<table>
<thead>
<tr>
<th>Virus</th>
<th>WT</th>
<th>Mutant*</th>
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<tbody>
<tr>
<td></td>
<td>At-eIF4E1</td>
<td>At-eIF4E2</td>
</tr>
<tr>
<td>UK1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>UK1-E116Q</td>
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<td>+</td>
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<tr>
<td>UK1-N163Y</td>
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<tr>
<td>CDN1</td>
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<td>+</td>
</tr>
<tr>
<td>CDN1- E116Q</td>
<td>+</td>
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<tr>
<td>CDN1- N163Y</td>
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*At-eIF4E1 (Arabidopsis Genome Initiative identifier At4G18040) mutant = cum1-1 mutant kindly provided by M. Ishikawa (Yoshii et al., 2004), SALK067430; At-eIF4E2 (At1G29590) mutant = SALK103888; At-eIF4E3 (At1G29550) mutant = SALK101805; At-eIF(iso)4E (At5G35620) mutant = d8pm insertion mutant kindly provided by C. Robaglia (Duprat et al., 2002); At-nCBP (At5G18110) mutant = SALK053948; At-eIF4G (At3G0240) mutant = SALK12882; At-eIF(iso)4G1 (At5G57870) mutant = SALK009905; At-eIF(iso)4G2 (At2G24050) mutant = SALK076633, At-eIF(iso)4G1 × At-eIF(iso)4G2 = obtained after crossing of the single mutants SALK009905 and SALK076633 as described by Nicaise et al. (2007). All A. thaliana Columbia-0 plants (wild type) and the derived mutants or T-DNA insertion lines were grown in a greenhouse or in growth chambers at 25 °C with a 16 h light period. WT, Wild type.
located in the central region of the VPg, previously shown to be involved in potato virus Y virulence towards pot-1 and pvr2 resistance mediated by elf4E in tomato and pepper, respectively (Moury et al., 2004), the second mutation, N163Y, is located outside this region, in the C-terminal part of the VPg. Furthermore, we showed that these single amino acid changes also allowed the virus to overcome the resistance conferred by disruption of both the At-elf(iso)4G1 and At-elf(iso)4G2 genes in the double A. thaliana mutant. This is consistent with our previous data showing that TuMV infection of A. thaliana relies on the recruitment of elf(iso)4E and, in parallel, either elf(iso)4G1 or elf(iso)4G2 components of the elf(iso)4F complex (Nicaise et al., 2007). Likewise, the virulent viruses isolated in this study bypass the requirement for both components of the elf(iso)4F complex.

Although TuMV VPg has been shown previously to interact with At-elf(iso)4E (Léonard et al., 2000), we present here its interaction pattern with all elf4E isoforms and nCBP. This is the first time that a complete elf4E family has been assessed for interaction with the viral VPg protein in a plant species. Despite strong homologies between the five members of this family in A. thaliana, TuMV VPg interacted solely with elf(iso)4E. This is in total agreement with our previous mutant studies, which showed that plants deficient in this protein, but not in the other elf4Es, were resistant to TuMV (Nicaise et al., 2007). These data further confirm the link between elf4E–VPg interaction and virulence (Charron et al., 2008).

As the At-elf(iso)4E KO mutant completely lacks both elf(iso)4E mRNA and protein (Duprat et al., 2002), a simple hypothesis would be that TuMV variants of this mutant rely on recruitment of the elf4F complex as a whole. However, we showed that At-elf4E and At-elf4G KO mutants are susceptible to TuMV-E116Q and TuMV-N163Y infection. Hence, rather than switching from using one complex to the other, it is more likely that the virus has extended its range by being able to recruit both complexes: it is likely that, even if TuMV variants recruit the elf4F complex in ‘elf(iso)4F KO’ plants, they are still able to recruit elf(iso)4F in ‘elf4F KO’ plants. However, our two-hybrid assays results do not argue in favour of the use of the elf4F complex by the TuMV variants, as we revealed only an At-elf(iso)4E–VPg interaction, independent of the nature (wild type or mutant) of the VPg tested. We cannot exclude the possibility that the two-hybrid system used in this study did not allow the detection of a weak interaction between TuMV VPg variants and another At-elf4E isoform, which may occur in planta. Indeed, in a natural elf4E–potyvirus pathosystem where elf4E is known to be the resistance gene, attempts to demonstrate an interaction between pea seed-borne mosaic virus (PsBMV) VPg and elf4E from a susceptible pea genotype were unsuccessful (Gao et al., 2004). In our experiments, occasionally a weak interaction could be detected between elf4E3 and either TuMV UK1-E116Q VPg or UK1-N163Y VPg, we assumed it was not a bona fide interaction.

Another hypothesis is that TuMV variants could recruit elf4G, independently of any elf4E isoform, in both At-elf(iso)4E KO and At-elf(iso)4G1 × At-elf(iso)4G2 double KO plants, suggesting that the role of elf4G in TuMV variants infection could be elf4E-independent. Indeed, it has been shown previously that cap-independent translation of tobacco etch virus (TEV) potyvirus is mediated only by the association between an internal ribosome entry site (IRES) element, elf4G and ribosomes (Gallie, 2001). Furthermore, Basso et al. (1994) provided evidence for an IRES within the 5′ non-translated region of TuMV. In addition, Khan et al. (2008) recently showed that an elf4F–VPg interaction enhances cap-independent translation by increasing the affinity of elf4E for TEV RNA, and that VPg can also interact with elf4G, suggesting a direct participation of VPg in translation initiation.

Finally, it remains possible that amino acid changes in the VPg of TuMV-E116Q and TuMV-N163Y may allow breaking of the resistance phenotype in At-elf(iso)4E KO plants by interaction with an alternative, as yet unidentified partner in planta. Indeed, an interaction between poly(A)-binding protein and the TuMV VPg-Pro has been demonstrated in planta (Léonard et al., 2004), suggesting that potyvirus RNA circularization could bypass elf4E, leaving another functional meaning for the binding of elf4E to VPg. Furthermore, the interaction between VPg and elf4E in planta is probably influenced by interactions with other plant and/or viral proteins. Indeed, although VPg has been shown to be solely responsible for breaking elf4E-mediated resistance in many plant–potyvirus pathosystems (Ayme et al., 2006; Charron et al., 2008; Kang et al., 2005), the involvement of other viral factors such as the cylindrical inclusion protein of lettuce mosaic potyvirus and the P3 protein of PsBMV was recently demonstrated to be involved in the breaking of elf4E-mediated resistance in lettuce and pea, respectively (Abdul-Razzak et al., 2009; Hjulsager et al., 2006).

The isolation of these new TuMV virulent variants and deciphering of how they overcome resistance as a result of mutations in the elf(iso)4F complex should prove useful in further unravelling the relationships existing between viral ligands and the plant cell machinery.

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