Trans-species synthetic gene design allows resistance pyramiding and broad-spectrum engineering of virus resistance in plants

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Summary
To infect plants, viruses rely heavily on their host’s machinery. Plant genetic resistances based on host factor modifications can be found among existing natural variability and are widely used for some but not all crops. While biotechnology can supply for the lack of natural resistance alleles, new strategies need to be developed to increase resistance spectra and durability without impairing plant development. Here, we assess how the targeted allele modification of the Arabidopsis thaliana translation initiation factor eIF4E1 can lead to broad and efficient resistance to the major group of potyviruses. A synthetic Arabidopsis thaliana eIF4E1 allele was designed by introducing multiple amino acid changes associated with resistance to potyviruses in naturally occurring Pism sativum alleles. This new allele encodes a functional protein while maintaining plant resistance to a potyvirus isolate that usually hijacks eIF4E1. Due to its biological functionality, this synthetic allele allows, at no developmental cost, the pyramiding of resistances to potyviruses that selectively use the two major translation initiation factors, eIF4E1 or its isoform eIFiso4E. Moreover, this combination extends the resistance spectrum to potyviruses for which no efficient resistance has so far been found, including resistance-breaking isolates and an unrelated virus belonging to the Luteoviridae family. This study is a proof-of-concept for the efficiency of gene engineering combined with knowledge of natural variation to generate trans-species virus resistance at no developmental cost to the plant. This has implications for breeding of crops with broad-spectrum and high durability resistance using recent genome editing techniques.

Introduction
Synthetic biology has emerged as a promising tool to provide advantageous new functions for crop improvement using plant biotechnology (Liu and Stewart, 2015). One aspect of synthetic biology is the possibility of editing the plant’s own genes to induce changes in the way the plant interacts with its environment (Baltes and Voytas, 2015). This approach was successfully used to improve water use and drought stress tolerance in tomato (Solanum lycopersicum) and Arabidopsis thaliana by modifying the abscisic acid (ABA) receptor PYRABACTIN RESISTANCE 1 to make it responsive to an agrochemical ligand (Park et al., 2015). Resistance to pathogens is also an important field in which gene engineering can play a major role by restricting the way pathogens hijack host factors essential for their infectious cycle. For example, targeted modification of the JA hormone receptor in Arabidopsis maintains efficient resistance to the bacterial effector from Pseudomonas syringae without impairing the JA recognition pathway (Zhang et al., 2015). It is of particular interest to understand how specific modifications of plant genes, often resulting from nonsynonymous Amino Acid (AA) changes, can generate new functions. These modifications can then be implemented through high-throughput allele mining (Barabaschi et al., 2016) or new genome editing technologies such as CRISPR/Cas9 (Ma et al., 2016). Overall, gene editing technologies appear as a promising tool to enhance and accelerate plant breeding (Østerberg et al., 2017; Palmgren et al., 2015).

Viruses are obligate intracellular parasites encoding very few proteins and are thus highly dependent on host factors for successful infection. A widely developed strategy to counter viral infections in crops is to modify these factors or inhibit their expression, therefore causing resistance by loss-of-susceptibility (Pavan et al., 2010; van Schie and Takken, 2014). The eukaryotic initiation factor 4E (eIF4E) and its isoform (eIFiso4E) have been proven to be susceptibility factors to several economically important single-stranded positive sense RNA (ssRNA+) viruses
Natural diversity is a great reservoir of eIF4E resistance alleles in crops and their related wild species. Mostly, these resistance alleles encode modified eIF4E proteins, which still retain a functional role in translation initiation while carrying nonsynonymous AA changes, often located in two conserved regions of the protein (Robaglia and Caranta, 2006). Such resistance alleles have been successfully deployed in many crop species such as tomato, barley (Hordeum vulgare), lettuce (Lactuca sativa), melon (Cucumis melo) and pepper (Capsicum annuum) (Nicaise et al., 2003; Nieto et al., 2006; Ruffel et al., 2002, 2005; Stein et al., 2005). However, such natural virus resistance alleles are not available in some other important crops such as papaya (Carica papaya), Prunus species or cassava (Manihot esculenta), which are challenged by economically important viruses such as Papaya ringspot virus, Plum pox virus and Cassava brown streak virus, respectively (Bart and Taylor, 2017; García et al., 2014; Gonzales, 1998). Moreover, even if resistance alleles are present in related wild species, their introgression into cultivated accessions can be difficult in the case of reproductive incompatibility or genetic linkage drag bringing unwanted traits (Lin et al., 2014). To compensate for this lack of natural alleles, eIF4E gene disruption can be used to induce virus resistance. Such approaches have been successfully used in some species such as Arabidopsis and cucumber (Cucumis sativus L.) (Chandrasekar et al., 2016; Duprat et al., 2002; Pyott et al., 2016). Because of redundancy among the eIF4E gene family, plants knocked out (KO) for a single translation initiation factor mostly exhibit normal growth (Bastet et al., 2017). However, as ssRNA+ viruses recruit selectively distinct members of the eIF4E gene family, a single KO is often associated with a limited resistance spectrum while simultaneous KO of several members of the eIF4E gene family often leads to lethality or impaired growth (Callot and Gallois, 2014; Gauffier et al., 2016). Consequently, pyramiding resistances by knocking out several factors to extend the virus resistance spectrum can be impeded by the induction of developmental defects as shown in Arabidopsis (Callot and Gallois, 2014) and tomato (Gauffier et al., 2016). It is also expected that redundancy among 4E (i.e. eIF4E or eIFiso4E) genes could reduce eIF4E-based resistance durability by making other 4E factors available to viruses (Bastet et al., 2017). Finally, a comparison between natural functional eIF4E resistance alleles and engineered loss-of-function KO alleles in tomato unveiled unexpected regulatory processes between the members of the eIF4E family making this approach less effective (Gauffier et al., 2016).

In the light of these observations, the best strategy to develop eIF4E-based resistance would be to design functional alleles by introducing point mutations in the gene, mimicking naturally occurring resistances, instead of knocking them out (Bastet et al., 2017). In this regard, several studies have shown that eIF4E or eIFiso4E could be engineered as resistance alleles, in tomato, potato (Solanum tuberosum) and Chinese cabbage (Brassica rapa), as attested by the transgenic ectopic expression of resistance alleles under the control of a strong constitutive promoter in a susceptible genetic background (Cavataorta et al., 2011; Kang et al., 2007; Kim et al., 2014).

In the present paper, we aimed at extending this approach by mimicking allele replacement and addressing the functional role of the modified eIF4E protein using the well-characterized Arabidopsis thaliana/potyvirus pathosystem (Ouibrahim and Caranta, 2013).

We report for the first time the construction of a synthetic resistance allele of eIF4E1 by introducing six nonsynonymous mutations deduced from pea eIF4E1 allelic variability. We show that a phenotypic defect associated with eIF4E1 loss of function, a bolting delay, can be complemented by the synthetic allele, while maintaining the resistance to the potyvirus Clover yellow vein virus (CYVV). Significantly, by maintaining function in planta, this synthetic resistance allele allows resistance pyramiding with an eIFiso4E loss-of-function allele and confers new resistances to another important potyvirus species as well as to previously described eIFiso4E-resistance-breaking (RB) isolates. In addition, these double-mutated plants displayed resistance to a polerovirus species. This study therefore establishes a proof-of-concept for editing susceptibility genes to create new genetic resistances and expand resistance spectrum without affecting the plant development and agronomic traits.

Results
Design of a synthetic eIF4E1n resistance allele in Arabidopsis

The wild-type Arabidopsis eIF4E1 gene from the Columbia (Col) accession was chosen as a target to design a synthetic resistance allele. eIF4E1 encodes a susceptibility factor to the potyvirus Clover yellow vein virus (CYVV) and, consequently, inactivation of eIF4E1 (eIF4E1KO) is associated with resistance to CYVV (Sato et al., 2005). No natural eIF4E1 resistance allele is known in Arabidopsis, so we looked for data on genetic resistance determinants in other species. We focused on eIF4E1 natural variation in pea (Pisum sativum), because the natural eIF4E resistance allele (known as sbm-1, cvy-2 or wlv) from pea accession J1405 confers resistance to several potyviruses, notably CYVV (Andrade et al., 2009; Bruun-Rasmussen et al., 2007; Gao et al., 2004). The eIF4E1 protein in Arabidopsis is highly similar to its counterpart in pea (81% homology/89% similarity) (Gao et al., 2004) (Figure 1a). Another resistance allele isolated from pea accession PI269818 and conferring resistance to one pathotype of Pea seed-borne mosaic virus (PsbMV) was also considered in this study. These two pea resistance alleles are associated with the presence of five AA substitutions and one deletion within the protein sequence: W62L (i.e. the tryptophan at position 62 is substituted by a leucine in the protein encoded by the resistance allele), A73D, A74D, G107R and N169K for J1405 line, as well as the deleted serine at position 77 (S77) in the PI269818 accession. These AA changes are mainly located in two regions (regions I and II) close to the cap-binding pocket according to the eIF4E 3D structure (Marcotrigiano et al., 1997; Robaglia and Caranta, 2006) (Figure S1). In Arabidopsis, the AA located at similar positions (AA 69, 80, 81, 114 and 176) are, in most cases, identical to the AA in the pea susceptible allele (Figure 1a). The synthetic Arabidopsis eIF4E1 allele was obtained by substituting the AA present in the five locations by the corresponding AA from the resistant pea accession J11405. The position of the deletion in the resistant pea accession PI269818 was substituted by an alanine in the synthetic Arabidopsis eIF4E1 sequence. The 3D predicted structure of the Arabidopsis eIF4E1 protein carrying the six modifications W69L, T80D, S81D, S84A, G114R, and N176K
mimics the structure of the pea resistant alleles. No drastic changes in the protein structure were predicted, hinting that the function of this mutated form of eIF4E1 in translation initiation should not be strongly affected (Figure 1b,c and Figure S1).

All six nonsynonymous substitutions were introduced by PCR-based directed mutagenesis on a 3.4-kb construct covering the whole Arabidopsis eIF4E1 gene including its endogenous promoter. This allele (called hereafter eIF4E1<sup>R</sup>) was introduced into Arabidopsis eIF4E1<sup>kO</sup> plants knocked out for eIF4E1 (eIF4E1<sup>kO</sup> lines), and three independent transgenic lines were selected for further analyses. The correct expression of the eIF4E1<sup>R</sup> transgene was checked in all three lines (Figure S2), as well as the sequence of

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**Figure 1** Design of a synthetic Arabidopsis resistance allele eIF4E1<sup>R</sup>. (a) Alignment of eIF4E protein sequences from pea susceptible (JI2009) and resistant (JI1405 and PI269818) accessions with the Arabidopsis thaliana eIF4E1 (AteIF4E1). The position of the amino acids that differ between susceptible and resistance pea accessions is highlighted in grey. Black triangles above the alignment indicate the mutations introduced in Arabidopsis to create the synthetic eIF4E1<sup>R</sup>. (b and c) Three-dimensional predicted structure of AteIF4E1 (b) and of eIF4E1<sup>R</sup> (c) based on homology modelling using the pea eIF4E 3D structure as a template (PDB ID: 2WMC-C). The modified amino acids between AtEIF4E1 and eIF4E1<sup>R</sup> are coloured in red with side chains shown.
the expressed elf4E1 mRNA in all lines. As controls, a construct harbouring a wild-type elf4E1 was introduced into the same elf4e1KO background, as well as an unrelated transgene, expressing the GUS reporter gene under control of the CaMV 35S promoter.

elf4E1R encodes a functional protein that complements elf4e1KO bolting delay

Although 4E translation initiation factors are largely redundant in their functions (Browning and Bailey-Serres, 2015; Combe et al., 2005; Duprat et al., 2002; Nicaise et al., 2002), the elf4e1KO Arabidopsis plants were found to display a consistent 7-day bolting delay when compared with the wild-type Columbia accession (Col WT) (Figure 2a,b, Bastet et al., 2017). To assess whether elf4E1R encodes a functional translation initiation factor, the ability of elf4e1KO to complement this bolting delay was tested. Indeed, elf4E1R restored the wild-type timing of bolting (Figure 2a,b). Furthermore, total protein extracts were subjected to cap analog-affinity assay and, on all independent transgenic lines expressing either elf4e1 or elf4E1R, elf4E1 proteins were shown to bind the cap analog, confirming that elf4E1R encodes a functional protein (Figure 2c).

elf4E1R is a resistance allele to CIYVV

We next addressed whether the six mutations introduced in the synthetic allele were sufficient to confer resistance to CIYVV, a potyvirus that requires an eIF4E1 factor for its viral cycle. All plants were mechanically inoculated with CIYVV and the viral accumulation was measured 30 days postinoculation (dpi) by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Figure 3). As expected, CIYVV accumulated in both Col WT and elf4e1KO-complemented plants while no virus accumulation was observed in elf4e1KO plants. Interestingly, no virus accumulation was detected in the elf4e1KO plants complemented by the elf4E1R construct suggesting that this mutated factor cannot be recruited by CIYVV. Altogether, these results show that the six AA changes introduced in elf4E1R are sufficient to convert elf4E1 into a resistance allele to CIYVV, while maintaining its physiological functionality. This is similar to the resistance genes that have been previously identified in crops or in their wild relatives (Robaglia and Caranta, 2006).

elf4E1R rescues the lethality associated with the double mutation elf4e1ko elfiso4eKO and does not impact plant yield

It was previously shown that the resistance spectra associated with either elf4e1ko or elfiso4eKO, to CIYVV and Turnip mosaic virus (TuMV), respectively (Duprat et al., 2002; Lellis et al., 2002), could not be combined in the same plant because of plant lethality (Callot and Gallois, 2014; Patrick et al., 2014). We speculated that since elf4E1R encodes a functional cap-binding factor, it may successfully be combined with loss-of-function alleles affecting both elf4E1 and elfiso4E and could therefore rescue the lethal phenotype. To verify this hypothesis, two previously described independent T2 transgenic lines [elf4e1ko elf4e1ko elf4E1R] (homozygous for elf4e1KO allele but complemented by elf4E1R) were crossed with [elf4e1ko elf4e1ko elfiso4eKO elfiso4eKO] (homozygous for elf4e1KO but heterozygous for elfiso4eKO) plants. The resulting F3 plants knocked out for elf4E1 and elfiso4E but complemented by elf4E1R were viable (Figures 4a and S3). The rescue of the lethal phenotype by elf4E1R further confirmed that this allele was fully functional in planta. Consistently, the elf4e1KO elfiso4eKO elfiso4eKO elfiso4eKO plants complemented by elf4E1R did not show any delayed bolting phenotype compared with wild-type plants (Figures 4a and S4). In addition, no decrease in dry weight or in seed yield was observed (Figure 4b,c and S4). In conclusion, the synthetic elf4E1R allele can fully complement the lack of both elf4E1 and elfiso4E isoforms in planta.

Cumulative resistance to several potyviruses in elf4E1R-complemented plants

As elf4E1 and elfiso4E are required for CIYVV and TuMV multiplication, respectively, elf4e1ko elfiso4eKO elf4E1R plants, together with control lines including single mutants affecting elf4e1 or elfiso4E alone, were separately challenged with these two viruses, and the accumulation of viruses was measured by ELISA (Figure 5). As expected, Col WT plants were susceptible to both viruses while elf4e1ko and elfiso4eKO plants were resistant to CIYVV and TuMV, respectively. Remarkably, CIYVV or TuMV did not accumulate in the elf4e1ko elfiso4eKO elf4E1R lines. Lettuce mosaic virus (LMV) and Plum pox virus (PPV) that both rely on the isoform elfiso4E (Duprat et al., 2002; Nicaise et al., 2003) were further inoculated onto the similar mutated lines, and the results showed that the elf4e1KO elfiso4eKO elf4E1R lines were fully resistant to these potyviruses as well (Figure S5). Therefore, by complementing the loss-of-function for both elf4E1 and elfiso4E, the synthetic elf4E1R allele allows combination of the resistance spectrum associated with each individual mutation, leading to an overall broad resistance spectrum to several potyviruses.

elf4E1R confers resistance to a potyvirus relying on both elf4E1 and elfiso4E

Watermelon mosaic virus (WMV) is a potyvirus that mainly affects cucurbit plants, but also Arabidopsis. Previously, the Arabidopsis Cap Verde island accession (Cvi) was identified as a source of partial resistance to WMV relying on the chloroplastic PHOSPHGLYCERATE KINASE 2 gene (cPGK2) (Oulibrham et al., 2014). Unlike most potyviruses, no resistance to WMV associated with loss-of-function alleles of either elf4E1 or elfiso4E has been shown so far (Figure S6). A hypothesis is that WMV could recruit either elf4E1 or elfiso4E as do Pepper vein mottle virus (PepMV) or Chilli vein mottle virus (ChiVMV) in Capsicum species (Hwang et al., 2009; Ruffel et al., 2006). In agreement with this assumption, the elf4e1ko elfiso4eKO elf4E1R lines were also fully resistant to WMV since no virus accumulated in the inoculated plants 3 weeks postinoculation (Figure 6). This result shows that, beyond allowing the pyramiding of the resistance spectra conferred by both mutated elf4E1 and elfiso4E, the strategy outlined here can extend the resistance to potyviruses using both factors.

elf4E1R restrains TuMV resistance-breaking isolates

The durability of resistance is a major issue for plant breeding and it is crucial to develop and release resistances that are efficient towards resistance-breaking (RB) emerging isolates. Significantly, elf4E1-mediated resistance can be overcome by RB potyviruses (Sanfracon, 2015; Wang and Krishnaswamy, 2012). Previously, two RB isolates of TuMV able to overcome elfiso4eKO-mediated resistance were isolated in Arabidopsis (Gallois et al., 2010). Each isolate, TuMV-EI16Q and TuMV-N163Y, possesses a single nonsynonymous mutation (mutation E116Q or N163Y, respectively) in the avirulence factor VPg (viral genome-linked protein),
that is responsible for the resistance overcoming. It had been hypothesized that the overcoming of eifiso4eKO-mediated resistance could be achieved by the ability of both isolates to recruit another translation initiation factor, namely eIF4E1 (Gallois et al., 2010). As often with resistance-breaking strains (Moury et al., 2004), TuMV-E116Q and TuMV-N163Y accumulate weakly in eifi-so4eKO lines (Figure S7). Therefore, to monitor plant infection, we inoculated the same plants as before with a viral isolate expressing the GFP reporter gene. The mutations E116Q and N163Y were each introduced into a TuMV-GFP infectious cDNA clone (Beauchemin et al., 2005), generating TuMV-E116Q-GFP and TuMV-N163Y-GFP, respectively. Plants were agro-inoculated, and the GFP accumulation in plants was detected 21 dpi using a GFP-imaging camera (Figure 7a). TuMV-E116Q-GFP and TuMV-N163Y-GFP, but not TuMV-GFP accumulated in eifi-so4eKO plants, confirming their ability to overcome the eifiso4eKO-mediated resistance. However, the two eif4e1KO eifiso4eKO eIF4E1R lines were fully resistant to both resistance-breaking TuMV isolates. The absence of virus accumulation in the eif4e1KO eifiso4eKO eIF4E1R lines was moreover confirmed by RT-PCR on total RNA extracted from the inoculated plants (Figure 7b). Altogether, our results show that one nonsynonymous mutation either at position 116 or 163 in the VPg allows RB-TuMV viruses to use a new translation initiation factor, eIF4E1, in addition to eIFiso4E, and that the engineered eif4e1KO eifiso4eKO eIF4E1R genotype could significantly improve virus resistance durability.

Figure 2 Functional complementation of eif4e1KO plants by eIF4E1R allele. (a) Bolting of eif4e1KO and complemented plants, 4 weeks after sowing. Columbia Wild-type plants (Col WT) are used as control. Results are shown for three independent transgenic lines (1 to 3) expressing the eIF4E1R construct in a eif4e1KO background. (b) Boxplot representation of the bolting time (in days after sowing) for the same genotypes as in (a). Results are averaged from 16 individual plants per genotype. (a) and (b) represent significantly different groups (**P < 0.05). (c) In planta cap-binding purification of eIF4E1 proteins. Total soluble protein extract from control and transgenic plants was purified on m7GTP-agarose beads. After purification, the output fraction was analysed by Western blot using anti-eIF4E1 antibody while equal loading control was checked on total protein (input) by Western blot for actin detection and by Ponceau staining for Rubisco protein detection.
**eIF4E1**<sup>R</sup>** confers resistance to one polerovirus species**

Translation initiation factors 4E are mostly associated with resistance to potyviruses but resistances to other ssRNA<sup>+</sup> viruses, harbouring different genomic features, have also been described (Robaglia and Caranta, 2006). For example, loss-of-function in eIF4E1 was shown to be implicated in a delay of susceptibility to the carmovirus Turnip crinkle virus (TCV) (Yoshii et al., 1998). We found that the engineered eIF4E1<sup>KO</sup> eIFiso4E<sup>KO</sup> eIF4E1<sup>R</sup> plants were fully susceptible to TCV, showing that the eIF4E1<sup>R</sup> allele does not constitute a resistance allele to TCV (Figure S8). Likewise, the engineered eIF4E1<sup>KO</sup> eIFiso4E<sup>KO</sup> eIF4E1<sup>R</sup> plants were fully susceptible to Grapevine fanleaf virus (GFLV) and Arabis mosaic virus (ArMV), two ssRNA<sup>+</sup> nepoviruses (Figure S9).

Finally, the role of translation initiation factors in resistance to polerovirus, such as Turnip yellows virus (TuYV), Beet mild yellowing virus (BMVY) and Beet western yellows virus-USA (BWYV-USA), had previously been investigated in Arabidopsis (Reinbold et al., 2013). TuYV was shown to require the Arabidopsis eIF4E1<sup>R</sup> to infect the plant, whereas BMVY and BWYV infection was compromised in plants devoid of functional eIF4E1, although the latter resistance was only partial. To assess whether eIF4E1<sup>R</sup> could confer resistance to viruses other than potyviruses, the eIF4E1<sup>KO</sup> eIFiso4E<sup>KO</sup> eIF4E1<sup>R</sup> lines were challenged with these three polerovirus species.

Aphid-mediated inoculation was performed on controls and eIF4E1<sup>KO</sup> eIFiso4E<sup>KO</sup> eIF4E1<sup>R</sup> plants. Viral accumulation was detected by DAS-ELISA 21 dpi (Figure 8). All genotypes were fully susceptible to TuYV, consistent with the virus requirement for eIFiso4G1 and not eIF4E1 or eIFiso4E. Intermediate resistance to BMVY and BWYV-USA associated with eIF4E1 loss-of-function was confirmed as described before (Reinbold et al., 2013) but interestingly while susceptibility to BMVY was restored in eIF4E1<sup>KO</sup> eIFiso4E<sup>KO</sup> eIF4E1<sup>R</sup> lines, this genotype was fully resistant to BWYV-USA. In conclusion, the presence of the synthetic resistance allele eIF4E1<sup>R</sup> in an eIF4E1<sup>KO</sup> eIFiso4E<sup>KO</sup> genetic background is associated with a full resistance to a polerovirus isolate.

**Discussion**

Natural eIF4E-based resistance alleles are largely used to introduce virus resistance in crops by breeding. Genetic engineering could help provide new sources of resistance in crops that are devoid of such natural resistance. In this study, we developed a synthetic allele, eIF4E1<sup>R</sup> that can make up for the lack of natural resistance alleles, and we gave evidence of its effectiveness in an Arabidopsis-based pathosystem. We showed that eIF4E1<sup>R</sup> mimics natural resistance alleles when replacing endogenous eIF4E while maintaining biochemical (binding to cap analog) and physiological (seed yield, biomass) functions. A full assessment of this genotype’s resistance to biotic or abiotic stresses as well as any changes in translatome could allow to analyse more subtle changes in the plant physiology that could be associated with this new allele.

By being fully functional, eIF4E1<sup>R</sup> allows combination of the resistance spectra associated with both loss-of-function alleles in eIF4E1 and eIFiso4E (i.e. the resistance to CIYVV associated with eIF4E1<sup>KO</sup> and the resistance to TuMV, PPV and LMV associated with eIFiso4E<sup>KO</sup>). Such association had been infeasible so far because of the lethality of the double mutant (Callot and Gallois, 2014; Patrick et al., 2014). Moreover, the same genetic combination involving eIF4E1<sup>R</sup> allowed us to extend the Arabidopsis resistance to WMV, a potyvirus for which no 4E-mediated resistance has been identified so far. Interestingly, this resistance is more efficient than the one previously described based on the plant host factor chloroplastic PHOSPHOGLYCERATE KINASE 2 (Oubrahim et al., 2014). This shows that WMV is able to use the two main translation initiation factors in Arabidopsis indifferently, namely eIF4E1 and eIFiso4E isoforms. This is a very similar situation to the one that has been described for susceptibility to PepVMV and ChiVMV in pepper, where resistance relies on both a Ca-eIF4E1-pvr2 functional resistance allele and a natural Ca-eIFiso4E-pvr6 KO allele (Hwang et al., 2009; Ruffel et al., 2006). We therefore suspect that the ability of certain viral species to recruit several isoforms could be more frequent than expected, and by generalization, the generality of 4E factors in susceptibility to ssRNA<sup>+</sup> viruses could be more universal than previously thought (Figure 9).

Finally, we described earlier that TuMV can overcome eIFiso4E loss-of-function through either one of two nonsynonymous mutations in the viral VPg coding sequence (Gallos et al., 2010). We hypothesized that because eIFiso4E was no longer available, the viral mutations associated with resistance breaking may extend the VPg’s ability to recruit new host factors, possibly
Figure 4 eIF4E1\textsuperscript{R} complements the eif4e1\textsuperscript{KO} eifiso4e\textsuperscript{KO} lethality phenotype at no developmental cost. (a) Phenotype of two independent eif4e1\textsuperscript{KO} eifiso4e\textsuperscript{KO} eIF4E\textsuperscript{R} lines, four weeks after sowing. (b) Dry weight analyses of 4-week-old controls and eif4e1\textsuperscript{KO} eifiso4e\textsuperscript{KO} eIF4E\textsuperscript{R} plants. Results were averaged from 20 plants for each genotype. (c) Fertility rate is the weight of seeds produced by plants of controls lines and eif4e1\textsuperscript{KO} eifiso4e\textsuperscript{KO} eIF4E\textsuperscript{R} plants. Results were averaged from 10 plants for each genotype. Kruskal–Wallis statistical tests were performed to identify statistically significant differences (a) and (b) represent significantly different groups (P < 0.05, standard error bars are represented on the graph).

other translation initiation factors. Indeed, we show here that eif4e1\textsuperscript{KO} eifiso4e\textsuperscript{KO} eIF4E\textsuperscript{R} lines were fully resistant to both RB-TuMV isolates. This means that E116Q and N163Y mutations in the VPG are two independent paths to the same RB mechanism, allowing the virus to recruit eIF4E1 as well as eIFiso4E in the plant. eIF4E-based resistance breaking has often been associated with the recruitment of the same eIF4E factor, as shown by the pepper/potyvirus co-evolution process (Charroux et al., 2008). This work presents for the first time RB events associated with the recruitment of another 4E isoform. This allows us to anticipate that resistance based on eIF4E loss-of-function could be easily overcome, leading to serious consequences for the design of durable genetic resistance (Bastet et al., 2017). In conclusion, the genetic combination using the synthetic allele designed in this study is associated with a full resistance to all seven potyviruses tested. Overall, our results show that combining mutations in several 4E translation initiation factors is highly likely to expand the resistance spectrum to other potyviruses, but might also extend the resistance durability.

More generally, translation initiation factors were also found in crops and in Arabidopsis to be involved in resistance to ssRNA+ viruses with different genome organizations to potyviruses (Robaglia and Caranta, 2006). This includes the resistance to viruses from genera such as Polerovirus (family Luteoviridae) and Sobemovirus (family Tombusviridae), harbouring a much shorter VPG (around 90 AA in length) displaying little homology with the Potyvirus VPG (around 190 AA). This also extends to Carmovirus (family Tombusviridae) members, including Melon necrotic mosaic virus, whose genomes are uncapped (Jiang and Laliberté, 2011). To demonstrate whether the efficiency of the eIF4E\textsuperscript{R} allele, specifically designed as a resistance allele to potyviruses, could be extended to other ssRNA+ viruses, we challenged the eIF4E\textsuperscript{R}–complemented plants with polerovirus species (TuYV, BMV, BWYV-USA), nepoviruses (GFLV-F13, GFLV-GHu, ArMV) and carmovirus (TCV). We did not observe any resistance to TCV, nor to the three nepoviruses assayed. However, we found a complete resistance to one polerovirus species (BWYV-USA) out of the three tested. It is worth noting that the same polerovirus species have been previously used to challenge loss-of-function mutants affecting translation initiation factors in Arabidopsis, with the eIF4E\textsuperscript{R} mutant showing partial resistance to BMV and BWYV-USA (Reinbold et al., 2013). Significantly, in our study, eIF4E\textsuperscript{R} complementation restores full susceptibility to BMV while it triggers full resistance to BWYV-USA, which could be of interest for sugarbeet breeding programmes. Recently, potyvirus VPG has been suggested to interact with host eIF4E through a conserved noncanonical 4E-binding domain (Miras et al., 2017) which interestingly does not seem to be conserved in VPG from other groups of ssRNA+ viruses. Other viruses are likely to recruit eIF4E through different structural binding domains and would therefore only be marginally affected by potyvirus-specific non-synonymous AA changes in eIF4E regions I and II as the ones introduced in the synthetic eIF4E\textsuperscript{R} allele (Robaglia and Caranta, 2006). For example, the Arabidopsis elfiso4E binds the viral protease rather than the VPG of the nepovirus Tomato ringspot virus (Léonard et al., 2002) and the carmovirus Melon necrotic spot virus (MNSV) binds eIF4E directly through an RNA motif at the 3’ end of the viral genome (Truniger et al., 2008). Consistent with this, the melon eIF4E natural allele rsv associated with resistance to MNSV displays a very idiosyncratic AA change at the C′terminal end of the eIF4E protein sequence (Nieto et al., 2006). This suggests that, to adjust to the different interaction patterns developed by these viruses, specific additional mutations—possibly outside regions I and II—should be characterized and introduced into eIF4E or elfiso4E protein sequences, to tailor specific resistances. Besides, ssRNA+ viruses can also use other translation initiation factors. For example, sobemovirus Rice yellow mottle virus in rice and polerovirus TuYV in Arabidopsis both use the large scaffolding translation initiation factors elfiso4G through the binding of their VPGs (Hébrard et al., 2010; Reinbold et al., 2013), suggesting that these factors may also be designed to trigger specific resistance.

The innovative approaches developed here can be fully translated to crops in order to develop amenable resistances to control losses associated with virus infection using environment-friendly strategies. Mostly, by targeting simultaneously
several translation initiation factors 4E and by combining loss-of-function alleles with modified functional alleles, our approach is likely to trigger resistance without impairing crop agronomical performance, a major requisite for plant breeding. By introducing six independent mutations in the elf4e1 coding sequence, similar to those naturally present in pea, the elf4e1 synthetic allele confers a very robust resistance to potyviruses. We show that mutations associated with resistance can be easily transferred from one species to another to design alleles based on the endogenous relevant genes, as previously shown in Solanaceae (Cavatorta et al., 2011; Kang et al., 2007). Furthermore, by expressing the modified elf4e1 under the control of its own regulatory sequences, the complementation of a knockout allele can be easily performed.
without the use of foreign DNA sequences such as strong constitutive promoters and terminators. This approach, also called cis-genesis, constitutes a major issue for public acceptance (Ilardi and Tavazza, 2015; Schouten et al., 2006) and a strategy similar to the one developed in this study in Arabidopsis could be easily transposed to any crops. This can also be carried out by new breeding techniques (NBT) such as CRISPR/Cas9 technology particularly with the use of Cas9-cytidine deaminase fusion, allowing precise base substitution in situ in the genome of plants of agronomical interest (Komor et al., 2016; Murovec et al., 2017; Shimatani et al., 2017). Genetic diversity could then be optimally exploited in a trans-species manner to provide new engineered sources of resistance.

**Experimental procedure**

**Plant materials and growth conditions**

*Arabidopsis thaliana* Columbia-0 (Col) plants were used in this study as a wild-type control for all tests. *A. thaliana* accessions

**Figure 7** *eif4e1KO* *eifiso4eKO* *eIF4E1R* plant resistance spectrum extends to resistance-breaking TuMV isolates. Controls and *eif4e1KO* *eifiso4eKO* *eIF4E1R* lines were challenged with either GFP-tagged TuMV, GFP-TuMV-E116Q (RB) or GFP-TuMV-N163Y (RB). Viral accumulation was assessed using GPPcam (PSI) camera-imaging (a). Fluorescence intensity is shown in false colour from blue (low) to red (high intensity) with the plant pot reflecting light, allowing visualization of the outline of the plant position. (b) Detection of TuMV mRNA by RT-PCR amplification of the VPg coding sequence for both TuMV-GFP and TuMV-N163Y-GFP 21 dpi in the noninoculated leaves. **Figure 8** *eif4e1KO* *eifiso4eKO* *eIF4E1R* plants resistance spectrum extends to polerovirus species BWYV-USA but not to TuYV and BMYV. BWYV-USA, TuYV and BMYV viral accumulation was measured by DAS-ELISA in control lines and in two *eif4e1KO* *eifiso4eKO* *eIF4E1R* lines. DAS-ELISA was performed 21 days after inoculation with TuYV (a), BMYV (b) or BWYV-USA (c). 12 plants per genotype were tested, and experiments were repeated twice. a, b and c represent significantly different groups, $P < 0.05$.
Landsberg erecta (Ler) and Cap Verde island (Cvi) were also used as susceptible and resistant controls, respectively, for WMV infection according to (Ouibrahim et al., 2014). eIF4E\textsuperscript{KO} and eIFiso4E\textsuperscript{KO} lines are, respectively, homozygous for the T-DNA insertion in eIF4E\textsuperscript{1} (At1g18040; SALK_145583) and the transposon DSm insertion in eIFiso4E \textsuperscript{1} (At1g35620; Duprat et al., 2002) in a Col background. For genetic crosses, immature flowers were emasculated and cross-pollinated manually.

For growth on plates, seeds were surface-sterilized for 10 min in 95% ethanol with 0.1% Tween 20 and plated on Murashige and Skoog (MS) medium, supplemented if necessary with 10 mg/L hygromycin B. Seedlings were transferred to soil after 2 weeks. For nepovirus, polerovirus, TCV, PPV and LMV resistance assays, seeds were directly sowed on soil and individually transferred 2 weeks later.

Plants were grown in a culture chamber at 20–24 °C with a cycle of 16-h light (fluorescent light at 100 μmol photon/m²/s) and 8-h dark for the flowering assay and nepovirus resistance assay, with a cycle of 8-h light and 16-h dark for CIYVV, TuMV, WMV, RB-TuMV, LMV, PPV, and TCV and 10-h light and 14-h dark for TuYV, BMVV-2tb and BWVV-USA resistance assays. For the flowering and fresh/dry weight assays, the different genotypes of plants were randomized in the culture chamber.

**Alignment data and 3D protein modelling**

The sequences for pea eIF4E from resistant and susceptible lines were collected from Gao et al. (2004) (GenBank ID AY611423, AY422375 and AY611425). Protein alignments were carried out using MultiAlin (http://multalin.toulouse.inra.fr) and Box Shade (http://www.ch.embnet.org/software/BOX_form.html). The 3D modelization of AteIF4E1 and eIF4E\textsuperscript{1} protein was constructed by homology prediction with YASARA software (http://www.yasara.org; Krieger and Vriend, 2014) using the crystal structure of *Pisum sativum* eIF4E \textsuperscript{1} (PDB ID: 2WMC-C) as a template. Protein structure was visualized with PyMOL software (The PyMOL Molecular Graphics System, version 1.8, Schrödinger, 2015; https://www.pymol.org/; Schrödinger, 2015).

**Plasmid construction and plant transformation**

A 1569-bp genomic –At4g18040– eIF4E\textsuperscript{1} fragment (spanning 1500 bp of the promoter region and 150 bp of the 3’UTR) was amplified on Columbia genomic DNA using primers Z4148-F and Z4148-R (Table S1) and subcloned into pDONR207 using BP gateway recombination (Invitrogen), resulting in plasmid pJL631. Site-directed mutagenesis associated with the following AA changes—W69L T80D S81D S84A G114R and N176K—were iteratively introduced with the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) using specific primers. All primers are listed in Table S1. All constructs were sequence-checked. The resulting eIF4E\textsuperscript{1} genomic construct was then cloned into the binary vector pMDC99 (Curts and Grossniklaus, 2003) using gateway LR recombination and subsequently introduced in *Arabidopsis* genome by Floral Dip agrotropism (Clough and Bent, 1998). Transformants were selected on MS plates supplemented with 10 mg/L hygromycin B.

**Virus materials, inoculation and detection assays**

CIYVV provided by Ichiro Uyeda (Sato et al., 2005), TuMV (CDN1 isolate) and WMV (Fr isolate) were propagated on *Nicotiana benthamiana* cv Xanthi, turnip (Brassica rapa) and zucchini squash (*Cucurbita pepo*), respectively, prior to being rubbed-inoculated on leaves from 1-month-old *Arabidopsis* plants (Gallosi et al., 2010; Ouibrahim et al., 2014). Viral accumulation detection of CIYVV, TuMV and WMV was carried out 20–30 days after inoculation by ELISA using anti-CIYVV (DSMZ), anti-potyvirus group (Agdia, Elkhart, Indiana) and anti-WMV (SEDIAG, Longvic, France, http://www.sediag.com) antisera and detection sets, respectively.

The LMV-AFVAR1 isolate, differing by a single amino acid change in the CP N-terminal region from its LMV-AF199 progenitor (Decroocq et al., 2009), was selected for its capacity to systemically infect Columbia-0. This virus was inoculated mechanically onto *Arabidopsis* rosette leaves 6 weeks after sowing. Virus accumulation in apical noninoculated leaves was analysed by DAS-ELISA assay with polyclonal antibodies anti-LMV (German-Retana et al., 2008) 21 days postinoculation (dpi).

The PPV-R isolate (Dideron strain) was agro-inoculated using the pBINPPVnkGFP construct (Fernández-Fernández et al., 2001) and ELISA assays were performed 21 dpi, for apical noninoculated leaves, using commercial anti-PPV antibodies (D+M polyclonal antibody, LCA Laboratory, Blanquefort, France).

RB-TuMV plasmids were constructed from pCambia TuMV-GFP, a gift from Jean-François Laliberté (Beauchemin et al., 2005), by introducing into the Vpg coding sequence the E116Q and N163Y mutations, respectively, by PCR-based mutagenesis using the QuikChange\textsuperscript{II} XL Site-Directed Mutagenesis kit (Agilent, Santa Clara, CA) with primers Z4653-F/Z4653-R for E116Q and Santa Clara, CA with primers Z4653-F/Z4653-R for E116Q and Z4654-F/Z4654-R for N163Y. Presence of the mutations was checked by sequencing (Genoscreen, Lille, France), and the resistance-breaking properties of these variants were assessed on eIFiso4E\textsuperscript{KO} plants. The plasmids were transformed into the C585 pMP90 agrobacterium strain and agro-inoculated in leaves.

GFP accumulation in plants was imaged 3 weeks after inoculation using a closed fluorometric camera FluorCam FC 800-C/1010-GFP (Photon System Instruments, Drasov, Czech Republic) equipped with a GFP filter. Fluorescence is represented in false colours.

Polarovirus inoculations were carried out as previously described (Reinbold et al., 2013). Briefly, BMVV (isolate 2TB), BWVV-USA and TuYV were inoculated using *Myzus persicae* membrane-fed on purified virus for 24 h. Ten viruliferous aphids were then transferred onto each test plant for an inoculation period of 4 days. Detection of viral infection was performed by DAS-ELISA (Loewe, Kronach, Germany) 21 dpi.

For nepoviruses, plants were mechanically inoculated with crude sap from *Chenopodium quinoa* infected separately with GFLV-F13, GFLV-GHu and ArMV-Tanat isolates. Detection of viral infection was performed by DAS-ELISA using anti-GFLV or anti-
ArMV polyclonal antibodies (Bioreba AG, Switzerland) 19 dpi for GFLV isolates and 24 dpi for ArMV.

TCV isolate M (Oh et al., 1995) inoculation with infectious clone contained in pBln61 was carried out by agro-infiltration on 5-week-old plants. Infection was detected by symptom development 23 dpi.

All results presented are mean values from at least 15 independent plants per genotype, unless indicated otherwise, and error bars represent standard error. The threshold for susceptibility is represented by a line on each graph and refers to absorbance value at 405 nm in ELISA equal to three times the mean value for healthy controls.

Expression analysis by Reverse transcription

Total RNA was extracted from young noninoculated leaves of 1-month-old plants using TRI-Reagent (Sigma-Aldrich, St Louis, MO). RT-PCR was performed with AMV reverse transcriptase (Avian myeloblastosis virus, Promega) on 1 μg of RNA according to the supplier’s instructions. eIF4E1 (A14g18040) and, as a control, ADENINE PHOSPHORYBOSYL TRANSFERASE 1 (APT1, A1tg27450) cDNAs were amplified with primers Z3135-F/Z3135-R and Z1734/Z1735, respectively.

A 575-bp fragment covering the central region of the TuMV VPg coding sequence was similarly amplified by RT-PCR from inoculated plants with primers Z4735-F/Z4735-R.

Antibodies and Western blot analysis

Total protein extracts obtained by grinding 4-week-old plants were used for Western blot analysis. Electrophoresis by SDS-PAGE was performed on equal amount of protein extracts, and proteins were transferred to Amersham (GE Healthcare, Chicago, Illinois, United States) using the method previously described (Estevan et al., 2011). Total RNA was extracted from young noninoculated leaves of 1-month-old plants using TRI-Reagent (Sigma-Aldrich, St Louis, MO) using the method previously described (Estevan et al., 2011). Total seed production of 10 plants per genotype was assayed by collecting and weighing all the seeds of each plant. A pool of 100 seeds per genotype was also weighed to ensure that there was no difference in seed average weight between genotypes.

Statistical analyses

Kruskal–Wallis statistical tests were performed using the pgirmess package on the free software R (http://www.r-project.org/).

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References

Andrade, M., Abe, Y., Nakahara, K.S. and Uyeda, I. (2009) The cv-2 resistance to Clover yellow vein virus in pea is controlled by the eukaryotic initiation factor 4E. J. Gen. Plant Pathol. 75, 241–249.

Baltes, N.J. and Voytas, D.F. (2015) Enabling plant synthetic biology through genome engineering. Trends Biotechnol. 33, 120–131.

Barbasch, D., Tondelli, A., Desiderio, F., Volante, A., Vaccino, P., Valè, G. and Cavatorta, J. (2014) Pyramiding resistances based on translation variation should guide gene editing. Trends Biotechnol. 32, 411–419.

Beauchemin, C., Bougie, V. and Laliberté, J.F. (2005) Simultaneous production of two foreign proteins from a polyvirus-based vector. Virus Res. 112, 1–8.

Browning, K.S. and Bailey-Serres, J. (2015) Mechanism of cytoplasmic mRNA translation. Arabidopsis Book, 13, e0176.

Brunn-Rasmussen, M., Møller, I.S., Tulinius, G., Hansen, J.K., Lund, O.S. and Johansen, I.E. (2007) The same allele of translation initiation factor 4E mediates resistance against two Potyviridae spp. in Psm sativum. Mol. Plant Microbe Interact. 20, 1075–1082.

Callot, C. and Gallois, J.L. (2014) Pyramiding resistances based on translation initiation factors in Arabidopsis is impaired by male gametophyte lethality. Plant Signal. Behav. 9, e27940.

Cavataorta, J., Perez, K.W., Gray, S.M., Van Eck, J., Yeam, I. and Jahn, M. (2011) Engineering virus resistance using a modified potato gene. Plant Biotechnol. J. 9, 1014–1021.

Chandrasekaran, J., Brumin, M., Wolf, D., Leibman, D., Klap, C., Pearlsman, M., Sherman, A. et al. (2016) Development of broad virus resistance in non-transgenic cucumber using CRISPR/Cas9 technology. Mol. Plant Pathol. 17, 1140–1153.
Charron, C., Nicolai, M., Gallois, J.L., Robaglia, C., Mouny, B., Palloix, A. and Caranta, C. (2008) Natural variation and functional analyses provide evidence for co-evolution between plant eIF4E and potyviral VPg. Plant J. 54, 56–68.

Clough, S.J. and Bent, A.F. (1998) Floral dip: A simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. 16, 735–743.

Combé, J.P., Petracek, M.E., van Eldik, G., Meulewaeter, F. and Twel, D. (2005) Translation initiation factors eIF4E and eIFiso4E are required for polysome formation and regulate plant growth in tobacco. Plant Mol. Biol. 57, 749–760.

Curta, M.D. and Grossniklaus, U. (2003) A gateway cloning vector set for high-throughput functional analysis of genes in plants. Plant Physiol. 133, 462–469.

Decroo, V., Salvador, B., Sicard, O., Glasa, M., Cosson, P., Svanella-Dumas, L., Revers, F. et al. (2009) The determinant of potyvirus ability to overcome the RTM resistance of Arabidopsis thaliana maps to the N-terminal region of the coat protein. Mol. Plant Microbe Interact. 22, 1302–1311.

Duprat, A., Caranta, C., Revers, F., Menand, B., Brownung, K.S. and Robaglia, C. (2002) The Arabidopsis eukaryotic initiation factor iso4E is dispensable for plant growth but required for susceptibility to potyviruses. Plant J. 32, 927–934.

Esteban, J., Maréna, A., Callot, C., Lacombe, S., Moretti, A., Caranta, C. and Gallois, J.L. (2014) Specific requirement for translation initiation factor 4E or its isoform drives plant host susceptibility to Tobacco etch virus. BMC Plant Biol. 14, 67.

Fernández-Fernández, M.R., Mouriño, M., Rivera, J., Rodrigue, F., Plana-Durán, J. and García, J.A. (2001) Protection of rabbits against rabbit hemorrhagic disease virus by immunization with the VP60 protein expressed in plants with a potyvirus-based vector. Virology, 280, 283–291.

Gallois, J.L., Charron, C., Sánchez, F., Pagny, G., Houvenaghel, M.C., Moretti, A., Ponz, F. et al. (2010) 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 factor iso4E and iso4G. J. Gen. Virol. 91, 288–293.

Gao, Z., Johansen, E., Eyers, S., Thomas, C.L., Noel Ellis, T.H. and Maule, A.J. (2004) The potyvirus resistance gene, sbm1, identifies a novel role for translation initiation factor 4E in cell-to-cell trafficking. Plant J. 40, 376–385.

Garcia, J.A., Glasa, M., Cambra, M. and Candresse, T. (2014) Plum pox virus and sharka: A model potyvirus and a major disease. Mol. Plant Pathol. 15, 226–241.

Gaufﬁer, C., Lebaron, C., Moretti, A., Constant, C., Moquet, F., Bonnet, G. and Caranta, C. et al. (2016) A TILLING approach to generate broad-spectrum resistance to potyviruses in tomato is hampered by eIF4E gene redundancy. Plant J. 85, 717–729.

German-Retana, S., Johansen, R., Dubrana, M.P., Mazier, M., Maisonneuve, B. and Candresse, T. et al. (2003) The eukaryotic translation initiation factor 4E controls lettuce susceptibility to the Potyvirus Lettuce mosaic virus. Plant Physiol. 132, 1272–1282.

Nicaise, V., Gallois, J.L., Chaffia, F., Allen, L.M., Schurdi-Levraud, V., Newling, K.S., Candresse, T. et al. (2007) Mutations in potato virus Y genome-linked protein determine virulence toward recessive resistances in Capsicum annuum and Lycopersicon hirsutum. Mol. Plant Microbe Interact. 17, 322–329.

Murovec, J., Pirc, Z. and Yang, B. (2017) New variants of CRISPR RNA-guided genome editing enzymes. Plant Biotechnol. J. 15, 917–926.

Marcotrigiano, J., Gingras, A.C., Sonenberg, N. and Burley, S.K. (1997) Costructural content of the messenger RNA 5′ cap-binding protein (eIF4E) bound to 7-methyl-GDP. Cell, 89, 951–961.

Mira, M., Truniger, V., Silva, C., Verdaguer, N., Aranda, M.A. and Querol-Audi, J. (2017) Structure of eIF4E in complex with an eIF4G peptide supports a universal bipartite binding mode for protein translation. Plant Physiol. 174, 1476–1491.

Moury, B., Morel, C., Johansen, E., Guibaud, L., Souche, S., Ayme, V., Caranta, C. et al. (2004) Mutations in potato virus Y genome-linked protein determine virulence toward recessive resistances in Capsicum annuum and Lycopersicon hirsutum. Plant Mol. Biol. 581, 0536–0545.

Nicolet, C., Morales, M., Orjeda, G., Clepet, C., Monfort, A., Sturbois, B., Puigdoménech, P. et al. (2006) An eIF4E allele confers resistance to an uncapped and non-polyadenylated RNA virus in melon. Plant J. 48, 452–462.

Oh, J.W., Kong, Q., Song, C., Carpenter, C.D. and Simon, A.E. (1995) Open reading frames of turnip crinkle virus involved in satellite symptom expression and incompatibility with Arabidopsis thaliana ecotype Dijon. Mol. Plant Microbe Interact. 8, 979–987.

Østerberg, J.T., Xiang, W., Olsen, L.I., Edenbrandt, A.K., Vedel, S.E., Christiansen, A., Landes, X. et al. (2017) Accelerating the domestication of new crops: Feasibility and approaches. Trends Plant Sci. 22, 373–384.

Oubrahim, L. and Caranta, C. (2013) Exploitation of natural genetic diversity to study plant-virus interactions: What can we learn from Arabidopsis thaliana? Mol Plant Pathol. 14, 844–854.

Oubrahim, L., Mazier, M., Esteven, J., Pagny, G., Decroo, V., Desbiez, C., Moretti, A. et al. (2014) Cloning of the Arabidopsis rvm1 gene for resistance to Watermelon mosaic virus points to a new function for natural virus resistance genes. Plant J. 79, 705–716.

Palmgren, M.G., Edenbrandt, A.K., Vedel, S.E., Andersen, M.M., Landes, X., Østerberg, J.T., Falhof, J. et al. (2015) Are we ready for back-to-nature crop breeding? Trends Plant Sci. 20, 155–164.

Patrick, R.M., Mayberry, L.K., Choy, G., Woodard, L.E., Liu, J.S., White, A., Mullen, R.A. et al. (2014) Two Arabidopsis loci encode novel eukaryotic show broad-spectrum Turnip mosaic virus (TuMV) resistance. Mol Plant Pathol. 15, 615–626.

published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd., 16, 1569–1581.
initiation factor 4E isoforms that are functionally distinct from the conserved plant eukaryotic initiation factor 4E. Plant Physiol. 164, 1820–1830.
Pawan, S., Jacobsen, E., Visser, R.G. and Bai, Y. (2010) Loss of susceptibility as a novel breeding strategy for durable and broad-spectrum resistance. Mol. Breed. 25, 1–12.
Pyott, D.E., Sheehan, E. and Molnar, A. (2016) Engineering of CRISPR/Cas9-mediated potyvirus resistance in transgene-free Arabidopsis plants. Mol. Plant Pathol. 17, 1276–1288.
Reinhold, C., Lacombe, S., Ziegler-Graff, V., Scheidecker, D., Wiss, L., Beuve, M., Caranta, C. et al. (2013) Closely related poleroviruses depend on distinct translation initiation factors to infect Arabidopsis thaliana. Mol. Plant Microbe Interact. 26, 257–265.
Robaglia, C. and Caranta, C. (2006) Translation initiation factors: A weak link in plant RNA virus infection. Trends Plant Sci. 11, 40–45.
Ruffel, S., Dussault, M.H., Palloix, A., Mouri, B., Bendahmane, A., Robaglia, C. and Caranta, C. (2002) A natural recessive resistance gene against potato virus Y in pepper corresponds to the eukaryotic initiation factor 4E (eif4E). Plant J. 32, 1067–1075.
Ruffel, S., Gallois, J.L., Lesage, M.L. and Caranta, C. (2005) The recessive potyvirus resistance gene pot-1 is the tomato orthologue of the pepper prv2-eif4E gene. Mol. Genet. Genom. 274, 346–353.
Ruffel, S., Gallois, J.L., Mouri, B., Robaglia, C., Palloix, A. and Caranta, C. (2006) Simultaneous mutations in translation initiation factors eIF4E and eIF(iso)4E are required to prevent pepper vein mottle virus infection of pepper. J. Gen. Virol. 87, 2089–2098.
Sanfaçon, H. (2015) Plant translation factors and virus resistance. Viruses, 7, 3392–3419.
Sato, M., Nakahara, K., Yoshii, M., Ishikawa, M. and Uyeda, I. (2005) Selective involvement of members of the eukaryotic initiation factor 4E family in the infection of Arabidopsis thaliana by potyviruses. FEBS Lett. 579, 1167–1171.
v van Schie, C.C. and Takken, F.L. (2014) Susceptibility genes 101: How to be a good host. Annu. Rev. Phytopathol. 52, 551–581.
Schouten, H.J., Krens, F.A. and Jacobsen, E. (2006) Cisgenic plants are similar to traditionally bred plants: International regulations for genetically modified organisms should be altered to exempt cisgenesis. EMBO Rep. 7, 750–753.
Schrödinger, L.L.C. (2015) The PyMOL Molecular Graphics System Version 1.8.
Shimatani, Z., Kashojiya, S., Takayama, M., Terada, R., Arazoe, T., Ishii, H., Taramura, H. et al. (2017) Targeted base editing in rice and tomato using a CRISPR-Cas9 cytidine deaminase fusion. Nat. Biotechnol. 35, 441–443.
Stein, N., Perovic, D., Kulemhein, J., Pello, B., Stracke, S., Streng, S., Ordon, F. et al. (2005) The eukaryotic translation initiation factor 4E confers multiallelic recessive Bymovirus resistance in Hordeum vulgare (L.). Plant J. 42, 912–922.
Truniger, V., Nieta, C., González-Ibeas, D. and Aranda, M. (2008) Mechanism of plant eIF4E-mediated resistance against a Carnovirus (Tombusviridae): Cap-independent translation of a viral RNA controlled in cis by an (a)virulence determinant. Plant J. 56, 716–727.
Wang, A. and Krishnaswamy, S. (2012) Eukaryotic translation initiation factor 4E-mediated recessive resistance to plant viruses and its utility in crop improvement. Mol. Plant Pathol. 13, 795–803.
Yoshii, M., Yoshioka, N., Ishikawa, M. and Naito, S. (1998) Isolation of an Arabidopsis thaliana mutant in which the multiplication of both cucumber mosaic virus and turnip crinkle virus is affected. J. Virol. 72, 8731–8737.
Zhang, L., Yao, J., Withers, J., Xin, X.F., Banerjee, R., Fariduddin, Q., Nakamura, Y. et al. (2015) Host target modification as a strategy to counter pathogen hijacking of the jasmonate hormone receptor. Proc. Natl. Acad. Sci. USA, 112, 14354–14359.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Three-dimensional predicted structure of Pisum sativum eIF4E proteins.
Figure S2 elf4E1 expression analysis in controls and complemented lines.
Figure S3 Genotyping of elf4e1KO elfiso4eKO elf4E1R plants.
Figure S4 Phenotype of the elf4e1KO elfiso4eKO elf4E1R plants.
Figure S5 elf4e1KO elfiso4eKO elf4E1R plants resistance spectrum extends to LMV and PPV.
Figure S6 The Cvi accession is partly resistant to WMV, but elf4e1KO and elfiso4eKO single mutants are both susceptible.
Figure S7 Low accumulation of resistance-breaking TuMV isolates in elf4e1KO elfiso4eKO plants as detected by DAS-ELISA.
Figure S8 elf4e1KO elfiso4eKO elf4E1R plants resistance spectrum does not extend to carmovirus TCV.
Figure S9 elf4e1KO elfiso4eKO elf4E1R plants resistance spectrum does not extend to nepoviruses.

Table S1 List of oligonucleotides used in this study.