Research Paper

Loss-of-function of *Nicotiana tabacum* L. eukaryotic translation initiation factors eIF4E1-S and eIF(iso)4E-T synergistically confers high-level resistance to both *Potato virus Y* (PVY) and resistance-breaking PVY

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The plant eukaryotic translation-initiation factors eIF4E and eIF(iso)4E play key roles in infection by plant RNA viruses, especially potyviruses. Mutations in the genes that encode these factors reduce susceptibility to the viruses. In the amphidiploid plant tobacco (*Nicotiana tabacum* L.), eIF4E1-S deletion mutants resist *Potato virus Y* (PVY), but resistance-breaking strains (RB-PVY) have appeared. In an earlier study, we demonstrated that the loss-of-function of eIF(iso)4E-T reduces susceptibility to RB-PVY. Here, we show that simultaneous inhibition of eIF4E1-S and eIF(iso)4E-T synergistically confers enhanced resistance to both PVY and RB-PVY without host growth or development defects. PVY symptoms and accumulation in a tobacco line lacking eIF4E1-S were detected at 14 days post-inoculation (dpi) and RB-PVY symptoms in lines without functional eIF(iso)4E-T were observed at 24 dpi. RB-PVY emerged in a PVY-infected tobacco line lacking eIF4E1-S. In contrast, lines without functional eIF4E1-S and eIF(iso)4E-T were nearly immune to PVY and RB-PVY, and little accumulation of either virus was detected even at 56 dpi. Thus, the lines will be promising for PVY-resistance breeding. This study provides a novel strategy to develop tobacco highly resistant to PVY and RB-PVY, and insights into the mechanisms responsible for high-level resistance.

**Key Words:** durable resistance, eIF4E, eIF(iso)4E, homeologous gene, *Nicotiana tabacum*, *Potato virus Y*, resistance-breaking.

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**Introduction**

*Potyviruses* is one of the largest genera of plant viruses. Potyviruses are positive-stranded RNA viruses and most of them are transmitted by aphids in a non-persistent manner (Revers and García 2015). Completion of the potyvirus infection cycle depends on complex interplays between factors encoded by the viral and host genomes (Thivierge et al. 2005). For example, the viral protein VPg, which mimics the 5’-cap structure of mRNA, interacts with the host eukaryotic translation-initiation factor 4E (eIF4E) or its isoform, eIF(iso)4E (Gingras et al. 1999, Wittmann et al. 1997).

Breeding plants for resistance is one way to reduce the damage caused by viral diseases. To accomplish this, it’s helpful to identify the host genes responsible for virus resistance. Screening following ethyl methanesulfonate and transposon mutagenesis of *Arabidopsis thaliana* for decreased susceptibility to *Turnip mosaic virus* identified eIF(iso)4E as the responsible gene (Duprat et al. 2002, Lellis et al. 2002). Since then, many natural recessive genes for resistance to potyviruses have been associated with mutations in the eIF4E/eIF(iso)4E protein family, and artificial mutations in eIF4E/eIF(iso)4E genes have been found to confer resistance in various crops (Robaglia and Caranta 2006, Sanfaçon 2015, Wang and Krishnaswamy 2012).

*Potato virus Y* (PVY), which belongs to the genus *Potyviruses*, shows a wide host range and is distributed worldwide (Quenouille et al. 2013). It causes serious crop losses in Solanaceae species such as potato (*Solanum tuberosum* L.), peppers (*Capsicum annuum* L.), tomato (*Solanum lycopersicum* L.), and tobacco (*Nicotiana tabacum* L.) (Scholthof et al. 2011). In tobacco, PVY is one of major viral pathogens. Especially the strains of PVY that induce vein necrosis are a major concern for tobacco production because they can cause severe yield and quality losses. The main genetic source of resistance to PVY is the *va* locus, which originates from the ‘Virgin A Mutant’ (‘VAM’) that was obtained by x-irradiation-induced mutagenesis (Koelle 1961). The recessive *va* allele has been
introduced into several tobacco cultivars, including ‘TN90’ (Miller 1991). ‘VAM’ contains a deletion longer than 1 Mbp at the Va locus on chromosome 21 (Dlugé et al. 2018, Noguchi et al. 1999). Recently it has been reported that an eIF4E gene (GenBank accession number KF155696) is located at the Va locus and that its deletion confers resistance to PVY (Julio et al. 2015). Tobacco is an amphidiploid plant whose genome consists of two types of the genome originated from ancestors; N. sylvestris (S-type) and N. tomentosiformis (T-type). The Va locus eIF4E is encoded in the S-type and is designated eIF4E1-S.

The resistance conferred by the va allele has been occasionally broken by variants of PVY (Blancard et al. 1999, Lacroix et al. 2010, 2011), although ‘VAM’ shows more durable resistance than cultivars with an introduced va locus (Acosta-Leal and Xiong 2013, Michel et al. 2019). The breaking of resistance based on va is associated with mutations (for example, a K105E mutation) in the VPg central domain of PVY (Acosta-Leal and Xiong 2013, Janzac et al. 2014, Masuta et al. 1999, Takakura et al. 2018), as is the case in other potyviruses. Because an outbreak of the resistance-breaking type of PVY (RB-PVY) is a serious threat to tobacco production, it’s urgent to find a way to suppress outbreaks. Recently, we reported that a tobacco mutant for a double mutation of eIF(iso)4E-T, eIF(iso)4E-T, showed reduced susceptibility to RB-PVY (Takakura et al. 2018). Furthermore, we showed in a yeast two-hybrid assay that VPg with a K105E mutation in RB-PVY physically interacts with eIF(iso)4E-T as well as with eIF4E-S, whereas VPg of PVY interacts only with eIF4E1-S (Takakura et al. 2018).

The objectives of the present study are to determine whether tobacco with double mutations of eIF4E1-S and eIF(iso)4E-T would resist both PVY and RB-PVY without growth or development defects and whether the plants would show higher resistance to RB-PVY than plants with eIF(iso)4E-T single mutation. Here, we examined the effect of eIF4E1-S and eIF(iso)4E-T double mutations on resistance to PVY and RB-PVY. This study provides the first evidence of a synergistic effect of homeologous genespecific inhibition of host eIF4E and eIF(iso)4E proteins on viral resistance and a novel breeding strategy for both PVY and RB-PVY resistance.

**Materials and Methods**

**Plant materials**

An elite burley tobacco cultivar ‘TN90’, and ‘VAM’ were used as PVY-resistant cultivars. Since both lack eIF4E1 in the S-type genome (eIF4E1-S) and have eIF4E1 in the T-type genome (eIF4E1-T), we designated the genotype of those plants as eIF4E1-SS/TT. ‘Tsukuba 1’, a fluecured tobacco, was used as a PVY-susceptible cultivar. A tobacco mutant for eIF(iso)4E in the T genome (eIF(iso)4E-SS/TT; hereafter, “iso-t”) and a double mutant for both eIF(iso)4E genes (eIF(iso)4E-ss/TT; hereafter, “iso-st”) were used as RB-PVY-resistant lines (Takakura et al. 2018). These mutants were obtained from an ethyl methanesulfonate induced tobacco mutant population of ‘Tsukuba 1’. Iso-t had a nonsense mutation in the first exon of eIF(iso)4E-T and iso-st had nonsense mutations in the first exons of both eIF(iso)4E-S and eIF(iso)4E-T (Takakura et al. 2018). A line with a genotype of eIF4E1-SS//TT and eIF(iso)4E-Ss//TT was produced by a cross between iso-s, a tobacco mutant for eIF(iso)4E in the S genome (eIF(iso)4E-ss/TT), and iso-t. The line was crossed with ‘TN-90’ (eIF4E1-ss//TT and eIF(iso)4E-SS//TT) and selected to produce an F1 with eIF4E1-Ss//TT and eIF(iso)4E-SS//TT genotype (Fig. 1). The F1 were successively backcrossed with ‘TN-90’ five times to produce a BC2F1 with an eIF4E1-ss//TT and eIF(iso)4E-Ss//TT genotype. The BC2F1 was selfed two times to produce four lines with the following genotypes; eIF4E1-ss//TT and eIF(iso)4E-ss//TT (TN90-iso-s), eIF4E1-ss//TT and eIF(iso)4E-SS//TT (TN90-iso-t), eIF4E1-ss//TT and eIF(iso)4E-SS//TT (TN90-iso-St), and eIF4E1-ss//TT and eIF(iso)4E-SS//TT (TN90-iso-ST). Genotyping for each alleles were conducted according to the following section, ‘Genotyping of eIF4E1 and eIF(iso)4E’. Plants were grown in a greenhouse at 22 to 25°C.

**Virus materials**

PVY, which causes necrosis on ‘Tsukuba 1’, and RB-PVY, which causes necrosis on ‘VAM’, ‘TN90’, and ‘Tsukuba 1’, were used. Both viruses were isolated in the leaf Tobacco Research Center, Japan Tobacco Inc. Both PVY and RB-PVY strains were classified as PVY<sup>NTN</sup> types.

![Fig. 1. Backcross scheme for producing the tobacco lines without functional eIF4E1-S and eIF(iso)4E-T. M means marker-assisted selection.](image-url)
belonging to the PVYN group by multiplex PCR analysis (Takakura et al. 2018). PVY was maintained on the leaves of ‘Tsukuba 1’, and RB-PVY was maintained on the leaves of ‘VAM’. VPg of the RB-PVY strain has a K105E mutation (Takakura et al. 2018).

Genotyping of eIF4E1 and eIF(iso)4E

Genomic DNA was extracted from tobacco leaves using a Gentra Puregene kit (QIAGEN, Hilden, Germany). The genotypes of eIF4E1-S were analyzed by the presence or absence of a PCR product amplified with gene-specific primers (5ʹ-GAATTGGACAATGAGCTTTAGT-3ʹ and 5ʹ-CTGGGCTACCATAAATAATTCC-3ʹ) for eIF(iso)4E-T and eIF(iso)4E-S as a positive control in PCR. Duplex PCR was performed by using a Multiplex PCR kit (QIAGEN) and the amplicons (473 bp for Va and 108 bp for N) were separated by electrophoresis in a QIAxcel System (QIAGEN) (Supplemental Fig. 1A). For genotyping of eIF(iso)4E genes, eIF(iso)4E-S and eIF(iso)4E-T were amplified by multiplex PCR with a mix of primers (5ʹ-GTCTGCTGGGCTACCATAAATAATTCC-3ʹ and 5ʹ-ACCTGTGAGCCCAGCAGGCTCTCAAT-5ʹ) as a positive control in PCR. Duplex PCR was performed by using a Multiplex PCR kit (QIAGEN) and the amplicons (473 bp for Va and 108 bp for N) were separated by electrophoresis in a QIAxcel System (QIAGEN) (Supplemental Fig. 1A). Genotypes were decided by fragment size as described in Udagawa et al. (2020).

Virus inoculation assay and sampling

Virus-infected leaves were ground into five parts (w/v) of 0.01 M potassium phosphate buffer (pH 7.0) with a mortar and pestle to produce inoculum and the most expanded leaves of healthy 5-week-old plants were mechanically inoculated with the extract using the tip of a cotton swab and Carborundum. Although the typical symptoms of PVY were vein necrosis and leaf mottling, we focused on vein necrosis and resistance. The systemic leaves were sampled from independent plants (n = 4 or 5) at each time point (0 to 56 days post-inoculation, dpi), powdered by a mortar and pestle in liquid nitrogen, and stored at –80°C until use.

Enzyme-linked immunosorbent assay

Total soluble protein was extracted from systemic leaves in General Extraction Buffer (Agdia, Inc., Elkhart, IN), and tested by enzyme-linked immunosorbent assay (ELISA) using anti-PVY antibodies (a PVY PathoScreen kit, Agdia, Inc.). The values of average absorption at 405 nm (A405) of four or five biological samples are calculated. Statistical analyses were performed with Statcel3 software (OMS Ltd., Saitama, Japan).

RNA extraction and cDNA synthesis

RNA was purified from the leaves using an RNeasy Plant Mini kit (QIAGEN). cDNA was synthesized using a PrimeScript RT reagent kit (Takara Bio Inc., Shiga, Japan).

Next-generation sequencing of VPg

VPg DNAs of PVY were amplified using a set of primers (5ʹ-TGCTGGGCTGGGCTACCATAAATAATTCC-3ʹ and 5ʹ-CTGGGCTACCATAAATAATTCC-3ʹ) for eIF(iso)4E-T, and digested fragments were separated by electrophoresis in a QIAxcel System (QIAGEN). We firstly analyzed the VPg sequence of inoculum sample to use it for the reference. Then, sequence reads of other samples were mapped to the reference and mutations were detected.

General methods

DNA and amino acid sequences were analyzed with GENETYX Ver.12 software (Genetex Co., Tokyo, Japan).

Results

Resistance of the lines without functional eIF4E1-S and eIF(iso)4E-T to PVY

We investigated PVY resistance of ‘Tsukuba 1’, ‘TN90’, ‘VAM’, TN90-iso-t, TN90-iso-st, and TN90-iso-ST. ‘Tsukuba 1’ (without mutation in neither eIF4E1 nor eIF(iso)4E) was susceptible to PVY; vein necrosis was observed in 75% of the plants at 10 days post-inoculation (dpi) and all of the plants at 14 dpi (Table 1). On the other hand, ‘TN90’, ‘VAM’, and TN90-iso-ST, which have the same eIF4E1 and eIF(iso)4E genotypes (eIF4E1-ss///Tt/ eIF(iso)4E-SS///Tt), showed reduced susceptibility (partial resistance) to PVY. None of the plants showed symptoms at 10 dpi, but 8% (‘VAM’) and 28% (‘TN90’ and TN90-iso-ST) of plants showed symptoms at 14 dpi, and 40% (‘VAM’), 64% (‘TN90’), and 96% (TN90-iso-ST) of plants showed symptoms at 34 dpi. The ratio of ‘VAM’ plants showing symptoms to ‘TN90’ plants showing symptoms was approximately one-third at 14 dpi and two-thirds at 34 and 54 dpi. Thus, as expected, ‘VAM’ showed stronger PVY resistance than ‘TN90’. In contrast, in the plants with mutations in both eIF4E1-S and eIF(iso)4E-T, vein necrosis was observed in only 1 of 25 plants at 34 (TN90-iso-t) or
Fig. 2A shows no symptoms in TN90-iso-t at 42 dpi, but symptoms in ‘TN90’. ELISA revealed that viral accumulation in ‘V AM’ was significantly less than that in ‘TN90’ at 14 dpi, as expected (Fig. 3A). PVY accumulated much less in TN90-iso-t and TN90-iso-st than in ‘TN90’ (at 14 dpi) and in ‘VAM’ (at 35 and 56 dpi) (Fig. 3A). Thus, the plants without both functional elf4E1-S and elf(iso)4E-T genes were nearly immune to PVY.

Table 1. Effect of double mutations in tobacco elf4E1-S and elf(iso)4E-T on Potato virus Y (PVY) infection

| Tobacco line         | Genotype elf4E1 & elf(iso)4E-T | No. of plants showing symptoms/ no. analyzed |
|----------------------|---------------------------------|---------------------------------------------|
| ‘Tsukuba 1’          | SS/TT & SS/TT                  | 18/24 (75%)                                 |
| ‘TN90’               | ss/TT & SS/TT                  | 0/25 (0%)                                   |
| ‘VAM’                | ss/TT & SS/TT                  | 0/25 (0%)                                   |
| TN90-iso-t           | ss/TT & SS/TT                  | 0/25 (0%)                                   |
| TN90-iso-st          | ss/TT & SS/TT                  | 0/25 (0%)                                   |
| TN90-iso-ST          | ss/TT & SS/TT                  | 0/25 (0%)                                   |

a: Vein necrosis; b: days post-inoculation; c: not tested; d: ‘TN90’ (lacking elf4E1-S) with a nonsense mutation in elf(iso)4E-T; e: ‘TN90’ with nonsense mutations in both elf(iso)4E-S and elf(iso)4E-T; f: ‘TN90’ without a nonsense mutation in elf(iso)4E (i.e., wild-type segregant).

Resistance of the lines without functional elf4E1-S and elf(iso)4E-T to RB-PVY

We also investigated the RB-PVY resistance of ‘Tsukuba 1’, ‘TN90’, ‘VAM’, iso-t, iso-st, and TN90-iso-ST. Lines without a mutation in elf(iso)4E-T (‘Tsukuba 1’, ‘TN90’, ‘VAM’, and TN90-iso-ST) were susceptible to RB-PVY; vein necrosis was observed in 30% (‘VAM’) to 90% (‘TN90’) of the plants at 11 dpi (Table 2). On the other hand, iso-t and iso-st showed reduced susceptibility (partial resistance) to RB-PVY: None of the iso-t plants showed symptoms at 11 dpi, but 60% showed symptoms at 24 dpi. In iso-st, no plants showed symptoms at 11 dpi, but 10% showed symptoms at 24 dpi and 25% showed symptoms at 56 dpi. In contrast, TN90-iso-t and TN90-iso-st showed no vein necrosis even at 56 dpi. Fig. 2B shows no symptoms in TN90-iso-t at 21 dpi, but
Fig. 3. Viral accumulation in tobacco eIF4E1 and eIF(iso)4E mutant lines. Plants were inoculated with (A) Potato virus Y (PVY) or (B) resistance-breaking PVY (RB-PVY). The systemic leaves from inoculated plants (n = 4 to 5) at 0 to 56 days post-inoculation (dpi) were tested by enzyme-linked immunosorbent assay (ELISA) using anti-PVY antibodies. The values of average absorption at 405 nm (A\textsubscript{405}) are shown. Values are means ± standard error. Accessions: iso-t, eIF(iso)4E-T mutant; iso-st, eIF(iso)4E-S mutant; TN90-iso-t, eIF4E1-S/eIF(iso)4E-T double mutant; TN90-iso-st, eIF4E1-S/eIF(iso)4E-S/eIF(iso)4E-T triple mutant; TN90-iso-ST, segregants without mutation of eIF(iso)4E; ‘VAM’, ‘Virgin A Mutant’ (resistant to PVY). Significance of difference from the corresponding ‘TN90’ values (iso-t value in 56 dpi) is shown. A\textsubscript{405} values of the double (TN90-iso-t) and triple (TN90-iso-st) mutants did not increase significantly even at 56 dpi.

RB-PVY did not accumulate in the iso-t and iso-st mutants up to 24 dpi, but it did accumulate to a high level (A\textsubscript{405} = 2.70) in iso-t and to a moderate level (A\textsubscript{405} = 0.58) in iso-st at 56 dpi (Fig. 3B). In contrast, RB-PVY did not accumulate in TN90-iso-t or TN90-iso-st even at 56 dpi. Thus, the plants without both functional eIF4E1-S and eIF(iso)4E-T genes were immune to RB-PVY. The differences of the symptom development and viral accumulation observed between iso-t and iso-st were not found between TN90-iso-t and TN90-iso-st (Table 2, Fig. 3B). This is probably because both eIF4E1-S and eIF(iso)4E-T are more important than eIF(iso)4E-S for RB-PVY infection.

Table 2. Effect of double mutations in tobacco eIF4E1-S and eIF(iso)4E-T on resistance-breaking Potato virus Y (RB-PVY) infection

| Tobacco line | Genotype eIF4E1 & eIF(iso)4E | No. of plants showing symptoms\(^a\)/no. analyzed |
|--------------|-----------------------------|-------------------------------------------------|
| ‘Tsukuba 1’  | SS/TT & SS/TT    | 13/20 (65%)          | 20/20 (100%)  | N.T.   |
| ‘TN90’       | ss/TT & SS/TT    | 18/20 (90%)          | 20/20 (100%)  | N.T.   |
| ‘VAM’        | ss/TT & SS/TT    | 6/20 (30%)           | 20/20 (100%)  | N.T.   |
| iso-t\(^d\)  | SS/TT & SS/TT    | 0/20 (0%)            | 12/20 (60%)   | 20/20 (100%) |
| iso-st\(^d\) | SS/TT & ss/TT    | 0/20 (0%)            | 2/20 (10%)    | 5/20 (25%)     |
| TN90-iso-t\(^d\) | ss/TT & SS/TT | 0/20 (0%)           | 0/20 (0%)     | 0/20 (0%)     |
| TN90-iso-st\(^d\) | ss/TT & ss/TT | 0/20 (0%)           | 0/20 (0%)     | 0/20 (0%)     |
| TN90-iso-ST\(^d\) | ss/TT & SS/TT | 16/20 (80%)         | 20/20 (100%)  | N.T.   |

\(^a\) Vein necrosis; \(^b\) days post-inoculation; \(^c\) not tested; \(^d\) ‘Tsukuba 1’ mutant with a nonsense mutation in eIF(iso)4E-T (Takakura et al. 2018); ‘Tsukuba 1’ mutant with nonsense mutations in both eIF(iso)4E-S and eIF(iso)4E-T (Takakura et al. 2018); ‘TN90’ (lacking eIF4E1-S) with a nonsense mutation in eIF(iso)4E-T; ‘TN90’ with nonsense mutations in both eIF(iso)4E-S and eIF(iso)4E-T; ‘TN90’ without a nonsense mutation in eIF(iso)4E (wild-type segregant).
The VPg sequences of the PVY-infected leaves were verified sequences from the central part of VPg by means of reverse-transcription PCR with purified RNA obtained from infected leaves using a next-generation sequencer. The VPg sequences of the inoculum, of four ‘Tsukuba 1’ plants were not immune to PVY, only less susceptible; viral symptoms or accumulation were detected up to 14 dpi (PVY in ‘TN90’, Table 1, Fig. 3A) or 24 dpi (RB-PVY in iso-t and iso-st, Table 2). In contrast, virus accumulation was hardly detected even at 56 dpi in the lines without functional eIF4E1-S and eIF(iso)4E-T (Fig. 3). Our results therefore demonstrated that knockout of both eIF4E1-S and eIF(iso)4E-T conferred nearly complete immunity to both PVY and RB-PVY. Thus, the effect of simultaneous loss of functional eIF4E1-S and eIF(iso)4E-T genes on virus resistance was not additive, but rather synergistic.

On the basis of the results in the present study, we propose a mechanism for the enhanced resistance to both PVY and RB-PVY in the eIF(iso)4E-T-impeded va tobacco (Fig. 5). The VPg protein of PVY interacts with tobacco eIF4E1-S, whereas that of RB-PVY interacts with both eIF4E1-S and eIF(iso)4E-T (Takakura et al. 2018, Fig. 5A). In the va tobacco, which lacks eIF4E1-S (Fig. 5B), PVY cannot utilize eIF4E1-S, resulting in increase of selection pressure to favor mutant PVY strains, including RB-PVY. RB-PVY can utilize both eIF4E1-S and eIF(iso)4E-T, and can therefore infect va tobacco (Fig. 5B). In the eIF(iso)4E-T mutant (Fig. 5C), RB-PVY cannot use eIF(iso)4E-T, but can use eIF4E1-S. Since inhibition of eIF(iso)4E-T, but not of eIF4E1-S, affected RB-PVY accumulation even though the sequence of the parental PVY strains. To investigate whether the PVY that emerged in va tobacco in the present study has a characteristic variation observed in RB-PVY, we verified sequences from the central part of VPg by means of reverse-transcription PCR with purified RNA obtained from infected leaves using a next-generation sequencer. The VPg sequences of the PVY-infected leaves of four ‘TN90’ plants at 28 dpi had mutations characteristic to RB-PVY (Janzac et al. 2014); one was V108I and the others were K105E (Fig. 4A, 4B). These mutations were detected neither in the PVY used as the inoculum nor in ‘Tsukuba 1’ at 28 dpi. This result can be explained by assuming that the PVY genome underwent random mutations, and then a virus that was able to infect ‘TN90’ plants without the functional eIF4E1-S emerged. This is plausible because mutation rates in RNA viruses are high (Sanjuán et al. 2010). Another explanation is that the RB-type sequence was present in the inoculum at a very low level (1/10000) and selectively increased.

### Discussion

In the tobacco–PVY interaction, a nonsense mutation or complete deletion of eIF4E1-S reduced susceptibility to PVY (Julio et al. 2015), and a nonsense mutation of eIF(iso)4E-T reduced susceptibility to RB-PVY (Takakura et al. 2018). However, plants with these single mutants were not immune to PVY, only less susceptible; viral symptoms or accumulation were detected up to 14 dpi (PVY in ‘TN90’, Table 1, Fig. 3A) or 24 dpi (RB-PVY in iso-t and iso-st, Table 2). In contrast, virus accumulation was hardly detected even at 56 dpi in the lines without functional eIF4E1-S and eIF(iso)4E-T (Fig. 3). Our results therefore demonstrated that knockout of both eIF4E1-S and eIF(iso)4E-T conferred nearly complete immunity to both PVY and RB-PVY. Thus, the effect of simultaneous loss of functional eIF4E1-S and eIF(iso)4E-T genes on virus resistance was not additive, but rather synergistic.

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### Table 1

| Inoculum       | K105E | V108I |
|----------------|-------|-------|
| Tsukuba 1_1    | 0% (0/279) | 0% (0/279) |
| Tsukuba 1_2    | 0% (0/172) | 0% (0/172) |
| Tsukuba 1_3    | 0% (0/197) | 0% (0/197) |
| Tsukuba 1_4    | 0% (0/198) | 0% (0/198) |
| TN90_1        | 99.6% (285/289) | 99.6% (285/289) |
| TN90_2        | 99.8% (503/504) | 99.8% (503/504) |
| TN90_3        | 99.8% (451/452) | 99.8% (451/452) |
| TN90_4        | 99.4% (345/347) | 99.4% (345/347) |

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### Table 2

| Inoculum       | 105 | 108 | 120 | 188 |
|----------------|-----|-----|-----|-----|
| Inoculum       |     |     |     |     |
| Tsukuba 1_1    |     |     |     |     |
| Tsukuba 1_2    |     |     |     |     |
| Tsukuba 1_3    |     |     |     |     |
| Tsukuba 1_4    |     |     |     |     |
| TN90_1        |     |     |     |     |
| TN90_2        |     |     |     |     |
| TN90_3        |     |     |     |     |
| TN90_4        |     |     |     |     |

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**Fig. 4.** Mutations in the VPg protein of *Potato virus Y* (PVY) during the infection of susceptible and resistant tobacco lines. (A) Alignment of the VPg sequences of the inoculum, of four ‘Tsukuba 1’ plants (28 days post-inoculation: dpi), and of four ‘TN90’ plants (28 dpi). Note that specific amino acid changes (K105E, lysine-105 to glutamic acid, or V108I, valine-108 to isoleucine) were detected in all four ‘TN90’ plants, but not in the ‘Tsukuba 1’ plants. (B) Frequency of base change in the K105E and V108I mutants of VPg (A313G, adenine-313 to guanine, and G322A, guanine-322 to adenine, respectively). The number of mutant-type reads divided by the read depth are shown in parenthesis.

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**Fig. 5.** Proposed mechanism of the enhanced resistance to both *Potato virus Y* (PVY) and resistance-breaking PVY (RB-PVY) in the double-mutant tobacco lines without functional eIF4E1-S and eIF(iso)4E-T. (A) Molecular interaction between tobacco eukaryotic translation initiation factors and the VPg proteins of PVY suggested by Takakura et al. (2018). PVY interacts with eIF4E1-S, whereas RB-PVY interacts with both eIF4E1-S and eIF(iso)4E-T. Thickness of the arrows indicates the degree of importance for viral infection. (B) PVY cannot interact with eIF4E1-S in the eIF4E1-S mutant, which increases selection pressure to favor RB-PVY that interacts with both eIF4E1-S and eIF(iso)4E-T. Thus, RB-PVY can infect the mutant. (C) RB-PVY cannot interact with eIF(iso)4E-T in the eIF(iso)4E-T mutant, but can interact with eIF4E1-S. Thus, RB-PVY gradually multiplies in the mutant. (D) PVY cannot interact with eIF4E1-S, and emerging RB-PVY can interact with neither eIF4E1-S nor eIF(iso)4E-T in the eIF4E1-S and eIF(iso)4E-T double mutant; thus, neither PVY nor RB-PVY can infect the mutant.
the VPg protein of RB-PVY could interact with both factors (Takakura et al. 2018; Table 2, Fig. 3B), it is likely that the K105E mutation creates a trade-off between the ability to interact de novo with eIF(iso)4E-T and a partial loss of the ability to utilize eIF4E1-S. Thus, RB-PVY probably recruits eIF4E1-S less efficiently than eIF(iso)4E-T during its infection cycle, but it gradually multiplies in the eIF(iso)4E-T mutant (Fig. 5C). In the tobacco plants without functional eIF4E1-S and eIF(iso)4E-T (Fig. 5D), PVY cannot use eIF4E1-S, and the emerged RB-PVY can use neither eIF4E1-S nor eIF(iso)4E-T; thus, both types of PVY cannot infect (Fig. 5D). The effectiveness of this tobacco mutant for preventing infection by both PVY strains may provide durable PVY resistance. This study provides the first evidence of a synergistic effect of homeologous gene-specific inhibition of host eukaryotic translation-initiation factors eIF4E and eIF(iso)4E on viral resistance. Simultaneous inhibition of several members of the eIF-4E gene family often leads to lethality or impaired growth. For example, in Arabidopsis thaliana, a double knockout mutant of eIF4E1 and eIF(iso)4E could not be obtained due to its lethality in male gametophytes (Callot and Gallois 2014). In tomato, the F1 hybrid between RNAi-eIF4E and RNAi-eIF(iso)4E lines produced a dwarf phenotype (Mazier et al. 2011). In tobacco, the antisense downregulation of both eIF4E and eIF(iso)4E resulted in plants with a semi-dwarf phenotype (Combe et al. 2005), probably because the antisense RNA affected both S-type and T-type genes. In this context, natural resistance eIF4E alleles that still encode functional translation initiation factors are advantageous over loss-of-function alleles (Bastet et al. 2017). On the other hand, tobacco with both a deletion of eIF4E1-S and a nonsense mutation of eIF(iso)4E-T and a nonsense mutation of eIF(iso)4E-T showed no obvious growth inhibition under greenhouse conditions (Fig. 2D). This might be because tobacco is an amphidiploid plant and in many cases has two homeologous genes in its genome (one from N. sylvestris and the other from N. tomentosiformis). Tobacco lacking eIF4E1-S with a nonsense mutation of eIF(iso)4E-T still has functional homologous genes (eIF4E1-T and eIF(iso)4E-S). Therefore, both eIF4E1 and eIF(iso)4E continue to function at least partly in the plants. Even the tobacco lacking eIF4E1-S with nonsense mutations in both eIF(iso)4E-S and eIF(iso)4E-T (triple mutations) showed no growth inhibition (Fig. 2D). In this tobacco, eIF(iso)4E function is completely absent, whereas eIF4E1-T remains active. This can be explained by assuming that eIF(iso)4E is not essential for plant growth, as was suggested in Arabidopsis thaliana (Duprat et al. 2002, Lellis et al. 2002) and chili pepper (Ruffel et al. 2006). Indeed, tobacco eIF(iso)4E-S and eIF(iso)4E-T double mutants showed normal growth (Takakura et al. 2018). As suggested in the mesopolyploid plant Brassica rapa (Nellist et al. 2014), it’s possible that the presence of multiple copies of eukaryotic translation initiation factors may have led to redundancy in tobacco’s translational machinery, which enables mutations that impair the function of susceptibility factors without severely affecting the plant’s growth. PVY and RB-PVY require specific translation initiation factors (eIF4E1-S and eIF(iso)4E-T, respectively) for their infection cycle. A deleterious mutation in one homeologous eIF4E or eIF(iso)4E gene can be functionally compensated for by the other gene in tobacco plants, whereas viruses lose the specific gene they require for infection. The strategy suggested in the present study may open new perspectives to extend the resistance spectrum or to deploy and manage durable and broad spectrum resistance to potyviruses that selectively use the two major translation-initiation factors eIF4E and eIF(iso)4E in polyploid plant species that are common in crops (Renny-Byfield and Wendel 2014, Wood et al. 2009).

The eIF(iso)4E-T knockout variant tobacco is expected to be useful for breeding of tobacco with resistance to both PVY and RB-PVY. It will be interesting to confirm its effectiveness against other PVY strains and to determine whether it shows durable resistance without reductions of yield and quality under field conditions.

**Author Contribution Statement**

H.U. and Y.T. designed and planned the study. H.U. prepared the tobacco mutants, and performed the viral VPg sequencing. K.K. performed the virus inoculation test and assessed the resistance. A.S. contributed to the molecular analysis including sample preparation. Y.T. performed ELISA and assessed the plant growth. All authors interpreted the results. H.U. and Y.T. analyzed data, drafted the manuscript, and designed the figures. H.K. and Y.T. reviewed and edited the paper with input from all authors.

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