Research Paper

Reduced susceptibility to a tobacco bushy top virus Malawi isolate by loss of function in host eIF(iso)4E genes

Hisashi Udagawa1,2), Kazuharu Koga1), Akira Shinjo1), Hiroyasu Kitashiba2) and Yoshimitsu Takakura*1)

1) Leaf Tobacco Research Center, Japan Tobacco, Inc., 1900, Idei, Oyama, Tochigi 323-0808, Japan
2) Graduate School of Agricultural Science, Tohoku University, 468-1, Aza-Aoba, Aramaki, Aoba-ku, Sendai, Miyagi 980-0845, Japan

Tobacco bushy top disease (TBTD) is a viral disease of tobacco (Nicotiana tabacum L.) caused by mixed infection of Tobacco bushy top virus or Ethiopian tobacco bushy top virus and a helper virus. Despite its damage to tobacco, practical genetic resources for disease resistance have not been found. Here, we report that a mutation of tobacco eIF(iso)4E genes (eIF(iso)4E-S and eIF(iso)4E-T), which encode eukaryotic translation initiation factors, confers resistance (reduced susceptibility) to TBTD caused by a virus from Malawi (designated as tobacco bushy top virus Malawi isolate, TBTV-MW). RNAi lines in which eIF(iso)4E genes were silenced showed reduced susceptibility to TBTV-MW. We also tested chemically-induced single (eIF(iso)4E-S or eIF(iso)4E-T) and double (eIF(iso)4E-S and eIF(iso)4E-T) nonsense mutants for resistance to TBTV-MW. Suppression of eIF(iso)4E-S showed reduced susceptibility, and the resistance of the double mutant tended to be even stronger. eIF(iso)4E mutants also showed reduced susceptibility to TBTV-MW transmitted by aphids. To the best of our knowledge, the eIF(iso)4E-S mutant is the first genetic resource for TBTD resistance breeding in tobacco.

Key Words: eIF(iso)4E, Nicotiana tabacum, tobacco bushy top virus, translation initiation factor.

Introduction

Tobacco bushy top disease (TBTD) is a viral disease that damages tobacco (Nicotiana tabacum L.) in Africa and China (Abraham et al. 2014, Gates 1962, Mo et al. 2002). Infected tobacco shows symptoms such as growth delay, mottling (often with spotted necrosis/chlorosis), and abnormal sucker growth, which reduce quality and yield. Tobacco bushy top virus (TBTV), reported in China, and Ethiopian tobacco bushy top virus (ETBTV), reported in Ethiopia, both cause TBTD (Abraham et al. 2014, Mo et al. 2003). Based on the features of genomic RNA structure and/or viral morphology observed by an electron microscope, both viruses are categorized in the genus Umbravirus (Abraham et al. 2014, Mo et al. 2003, Taliansky and Robinson 2003). Umbraviruses, generally, have four major open reading frames (ORFs); ORF1 and ORF2 code for RNA dependent RNA polymerase, ORF3 is responsible for long-distance movement, and ORF4 is responsible for cell to cell movement (Gibbs et al. 1996, Mo et al. 2003, Ryabov et al. 1999, Taliansky et al. 1996).

As umbraviruses lack ORFs for the coat protein, they are unable to form virions by themselves (Falk et al. 1979, Murant et al. 1969). Instead, a coat protein for the umbraviruses is provided by a helper virus of the family Luteoviridae in the natural environment, where umbraviruses are transmitted via aphids (Demler et al. 1994, Murant et al. 1985). For example, Tobacco vein distorting virus and Potato leafroll virus are helper viruses for TBTV and ETBTV, respectively (Abraham et al. 2014, Gates 1962, Mo et al. 2010).

The management of aphids is one practical way to control TBTD (Abraham 2019). Pesticides can reduce aphids directly, and, by adjusting the transplantation period, tobacco can be cultivated avoiding the timing of the virus infection by aphids. However, these cultivated ways are much laborious and costly. The development of resistant cultivars is another way to reduce damages by the virus. Although the genetic resources that might show resistance to TBTD have been reported in wild Nicotiana relatives (Briu 1990, Gates 1962), resistant cultivars have never been developed in N. tabacum to date.

Studies on virus–host interactions provide clues to virus resistance. Viral proteins or genome of certain viruses interact with eukaryotic translation initiation factors (eIFs) in host-plants for their replication and for cell-to-cell movement (Thivierge et al. 2005). Many potyviruses interact...
with eIF4E, a host cap-binding protein, or its isoform eIF(iso)4E, through their viral genome-associated protein (VPg) (Robaglia and Caranta 2006, Wittmann et al. 1997). The 5’ leader of Tobacco etch virus (genus Potyvirus) genomic RNA and the VPg of Rice yellow mottle virus (genus Sobemovirus) interact directly with eIF4G, a scaffold protein for other eIFs, and its isoform eIF(iso)4G, respectively (Gallie 2001, Hébrard et al. 2010). Since eIFs are factors involved in the susceptibility of hosts, natural variation or artificially induced mutations in eIF genes enable to reduce the degree of susceptibility (Albar et al. 2006, Bastet et al. 2017, Diaz-Pendon et al. 2004, Nieto et al. 2006, Piron et al. 2010, Yoshii et al. 2004). In the case of tobacco, resistance to potato virus Y (PVY), a member of genus Potyvirus, which causes one of the most serious diseases of tobacco, was conferred by the deletion of eIF4E (Julio et al. 2015), and the deletion is widely used in tobacco breeding. Although Resistance-Breaking PVY (RB-PVY) strains can break this resistance (e.g., Masuta et al. 1999), our group recently reported that the VPgs of RB-PVY strains interacted with eIF(iso)4E in their infection cycle and that a nonsense mutation of eIF(iso)4E conferred RB-PVY resistance (Takakura et al. 2018).

*N. tabacum* is an allopolyplid (amphidiploid) species that combines the S and T genomes derived from ancestral *Nicotiana sylvestris* and *Nicotiana tomentosiformis*. Genomic analysis revealed that *N. tabacum* has both S genome derived type (S-type) and T genome derived type (T-type) of eIF4E1, eIF4E2, and eIF(iso)4E (Sierro et al. 2014). In the present study, we investigated the involvement of tobacco eIF4E and eIF(iso)4E in the susceptibility to a virus causing TBTD in Malawi (tobacco bushy top virus Malawi isolate, TBTV-MW) to find genetic resources for TBTD resistance.

**Materials and Methods**

**Virus, sequencing of viral genome, and assembly**

TBTV-MW derived from infected leaf collected in Malawi has been maintained by recurrent sap inoculation into tobacco (*N. tabacum*) cv. Tsukuba 1 (we used “tobacco bushy top virus Malawi isolate” or “TBTV-MW” as the virus isolate name in the present study, but not as the species name, because classification of the virus isolate was not clear). Total RNA was extracted by using a Turbo DNA-free Kit (Thermo Fisher Scientific). Following library preparation by a TruSeq Stranded Total RNA with Ribo-Zero Plant Kit (Illumina), sequencing was performed with a NextSeq 500/550 Mid Output Kit v2 (2 × 150 bases, Illumina). Next-generation sequencing data was processed in CLC Genomics Workbench v. 11.0 software. Trimmed reads were mapped to the tobacco cv. K326 reference genome (GenBank accession PRJNA208210), and contigs were obtained by *de novo* assembly of unmapped reads. Nucleotide sequence data of TBTV-MW genome has been deposited in DDBJ/EMBL/GenBank databases with the LC494673 accession code.

**Preparation of RNAi-tobacco transformants**

We generated eIF4E1-RNAi lines by the same procedure as Takakura et al. (2018): eIF4E1-S (GenBank accession KF155696) was amplified from cDNA of ‘Tsukuba 1’ leaf with the primers in Supplemental Table 1. The amplicon was purified by using a MinElute PCR Purification Kit (QIAGEN). After confirmation of sequences as eIF4E1-S, the amplified fragment was cloned into pENTR/D-TOPO vector (Life Technologies). The amplicon was inserted into the pSP231 binary vector, which has a green fluorescent protein expression cassette in pHELLSGATE12 (Helliwell and Waterhouse 2003, Wesley et al. 2001), by the use of Gateway LR Clonase II Enzyme Mix (Life Technologies). An eIF4E1-RNAi construct in which the trigger DNA fragments were cloned as a pair of inverted repeats driven by the Ca35S promoter was obtained by Gateway technology. Purified binary vector was transferred into Agrobacterium tumefaciens LBA4404 by electroporation, and the RNAi cassette was introduced into tobacco cv. SR1 Petit Havana by standard *A. tumefaciens*-mediated transformation. In T1 progeny obtained by self-pollination of the primary transformants (T0), the presence or absence of the cassette was checked by fluorescence in a ChemiDocMP Imaging System (Bio-Rad Laboratories). In each T1 line, the presence or absence of the cassette was checked again by PCR of neomycin phosphotransferase II (Supplemental Table 1).

**Preparation of tobacco mutants**

eIF(iso)4E-S and eIF(iso)4E-T nonsense mutants (ssTT and SStt, with a point mutation in the first exon of the gene; Supplemental Fig. 1), the double mutant (ssTT), and the wild-type segregant (SSTT) were selected by Takakura et al. (2018) from an ethyl methanesulfonate induced tobacco population. These plants had ‘Tsukuba 1’ genetic background. To prepare ‘K326’ background lines, the double mutant (ssTT) was crossed with ‘K326’, followed by two more backcrosses, to generate BC2F1. After genotyping of BC2F2 individuals, homozygotes for both eIF(iso)4E genes were self-pollinated, and the K326-ssTT, K326-SStt, K326-sstt, and K326-SSTT lines were fixed at the BC2F2 generation. Genotyping was performed by using derived Cleaved Amplified Polymorphic Sequence (dCAPS) maker as describe later.

**RT-qPCR**

Total RNA was extracted from leaf samples with an RNeasy kit (QIAGEN) and cDNA was synthesized with the use of a PrimeScript RT reagent Kit (Takara). RT-qPCR was performed using TaqMan Fast Advanced Master Mix (Life Technologies) and the primers and probes in Supplemental Table 1 to calculate the expression values of eIF genes and the accumulation of TBTV-MW. Elongation
factor 1-α was used as an internal standard. Analysis was done in StepOne Real-Time PCR System v. 2.2 software (Life Technologies).

Sap inoculation test
TBTV-MW-infected ‘Tsukuba 1’ leaves were ground in two volumes of 0.01 N phosphate buffer (pH 7.0). The homogenate was inoculated with carborundum into half of the fifth or sixth leaf from the bottom in each tobacco seedling at about 30 days after sowing. Inoculated plants were grown in a greenhouse at 22 ± 2°C. The presence or absence of mottling symptoms in each plant was recorded to evaluate TBTV-MW resistance. For ‘K326’ background lines, another inoculated plant set different from the resistance evaluation test set was prepared. All the upper leaves other than the apical meristem were sampled at 13 days post-inoculation (DPI) to quantify the relative virus amount in the inoculated plants.

Aphid transmission test
Leaves were sampled from ‘Tsukuba 1’ infected with TBTV-MW and Tobacco vein distorting virus as a helper virus. Virus-free aphids (Myzus persicae) were fed on infected leaves for 1 day before transfer. Ten aphids per plant were placed on seedlings at 30 days after sowing, which was set as 0 DPI. Plants with aphids were grown in a growth chamber at 25°C, and aphids were removed at 7 DPI by a soil application of imidacloprid. Virus resistance was evaluated at 14, 19, and 25 DPI by observing the presence or absence of mottling symptoms. The topmost fully developed leaf of each plant was sampled at 25 DPI to quantify the virus amount.

dCAPS markers to detect eIF(iso)4E mutation
eIF(iso)4E-S and eIF(iso)4E-T were amplified by multiplex PCR with a mix of specific primers (Supplemental Table 1) and a Multiplex PCR kit (QIAGEN), which includes a DNA polymerase that does not have 3’ to 5’ exonuclease activity. Restriction enzymes NlaIII for eIF(iso)4E-S and MboI (both from New England Biolabs) for eIF(iso)4E-T were added to the PCR amplicons to digest each wild-type allele. Digested fragments were separated by electrophoresis in a QIAxcel System (QIAGEN), and genotypes were decided by fragment size.

Results
Characterization of TBTV-MW genome and sequence comparison
Genomic RNA of TBTV-MW was sequenced by using next-generation sequencer. Out of 1,560 contigs generated by de novo assembly, one contig (#13) of 4,186 nt had 87.7% identity with genome sequences of ETBTV (GenBank accession KJ918748.1), 51.6% with that of TBTV (AF402620.2), and 59.7% with that of Groundnut rosette virus (Z69910.1) (Supplemental Table 2). No other contig showed distinct identity to known viruses. Amino acid sequences deduced from four predicted ORFs in the contig genome sequence also showed the highest identity (79.1%–94.6%) to those of ETBTV (Supplemental Table 2). Molecular phylogenetic analysis showed that the genome sequence of #13 contig was placed closest to that of ETBTV in the same cluster (Fig. 1). We concluded that the genome sequence of #13 contig corresponded to the TBTV-MW genome, and used it in the following experiments.

Reduced susceptibility to TBTV-MW by RNAi suppression of eIF(iso)4E
To find genes responsible for TBTV-MW resistance, we focused on six known eIF genes that encode eIF4E or eIF(iso)4E in tobacco, i.e., eIF4E1-S (GenBank accession KF155696), eIF4E1-T (KM202068), eIF4E2-S (AY702653), eIF4E2-T (KM202067), eIF(iso)4E-S (AY699609), and eIF(iso)4E-T (EB683576), and produced RNAi suppression lines for each gene. For eIF4E1-RNAi suppression lines, the RNAi cassette to suppress both S- and T-type of eIF4E1 genes at the same time was constructed. eIF4E2- and eIF(iso)4E-RNAi lines were previously prepared by Takakura et al. (2018). Expression levels of both eIF4E1s in leaves were evaluated by RT-qPCR in T2 generations of each eIF4E1-RNAi transgenic line (Supplemental Fig. 2). The expression of both genes in each RNAi line was significantly reduced compared with that in non-transformants. The target genes were significantly suppressed in eIF4E2-RNAi and eIF(iso)4E-RNAi lines as well (Supplemental Fig. 2).
eIF4E1-RNAi(+) lines (with the RNAi cassette) were as susceptible to TBTV-MW as eIF4E1-RNAi(–) lines (without the cassette) (Fig. 2). eIF4E2-RNAi lines showed different responses: lines RNAi(+) #1 and RNAi(–) #1 were comparably susceptible, whereas line RNAi(+) #8 had no symptoms even at 18 DPI. In contrast, eIF(iso)4E-RNAi(+) lines showed clear and stable reduced susceptibility: all three lines showed <10% disease rate even at 18 DPI, lower than eIF(iso)4E-RNAi(–) and control non-transgenic lines. These results indicate that eIF(iso)4E genes could be involved in susceptibility to TBTV-MW.

Reduced susceptibility to TBTV-MW by nonsense mutation of eIF(iso)4E genes in the sap inoculation test

As eIF(iso)4E-RNAi(+) showed reduced susceptibility, we performed a sap-inoculation test for eIF(iso)4E mutants (‘Tsukuba 1’ background lines) to verify whether either or both of eIF(iso)4E-S and eIF(iso)4E-T are involved. In advance of the sap inoculation test, we measured expression levels of eIF(iso)4E-S and eIF(iso)4E-T in each single-nonsense mutant (ssTT and SStt), the double mutant (sstt), and the wild-type segregant (SSTT) by RT-qPCR. Transcription of the mutant gene was greatly reduced in each mutant, probably caused by nonsense-mediated mRNA decay (Nyikó et al. 2013, Shaul 2015) (Fig. 3A). The number of plants with symptoms was the most in SSTT and SStt genotypes, followed by ssTT genotype. In sstt double mutant, the number was remarkably decreased (Fig. 3B, 3C).

To test the effect of the mutations in a different genetic background (e.g., Palloix et al. 2009, Quenouille et al. 2013), we prepared ‘K326’ background lines. The dCAPS markers were developed for detection of the wild-type and mutant alleles of both eIF(iso)4E-S and -T (Fig. 4A). After marker selection (Fig. 4B shows an example), RT-qPCR was carried out for the selected mutants. Reduced expression of the mutant gene was confirmed in each ‘K326’ background line (Fig. 5A). Additionally, as in the ‘Tsukuba 1’ background lines, lines K326-sstt and K326-ssTT
showed reduced susceptibility, while K326-SSStt did not (Fig. 5B). These results indicate that the mutation of eIF(iso)4E-S greatly reduced susceptibility to TBTV-MW irrespective of genetic background. RT-qPCR revealed that the relative TBTV-MW accumulation was significantly lower in K326-sstt and K326-ssTT than in K326-SStt and K326-SSTT (Fig. 5C). Since there was a positive association between the ratio of plants with symptoms and virus accumulation, the reduction in the symptoms was thought to be due to suppression of virus accumulation.

**Reduced susceptibility to TBTV-MW by nonsense mutation of eIF(iso)4E genes in the aphid transmission test**

In general, umbravirus infects the host plant via aphids in natural environment. To evaluate the effect of the mutant eIF(iso)4E genes on TBTV-MW resistance via aphids transmission, K326-sstt and K326-ssTT, which showed reduced susceptibility in the sap inoculation test, were tested. No K326-sstt plants showed mottling symptoms even at 25 DPI, and few K326-ssTT plants showed symptoms up to 25 DPI (Fig. 6A). RT-qPCR at 25 DPI revealed significantly less accumulation of TBTV-MW in K326-sstt than in K326-SStt, and much less in K326-ssTT than in K326-SSTT, although significant difference was not detected (Fig. 6B). The proportion of plants with symptoms was associated with virus accumulation. These results were almost similar to those in the sap inoculation test, suggesting that the mutants with sstt genotype or ssTT in eIF(iso)4E gene is effective for reduction of TBTV-MW susceptibility and eIF(iso)4E-S is one of the major factors in TBTV-MW susceptibility.

**Discussion**

**Classification of TBTV-MW**

Phylogenetic analyses of viral genomic RNAs revealed that TBTV-MW is more closely related to ETBTV than to TBTV. Species demarcation criteria in the genus *Umbravirus* include natural host range, dsRNA pattern, nucleotide sequence identity being <70%, and little or no hybridization with cDNA probes of known viruses (Ryabov et al. 2012). Tobacco is a common natural host for both TBTV-MW and ETBTV, and a nucleotide identity of 87.7% between them is clearly higher than the criterion. Abraham et al. (2014) reported that ETBTV is distributed in the
In inoculation tests, eIF(iso)4E-Malawi, supporting a close relationship between TBTV-TBTV-MW.

Further investigation of host plant range following infection with TBTV-MW by aphids (number of plants with symptoms at indicated days post-inoculation = 15 per line), the number of plants with symptoms at indicated days post-inoculation (DPI) was recorded. (B) Semi-quantification of TBTV-MW in each genotype at 25 DPI measured by RT-qPCR of viral RNA dependent RNA polymerase region from Ethiopia to Zimbabwe, which includes Malawi, supporting a close relationship between TBTV-MW and ETBTV. Further investigation of host plant range and dsRNA pattern may be necessary; however, these facts strongly suggest that TBTV-MW is related to ETBTV and is the causal virus of TBTD.

Involvement of eIF4E and eIF(iso)4E in TBTV-MW susceptibility

Our results showed that the suppression of eIF(iso)4E genes enabled a reduction of susceptibility to TBTV-MW. In inoculation tests, eIF(iso)4E-RNAi(+) plants showed significant reduction of susceptibility, indicating that eIF(iso)4E-S or -T, or both, is involved in susceptibility to TBTV-MW. Although we also tested for eIF4E1 and eIF4E2, a suppression of eIF4E1 did not show a reduction of susceptibility to TBTV-MW and the consistent results were not observed in eIF4E2 RNAi(+) lines. These results suggest that both eIF4E1 and eIF4E2 genes, in contrast to eIF(iso)4E, might not be involved in susceptibility to TBTV-MW.

In the inoculation test with knockout mutants, mutants that had the nonsense mutation in eIF(iso)4E-S showed reduced susceptibility to TBTV-MW. This result suggests that eIF(iso)4E-S plays an important role in the life cycle of TBTV-MW. On the other hand, the eIF(iso)4E-T mutant did not show significantly reduced susceptibility. However, the numbers of symptomatic plants of sstt and K326-sstt were always less than or equal to those of ssTT and K326-ssTT in the sap inoculation test, and clearer difference between sstt and ssTT genotypes was observed in the aphid transmission test. Therefore, eIF(iso)4E-T also might be involved or partly involved in susceptibility. In Arabidopsis thaliana, the eIF4E1 knockout plants shows partial resistance to Beet western yellows virus-USA (genus polerovirus), whereas eIF(iso)4E knockout plants do not (Reinbold et al. 2013); nevertheless, the double knockout of eIF4E1 and eIF(iso)4E shows enhanced resistance (Bastet et al. 2018). Similarly, in the susceptibility to TBTV-MW mediated by eIF(iso)4E, mutant eIF(iso)4E-S works as a major resistance gene, and mutant eIF(iso)4E-17-nt mutation.

Resistance to potyviruses due to eIF genes is well studied. The mechanism can be explained by the interaction between VPg and the host eIF4E or eIF(iso)4E. On the other hand, certain viruses that don’t have VPg use the host translation mechanism via 3' cap independent translation enhancers (Truniger et al. 2017). 3' cap independent translation enhancers are classified into several types (Truniger et al. 2017). Among them, the Barley yellow dwarf virus (BYDV; genus Luteovirus)-like translation element (BTE) has a characteristic 17-nt conserved sequence (5'-GGAUCCUGGGAAACAGG-3') (Kraft et al. 2013, Wang et al. 2010). Both TBTV and Groundnut rosette virus have a functional BTE (Wang et al. 2010). We have confirmed that the 17-nt sequence was also conserved in TBTV-MW, possibly indicating that TBTV-MW also uses the host translation mechanism via BTE. In BYDV, BTE does not directly interact with eIF(iso)4E (Treder et al. 2008). Nevertheless, it was recently reported that resistance to BYDV was conferred by the silencing of eIF(iso)4E in wheat (Fellers et al. 2018, Rupp et al. 2019). Thus, it is still possible that eIF(iso)4E functions in translation via BTE. Considering that eIF(iso)4E forms the complex with other eIFs (Robaglia and Caranta 2006), further study for elucidating the relationship between BTE and the eIF complex is necessary. It will reveal the molecular mechanism of the reduced susceptibility to TBTV-MW caused by loss of function in eIF(iso)4E.

Use of eIF(iso)4E mutants as a genetic resource in breeding

As the double mutant showed a tendency to be more resistant than the eIF(iso)4E-S single mutant, it would be preferable to introduce both eIF(iso)4E-S and T mutations into breeding lines for TBTD resistance. To our best

Fig. 6. Evaluation of susceptibility of eIF(iso)4E mutants (‘K326’ background) by aphid transmission. (A) Aphid transmission test. Following infection with TBTV-MW by aphids (n = 15 per line), the number of plants with symptoms at indicated days post-inoculation (DPI) was recorded. (B) Semi-quantification of TBTV-MW in each genotype at 25 DPI measured by RT-qPCR of viral RNA dependent RNA polymerase region. Bars show mean and SEM (n = 15) relative to K326-ssTT. Bars with the same letter are not significantly different by Tukey's test (P < 0.05).
knowledge, the genetic resources that might show TBTD resistance have been reported only in wild Nicotiana relatives, N. clevelandii, N. acuminata, N. excelsior, and N. suaveolens (Bruin 1990, Gates 1962). Therefore, the eIF(iso)4E mutants may be the first genetic resource in N. tabacum. In addition, our study shows the first example of reduced susceptibility to an umbravirus by the suppression of eIF genes. Because of the close relation with TBTV-MW, the same mutations have a higher possibility to confer resistance to ETBTV as well. It will be important to clarify the range of resistance in the eIF(iso)4E mutants to other umbraviruses, especially ETBTV and TBTV.

The developed selection marker in the present study, which can detect the single nucleotide polymorphisms (SNPs) in both eIF(iso)4E genes, offers a useful tool for the resistance breeding. Previously, dCAPS markers for both SNPs were already designed (Takakura et al. 2018), but there was still scope for improvement in labor and cost: they required independent nested PCR and restriction enzyme digestion to detect each SNP. To solve these problems, we redesigned the system; amplification of both genes in one PCR reaction followed by one double digestion. Marker-assisted breeding for TBTD resistance will be more promoted by the newly developed dCAPS marker.

In Arabidopsis thaliana, eIF(iso)4E mutant exhibited normal growth (Lellis et al. 2002). Similarly, we observed no abnormal growth in the tobacco eIF(iso)4E mutants. However, the influence of the mutations on plant quality and yield should be evaluated in detail. For practical breeding with TBTD resistance, we have continued further studies together with investigation for strength and stability of the resistance in field conditions.

Author Contribution Statement

H.U., H.K., and Y.T. designed and planned the study. Y.T. constructed the RNAi cassette and prepared the RNAi plants. H.U. performed the viral genome sequencing, prepared the tobacco mutants, and developed the DNA marker. K.K. performed the virus inoculation test and assessed the resistance. A.S. contributed to the RT-qPCR including sample preparation. All authors interpreted the results. H.U. analyzed data, drafted the manuscript, and designed the figures. H.K. and Y.T. reviewed and edited the paper with input from all authors.

Acknowledgments

The authors acknowledge Kyoko Ishizono, Tomoko Morita, and Asako Kikuchi for their excellent technical assistance.

Literature Cited

Abraham, A.D., W. Menzel, B. Bekele and S. Winter (2014) A novel combination of a new umbravirus, a new satellite RNA and potato leafroll virus causes tobacco bushy top disease in Ethiopia. Arch. Virol. 159: 3395–3399.

Abraham, A. (2019) Emerged plant virus disease in Ethiopian agriculture: causes and control options. Ethiop. J. Agric. Sci. 29: 39–55.

Albar, L., M. Bangratz-Reyser, E. Hébrard, M.N. Ndjomandjob, M. Jones and A. Ghesquière (2006) Mutations in the eIF(iso)4G translation initiation factor confer high resistance of rice to Rice yellow mottle virus. Plant J. 47: 417–426.

Bastet, A., C. Robaglia and J.L. Gallois (2017) eIF4E resistance: natural variation should guide gene editing. Trends Plant Sci. 22: 411–419.

Bastet, A., B. Lederer, N. Giovinazzo, X. Arnoux, S. German-Retana, C. Reinbold, V. Brault, D. Garcia, S. Dje n nane, S. Gersch et al. (2018) Trans-species synthetic gene design allows resistance pyramiding and broad-spectrum engineering of virus resistance in plants. Plant Biotechnol. J. 16: 1569–1581.

Bruin, D.J. de (1990) Sources of resistance in the genus Nicotiana to the virus causing bushy top disease in tobacco. Phytophylactica 22: 263–264.

Demler, S.A., D.G. Rucker, L. Nooruddin and G.A. de Zoeten (1994) Replication of the satellite RNA of pea enation mosaic virus is controlled by RNA 2-encoded functions. J. Gen. Virol. 75: 1399–1406.

Diaz-Pendon, J.A., V. Truniger, C. Nieto, J. Garcia-Mas, A. Bendahmane and M.A. Aranda (2004) Advances in understanding recessive resistance to plant viruses. Mol. Plant Pathol. 5: 223–233.

Falk, B.W., T.J. Morris and J.E. Duffus (1979) Unstable infectivity and sedimentable ds-RNA associated with lettuce speckles mottle virus. Virology 96: 239–248.

Fellers, J., H.N. Trick, L. Cruz and J. Rupp (2018) Plant germplasm resistant to RNA viruses. US Patent 9909,139. Issued 8 Mar. 2018.

Gallic, D.R. (2001) Cap-independent translation conferred by the 5' leader of tobacco etch virus is eukaryotic initiation factor 4G dependent. J. Virol. 75: 12141–12152.

Gates, L.F. (1962) A virus causing axillary bud sprouting of tobacco in Rhodesia and Nyasaland. Ann. Appl. Biol. 50: 169–174.

Gibbs, M.J., J.I. Cooper and P.M. Waterhouse (1996) The genome organization and affinities of an Australian isolate of carrot mottle umbravirus. Virology 224: 310–313.

Hébrard, E., N. Poulicard, C. Gérard, O. Traoré, H.C. Wu, L. Albar, D. Fargette, Y. Bessin and F. Vignols (2010) Direct interaction between the Rice yellow mottle virus (RYMV) VPg and the central domain of the rice eIF(iso)4G1 factor correlates with rice susceptibility and RYMV virulence. Mol. Plant Microbe Interact. 23: 1506–1513.

Hellwell, C. and P. Waterhouse (2003) Constructs and methods for high-throughput gene silencing in plants. Methods 30: 289–295.

Julio, E., J. Cotucheau, C. Decorps, R. Volpatti, C. Sentenac, T. Candresse and D.F. de Borne (2015) A eukaryotic translation initiation factor 4E (eIF4E) is responsible for the "va" tobacco recessive resistance to potyviruses. Plant Mol. Biol. Rep. 33: 609–623.

Kraft, J.J., K. Treder, M.S. Peterson and W.A. Miller (2013) Cation-dependent folding of 3' cap-independent translation elements facilitates interaction of a 17-nucleotide conserved sequence with eIF4G. Nucleic Acids Res. 41: 3398–3413.

Kumar, S., G. Stecher, M. Li, C. Knyaz and K. Tamura (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35: 1547–1549.

Lellis, A.D., K.D. Kasschau, S.A. Whitham and J.C. Carrington (2002) Loss-of susceptibility mutants of Arabidopsis thaliana reveal an essential role for eIF(iso)4E during potyvirus infection.

Bruin, D.J. de (1990) Sources of resistance in the genus Nicotiana to the virus causing bushy top disease in tobacco. Phytophylactica 22: 263–264.

Demler, S.A., D.G. Rucker, L. Nooruddin and G.A. de Zoeten (1994) Replication of the satellite RNA of pea enation mosaic virus is controlled by RNA 2-encoded functions. J. Gen. Virol. 75: 1399–1406.

Diaz-Pendon, J.A., V. Truniger, C. Nieto, J. Garcia-Mas, A. Bendahmane and M.A. Aranda (2004) Advances in understanding recessive resistance to plant viruses. Mol. Plant Pathol. 5: 223–233.

Falk, B.W., T.J. Morris and J.E. Duffus (1979) Unstable infectivity and sedimentable ds-RNA associated with lettuce speckles mottle virus. Virology 96: 239–248.

Fellers, J., H.N. Trick, L. Cruz and J. Rupp (2018) Plant germplasm resistant to RNA viruses. US Patent 9909,139. Issued 8 Mar. 2018.

Gallic, D.R. (2001) Cap-independent translation conferred by the 5' leader of tobacco etch virus is eukaryotic initiation factor 4G dependent. J. Virol. 75: 12141–12152.

Gates, L.F. (1962) A virus causing axillary bud sprouting of tobacco in Rhodesia and Nyasaland. Ann. Appl. Biol. 50: 169–174.

Gibbs, M.J., J.I. Cooper and P.M. Waterhouse (1996) The genome organization and affinities of an Australian isolate of carrot mottle umbravirus. Virology 224: 310–313.

Hébrard, E., N. Poulicard, C. Gérard, O. Traoré, H.C. Wu, L. Albar, D. Fargette, Y. Bessin and F. Vignols (2010) Direct interaction between the Rice yellow mottle virus (RYMV) VPg and the central domain of the rice eIF(iso)4G1 factor correlates with rice susceptibility and RYMV virulence. Mol. Plant Microbe Interact. 23: 1506–1513.

Hellwell, C. and P. Waterhouse (2003) Constructs and methods for high-throughput gene silencing in plants. Methods 30: 289–295.

Julio, E., J. Cotucheau, C. Decorps, R. Volpatti, C. Sentenac, T. Candresse and D.F. de Borne (2015) A eukaryotic translation initiation factor 4E (eIF4E) is responsible for the "va" tobacco recessive resistance to potyviruses. Plant Mol. Biol. Rep. 33: 609–623.

Kraft, J.J., K. Treder, M.S. Peterson and W.A. Miller (2013) Cation-dependent folding of 3' cap-independent translation elements facilitates interaction of a 17-nucleotide conserved sequence with eIF4G. Nucleic Acids Res. 41: 3398–3413.

Kumar, S., G. Stecher, M. Li, C. Knyaz and K. Tamura (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35: 1547–1549.

Lellis, A.D., K.D. Kasschau, S.A. Whitham and J.C. Carrington (2002) Loss-of susceptibility mutants of Arabidopsis thaliana reveal an essential role for eIF(iso)4E during potyvirus infection.
Masuta, C., M. Nishimura, H. Morishita and T. Hataya (1999) A single amino acid change in viral genome-associated protein of potato virus Y correlates with resistance breaking in ‘virgin a mutant’ tobacco. Phytopathology 89: 118–123.

Mo, X.H., X.Y. Qin, Z.X. Tan, T.F. Li, J.Y. Wu and H.R. Chen (2002) First report of tobacco bushy top disease in China. Plant Dis. 86: 74.

Mo, X.H., X.Y. Qin, J. Wu, C. Yang, J.Y. Wu, Y.Q. Duan, T.F. Li and H.R. Chen (2003) Complete nucleotide sequence and genome organization of a Chinese isolate of tobacco bushy top virus. Arch. Virol. 148: 389–397.

Mo, X.H., Z.B. Chen and J.P. Chen (2010) Complete nucleotide sequence and genome organization of a Chinese isolate of Tobacco vein distorting virus. Virus Genes 41: 425–431.

Murant, A.F., R.A. Goold, I.M. Roberts and J. Cathro (1969) Carrot mottle—a persistent aphid-borne virus with unusual properties and particles. J. Gen. Virol. 4: 329–341.

Murant, A.F., P.M. Waterhouse, J.H. Raschke and D.J. Robinson (1985) Carrot red leaf and carrot mottle viruses: observations on the composition of the particles in single and mixed infections. J. Gen. Virol. 66: 1575–1579.

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

Niyokó, T., F. Kerényi, L. Szabókai, A.H. Benkovics, P. Major, B. Sonkoly, Z. Mérá, E. Barta, E. Niemiec, J. Kufel et al. (2013) Plant nonsense-mediated mRNA decay is controlled by different autoregulatory circuits and can be induced by an EJC-like complex. Nucleic Acids Res. 41: 6715–6728.

Palloix, A., V. Ayme and B. Moury (2009) Durability of plant major resistance genes to pathogens depends on the genetic background, experimental evidence and consequences for breeding strategies. New Phytol. 183: 190–199.

Piron, F., M. Nicolaï, S. Minoïa, E. Piednoir, A. Moretti, A. Salgués, D. Zamir, C. Caranta and A. Bendahmane (2010) An induced mutation in tomato eIF4E leads to immunity to two potyviruses. PLoS ONE 5: e11313.

Quenouille, J., J. Montarroy, A. Palloix and B. Moury (2013) Farther, slower, stronger: how the plant genetic background protects a major resistance gene from breakdown. Mol. Plant Pathol. 14: 109–118.

Reinbold, C., S. Lacombe, V. Ziegler-Graff, D. Scheidecker, L. Wiss, M. Beuve, C. Caranta and V. Braut (2013) Closely related potyviruses depend on distinct translation initiation factors to infect Arabidopsis thaliana. Mol. Plant Microbe Interact. 26: 257–265.

Robaglia, C. and C. Caranta (2006) Translation initiation factors: a weak link in plant RNA virus infection. Trends Plant Sci. 11: 40–45.

Rupp, J.S., L. Cruz, H.N. Trick and J.P. Fellers (2019) RNAi-mediated silencing of endogenous wheat genes EiF(iso)4E-2 and EiF4G induces resistance to multiple RNA viruses in transgenic wheat. Crop Sci. 59: 2642–2651.

Ryabov, E.V., D.J. Robinson and M.E. Taliansky (1999) A plant virus-encoded protein facilitates long-distance movement of heterologous viral RNA. Proc. Natl. Acad. Sci. USA 96: 1212–1217.

Ryabov, E.V., M.E. Taliansky, D.J. Robinson, P.M. Waterhouse, A.F. Murant, G.A. de Zoiets, B.W. Falk, H.J. Vetten and M.J. Gibbs (2012) Genus Umbravirus. In King, A., M.J. Adams, E.C. Carstens and E.J. Lefkowitz (eds.) Virus taxonomy: ninth report of the International Committee on Taxonomy of Viruses. Elsevier, New York, pp. 1191–1195.

Shaoul, O. (2015) Unique aspects of plant nonsense-mediated mRNA decay. Trends Plant Sci. 20: 767–779.

Sierro, N., J.N. Battey, S. Ouadi, N. Bakaher, L. Bovet, A. Willig, S. Goepfert, M.C. Peitsch and N.V. Ivanov (2014) The tobacco genome sequence and its comparison with those of tomato and potato. Nat. Commun. 5: 3833.

Takakura, Y., H. Udagawa, A. Shinjo and K. Koga (2018) Mutation of a Nicotiana tabacum L. eukaryotic translation-initiation factor gene reduces susceptibility to a resistance-breaking strain of Potato virus Y. Mol. Plant Pathol. 19: 2124–2133.

Taliansky, M.E., D.J. Robinson and A.F. Murant (1996) Complete nucleotide sequence and organization of the RNA genome of groundnut rosette umbravirus. J. Gen. Virol. 77: 2335–2345.

Taliansky, M.E. and D.J. Robinson (2003) Molecular biology of umbraviruses: phantom warriors. J. Gen. Virol. 84: 1951–1960.

Thivierge, K., V. Nicaise, P.J. Dufresne, S. Cotton, J.F. Laliberté, O. Le Gall and M.G. Fortin (2005) Plant virus RNAs. Coordinated recruitment of conserved host functions by (+) ssRNA viruses during early infection events. Plant Physiol. 138: 1822–1827.

Tredter, K., E.L. Kneller, E.M. Allen, Z. Wang, K.S. Browning and W.A. Miller (2008) The 3’ cap-independent translation element of Barley yellow dwarf virus binds eIF4E via the eIF4G subunit to initiate translation. RNA 14: 134–147.

Truniger, V., M. Miras and M.A. Aranda (2017) Structural and functional diversity of plant virus 3’-Cap-Independent Translation Enhancers (3’-CITEs). Front. Plant Sci. 8: 2047.

Wang, Z., J.J. Kraft, A.Y. Hui and W.A. Miller (2010) Structural plasticity of Barley yellow dwarf virus-like cap-independent translation elements in four genera of plant viral RNAs. Virology 402: 177–186.

Wesley, S.V., C.A. Helliwell, N.A. Smith, M.B. Wang, D.T. Rouse, Q. Liu, P.S. Gooding, S.P. Singh, D. Abbott, P.A. Stoutjesdijk et al. (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J. 27: 581–590.

Wittmann, S., H. Chatel, M.G. Fortin and J.F. Laliberté (1997) Interaction of the viral protein genome linked of turnip mosaic potyvirus with the translational eukaryotic initiation factor (iso) 4E of Arabidopsis thaliana using the yeast two hybrid system. Virology 234: 84–92.

Yoshii, M., M. Nishikiori, K. Tomita, N. Yoshioka, R. Kozuka, S. Naito and M. Ishikawa (2004) The Arabidopsis cucumovirus multiplication I and 2 loci encode translation initiation factors 4E and 4G. J. Virol. 78: 6102–6111.