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BRIEF REPORT

Conferring virus resistance in tomato by independent RNA silencing of three tomato homologs of *Arabidopsis TOM1*

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Abstract

The *TOM1*/*TOM3* genes from *Arabidopsis* are involved in the replication of tobamoviruses. Tomato homologs of these genes, *LeTH1*, *LeTH2* and *LeTH3,* are known. In this study, we examined transgenic tomato lines where inverted repeats of either *LeTH1*, *LeTH2* or *LeTH3* were introduced by *Agrobacterium*. Endogenous mRNA expression for each gene was detected in non-transgenic control plants, whereas a very low level of each of the three genes was found in the corresponding line. Small interfering RNA was detected in the transgenic lines. Each silenced line showed similar levels of tobamovirus resistance, indicating that each gene is similarly involved in virus replication.

Keywords Tomato · Virus resistance · RNA silencing · *LeTH1* · *LeTH2* · *LeTH3* · *TOM1* · *TOM3* · *Tomato mosaic virus*

Viruses are small infective parasites which contain either an RNA or DNA genome. They are capable of directing their own replication and do not serve any essential function for their hosts [1]. Tomato mosaic virus (ToMV) is a well characterized tobamovirus with a positive-sense, single-stranded RNA of 6.4 kilobases (kb) in length. The genome encodes four proteins: a 130 kDa replicase protein (RP), a 180 kDa RP (a read-through product of the 130 kDa protein), a 30 kDa cell-to-cell movement protein (MP), and a 17.5 kDa coat protein (CP) [2–4]. Several host factors have been identified that specifically interact with the virus-associated RNA and are involved in viral intracellular multiplication [5–7]. Ishikawa et al. [8] reported that host factors are required for multiplication of ToMV Cg strain (TMV-Cg), a crucifer-infecting tobamovirus in *Arabidopsis*. *Tobacco mosaic virus multiplication 1* (*TOM1*) gene mutants in *Arabidopsis* demonstrate about 1/10 the amount of TMV-Cg replication when compared with wild-type plants. In addition, in a *TOM1 TOM3* double mutant, growth of TMV-Cg was completely suppressed [9]. Furthermore, TOM2A and TOM2B promote binding of TOM1 to the cell membrane, and both control the tobamovirus multiplication phenotype [2, 10]. TOM2A and TOM2B are not related to TOM1 and no similar protein is known so far [2]. *TOM1* and *TOM3* are conserved in a variety of plants including tomato, pepper, tobacco and melon [11, 12]. *NtTOM1* and *NtTOM3*, tobacco homologs of *TOM3* and *TOM1*, respectively, were suggested to be involved in tobamovirus multiplication, because silencing of both genes caused high resistance against several tobamoviruses [11]. Tomato homologs *LeTH1* (a homolog of *TOM3*), *LeTH2* and *LeTH3* (homologs of *TOM1*) are also thought to participate in the multiplication of tobamoviruses. By suppressing the expression of *LeTH1*, *LeTH2*, or *LeTH3* by RNA silencing, it could be possible to confer viral resistance in tomato against tomato mosaic virus (ToMV), depending upon the individual role of these genes.

RNA silencing comprises a number of sequence-specific mRNA degradation processes induced by double-stranded RNA (dsRNA) [13]. The dsRNA is cleaved by Dicer, an endonuclease, into 21–25 bp small interfering RNAs (siRNAs) [14]. The negative strand of the siRNA is then incorporated into an RNA-induced silencing complex (RISC) and acts as a guide to recognize mRNA for degradation [15].

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This mechanism can also be induced by viruses and acts as a host defense mechanism against them [16–19].

In this study, transgenic tomatoes carrying an inverted repeat of either *LeTH1*, *LeTH2*, or *LeTH3* (and thus silenced for each gene) were analyzed for associated inhibitory effects on the multiplication of ToMV.

Transgenic tomato (*Solanum lycopersicum* Mill. cv. Micro-Tom) LeTH1-IR, LeTH2-IR, and LeTH3-IR lines were produced using *Agrobacterium tumefaciens* as described in [20, 21] after plasmid construction (Fig. 1A) based on the method by Asano et al. [11]. Transgenic lines were selected with kanamycin (50 ug/mL). Plants were grown as described by Ali et al. [22] in growth chambers with the temperature set at 26 °C with 16 h light and 8 h dark. A cetyltrimethylammonium bromide-based (CTAB-based) procedure was used for DNA extraction from leaves

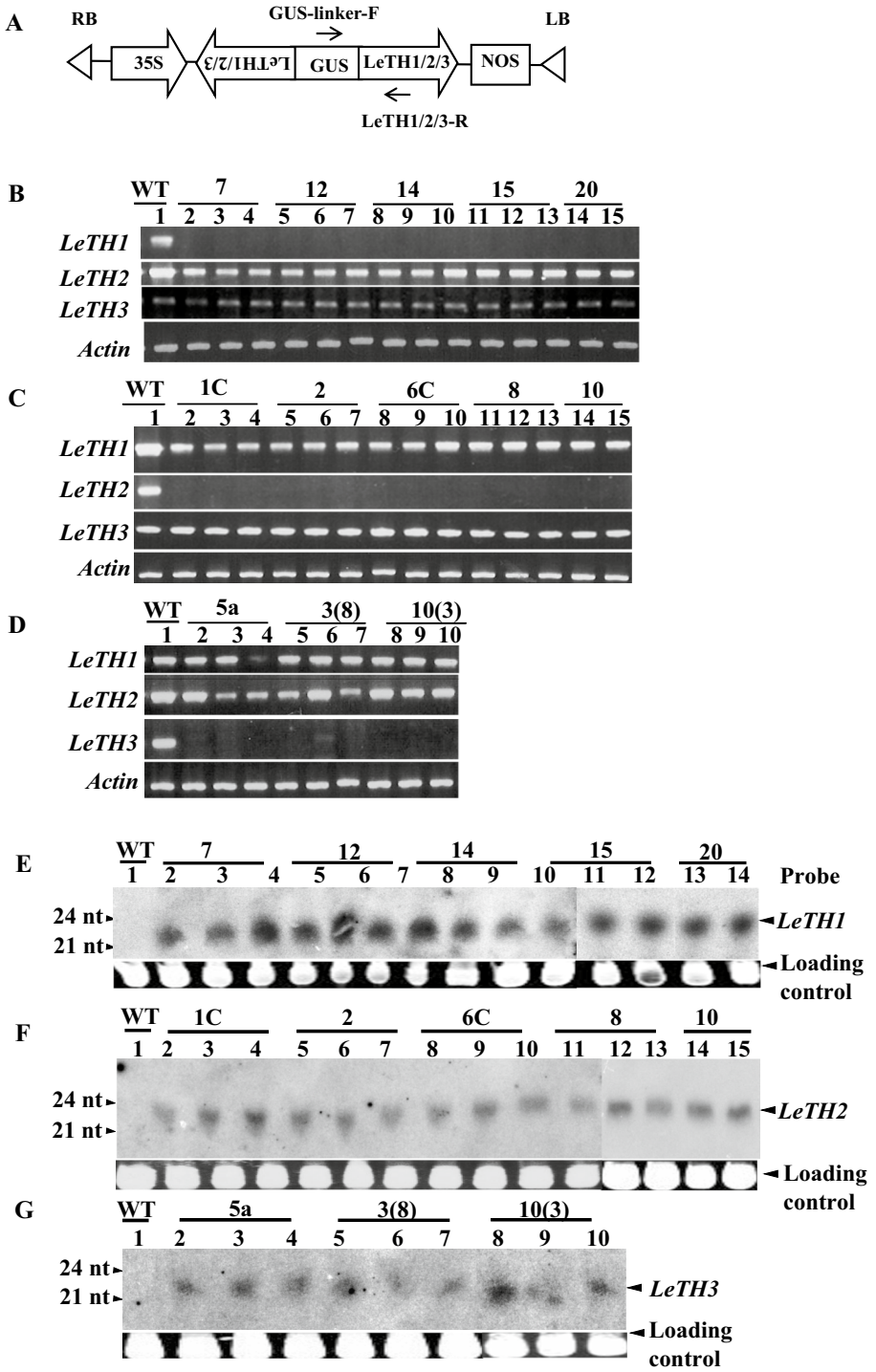

Fig. 1 Evaluation of expression of *LeTH1*, *LeTH2*, and *LeTH3*. **A.** Transgene construct for transgenic tomato. Horizontal closed arrows show primer positions. **B.** RT-PCR analysis of LeTH1-IR. Lane 1, WT; lanes 2–4, line 7; lanes 5–7, line 12; lanes 8–10, line 14; lanes 11–13, line 15; lanes 14–15, line 20. **C.** RT-PCR analysis of LeTH2-IR. Lane 1, WT; lanes 2–4, line 1C; lanes 5–7, line 2; lanes 8–10, line 6C; lanes 11–13, line 8; lanes 14–15, line 10. **D.** RT-PCR analysis of LeTH3-IR. Lane 1, WT; lanes 2–4, line 5a; lanes 5–7, line 3 (8); lanes 8–10, line 10 (3). The presence of the transgene was confirmed by PCR with genomic DNA extracted from leaves of transgenic lines. DNA from different transgenic plants (100 ng) was used as a template for PCR. Total RNA (1 µg) was reverse-transcribed with oligo-(dT) primer. The cDNA was amplified by PCR with the primers listed in Suppl. Table S1. Primers Actin-F/Actin-R (Suppl. Table S1) were used to amplify actin as an internal control. PCR products were analyzed by electrophoresis on a 1% agarose gel. **E.** siRNA detection of LeTH1-IR 1. Lane 1, WT; lanes 2–4, line 7; lanes 5–7, line 12; lanes 8–10, line 14; lanes 11–13, line 15; lanes 14–15, line 20. **F.** siRNA detection of LeTH2-IR. Lane 1, WT; lanes 2–4, line 1C; lanes 5–7, line 2; lanes 8–10, line 6C; lanes 11–13, line 8; lanes 14–15, line 10. **G.** siRNA detection of LeTH3-IR. Lane 1, WT; lanes 2–4, line 5a; lanes 5–7, line 3(8); lanes 8–10, line 10(3). Small RNA fractions (50 µg) were analyzed by northern hybridization with [α-^{32}P] dCTP-labeled cDNA probes

[23]. The quantity and purity of DNA was measured using a spectrophotometer (Gene Spec I; Hitachi Co. Ltd., Tokyo, Japan). Genomic DNA (100 ng) was used as a template for PCR. The forward primer GUS-linker-F in combination with the reverse primers LeTH1-1159R, LeTH2-RT-R or LeTH3-1158R were used to amplify the *LeTH1*, *LeTH2* or *LeTH3*-derived transgenes, respectively (Suppl. Table S1). Cycle conditions were: pre-heating for 2 min at 94 °C followed by a 25-cycle amplification program of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C, with a final extension at 72 °C for 5 min. Amplification products were confirmed by electrophoresis on a 1% agarose gel. Extraction of total RNA from tomato leaves was carried out using a TRI reagent kit (Molecular Research Centre, Cincinnati, OH, USA) according to the manufacturer's instructions. Residual genomic DNA was removed by DNase I treatment (Takara, Kyoto, Japan) for 30 min at 37 °C. One microgram (1 µg) of total RNA was reverse-transcribed using RevertAid reverse transcriptase (Fermentas, Hanova, CA, USA) in a 20 µl reaction mixture. The mixture containing RNA and primers was heated at 70 °C for 10 min and chilled for 10 min before adding 5X buffer and RNase inhibitor, and then incubated at 42 °C for 60 min followed by inactivation at 70 °C for 10 min. The cDNA was used as a template for PCR as mentioned above. RT-PCR products were confirmed by electrophoresis on a 1% agarose gel. Forward and reverse primers LeTH1-769F/LeTH1-1159R, LeTH2-RT-F/LeTH2-RT-R or LeTH3-664F/LeTH3-1158R (Suppl. Table S1) were used to amplify the *LeTH1*, *LeTH2* or *LeTH3*-derived transcripts, respectively.

*Bam*HI and *Sac*I were used to digest the cDNA clones pBI-LeTH1, pBI-LeTH2 and pBI-LeTH3, which contained PCR-amplified LeTH1, LeTH2 and LeTH3 fragments (1365, 1776, and 1256 bp, respectively) inserted into the same restriction sites of pBI221 after removing the *GUS* region. The gel-purified fragments were used as probes for *LeTH1*, *LeTH2* and *LeTH3*, respectively. A *GUS* probe (1.8 kbp fragment) was obtained from pBI121 digested with *Bam*HI and *Sac*I. The [α-^{32}P] dCTP-labeled probes were prepared using the Megaprime DNA Labeling System (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions.

DNA gel blot analysis was performed using genomic DNA (20 µg) from T2 transgenic lines as previously described with a few modifications [22]. Briefly, genomic DNA was subjected to gel electrophoresis and transferred to a nylon membrane (Hybond N$^+$, GE Healthcare, New York, USA). The membrane was hybridized with the *GUS* probe and analyzed using a Bio Image Analyzer (BAS-2000, Fuji Photofilm, Tokyo, Japan) as described by Ali et al. [24]. Total RNA from the ToMV-inoculated leaves of T3 plants was extracted using the TRI reagent kit according to the manufacturer's instructions, with a few modifications. The total RNA gel blot analysis for transcripts was hybridized with a [α-^{32}P] dCTP-labeled ToMV cDNA probe, which was prepared according to Ali et al. [25]. For siRNA detection, small RNAs (30 µg) were separated by 15% polyacrylamide gel electrophoresis and electro-transferred to a nylon membrane. Hybridization and detection were carried out with the labeled cDNA probes (*LeTH1*, *LeTH2* or *LeTH3*) as described [25].

To check whether virus resistance was conferred in transgenic plants, they were inoculated with purified ToMV-L (10 µg/ml; Ohno et al. [4], DDBJ accession X02144) in 10 mM sodium phosphate buffer, pH 7.0. Young detached leaves (3rd and 4th true leaves from the bottom) from one-month-old plants were inoculated with a suspension of virus. One-month-old plants were also inoculated in the adaxial surface of the first pair of true leaves with a suspension of virus. Virus infection was confirmed at 15 days post-inoculation (dpi) by ELISA and northern blot analysis. ELISA was performed using antiserum specific for ToMV (Japan Plant Protection, Tokyo, Japan) and an anti-rabbit IgG conjugated with alkaline phosphatase (Sigma-Aldrich, St. Louis, MO, USA). We considered a negative/positive threshold as twice the absorbance value of the negative control. Northern blot analysis was used in some experiments (as described above) to check the level of virus accumulation in the transgenic plants.

Sequence identities among *A. thaliana TOM1* and *TOM3* and their tomato and tobacco homologs were calculated with Blast (https://www.ebi.ac.uk/Tools/sss/ncbiblast/nucleotide.html). Accession numbers are shown in Table 1.

The presence of the transgenes was confirmed in kanamycin-resistant T2 plants by DNA gel blot analysis with a *GUS* probe (Suppl. Fig. S2). Seeds of the transgene-positive T2 lines were grown in the presence of kanamycin, and all T3 plants of each line were tested for the presence of the transgene by PCR. DNA products of expected sizes (1365, 1776 and 1256 bp) were amplified from the transgenic lines LeTH1-IR, LeTH2-IR and LeTH3-IR, respectively (Suppl. Fig. S1A, B and C), which confirmed the presence of the transgenes derived from *LeTH1*, *LeTH2* or *LeTH3* in the T3 plants. To detect endogenous mRNA expression of *LeTH1*, *LeTH2* and *LeTH3* in T3 plants, RT-PCR analysis was conducted. Endogenous mRNA expression of *LeTH1*, *LeTH2* and *LeTH3* was detected as a highly condensed DNA band in wild-type plants (Fig. 1B, C and D). However, only a very low level of the target gene was detected in the transgenic lines (Fig. 1B, C and D). These results indicate that endogenous mRNA was degraded in the transgenic lines. To detect siRNA in transgenic lines, RNA gel blot analysis was performed using the small RNA fraction purified from T3 plants. The analysis showed that siRNA was detected in all plants tested (Fig. 1E, F, G), but not in the non-transgenic

Table 1 Nucleotide sequence identity (%) of *TOM1*and *TOM3* homologs from tomato (*Solanum lycopersicum*), tobacco (*Nicotiana tabacum*) and *Arabidopsis thaliana*

	NtTOM1	*NtTOM3*	*LeTH1*	*LeTH3*	*LeTH2*	*TOM1*	*TOM3*	*TOM2A*	*TOM2B*
NtTOM1	100	58.1	57.5	90.3	61.0	68.3	59.8	31.2	40.7
NtTOM3		100	78.0	59.6	55.7	56.8	72.3	31.4	41.0
LeTH1			100	57.9	55.6	57.6	68.0	37.1	45.3
LeTH3				100	58.6	66.9	58.0	34.4	44.2
LeTH2					100	56.1	58.9	35.9	44.8
TOM1						100	58.5	36.8	44.8
TOM3							100	33.0	42.0
TOM2A								100	43.0
TOM2B									100

Nicotiana tabacum: *NtTOM1*, AB193039; *NtTOM3*, AB193040. *Solanum lycopersicum*: *LeTH1*, AB193041; *LeTH2*, AB193042; *LeTH3*, AB193043. *Arabidopsis thaliana*: *TOM1*, AB016925; *TOM3*, AB036427; *TOM2A*, AT1G32400; *TOM2B*, AT1G32370

control plants. From these results it is clear that RNA silencing was induced in each transgenic line.

To confirm whether ToMV resistance was conferred in these transgenic lines, whole plants or detached leaves were inoculated with ToMV-L. Inoculated non-transgenic tomato plants showed symptoms at 10 dpi, while no symptoms appeared in transgenic plants of each line at this time (Fig. 2). However, mild symptoms appeared at 15 dpi in transgenic plants of each transgenic line (data not shown). An extremely low amount of virus was detected in the transgenic lines, showing that virus resistance was conferred (Fig. 3, Suppl. Fig. S3 and S4). Each silenced transgenic line showed similar levels of resistance when compared to each other.

Understanding the role of host proteins that support virus replication will be helpful in advancing our knowledge of virus-host interactions. Studies on tobamoviruses have shown that host proteins involved in the viral infection process play a vital role in their pathogenicity by forming membrane-bound viral replication complexes in plant cells [2]. *TOM1*- and *TOM3*–like genes are conserved in *Capsicum annuum*, *Cucumis melo*, *Nicotiana tobacum*, *N. benthamiana*, *Oryza sativa* and *S. lycopersicum*, [11, 12, 26]. In this study, transgenic tomato plants silenced for *LeTH1*, a homolog of *TOM3*, and for *LeTH2* and *LeTH3*, homologs of *TOM1*, were examined for ToMV resistance.

LeTH1, *LeTH2*, and *LeTH3* in tomato have high sequence identities with *N. tabacum NtTOM1* and *NtTOM3* as well as with *Arabidopsis TOM1* and *TOM3* (Table 1). *LeTH3* has 90.3% and 59.6% sequence identity with *NtTOM1* and *NtTOM3*, respectively; *LeTH1* has 57.5% and 78% identity with *NtTOM1* and *NtTOM3*, respectively; and *LeTH2* has 61% and 55.7% identity with *NtTOM1* and *NtTOM3,* respectively. Although functional roles for *LeTH1*, *LeTH2*, and *LeTH3* in tomato are unknown, our silenced transgenic lines showed similar levels of virus resistance. It has been observed that cDNAs with higher than 70% identity are capable of silencing a target gene [27]. Recently, it was reported that silencing the pepper (*Capsicum annuum*) genes *CaTOM1* and *CaTOM3*, homologs of *TOM1* and *TOM3*, respectively, efficiently inhibited TMV infection [12]. Similar results were also obtained by silencing a *TOM1*-like gene in *N. benthamiana* (*NbTOM1*) [26]. Here, we examined the efficiency of LeTH1-IR, LeTH2-IR, and LeTH3- IR constructs in conferring resistance to ToMV. Higher resistance could be

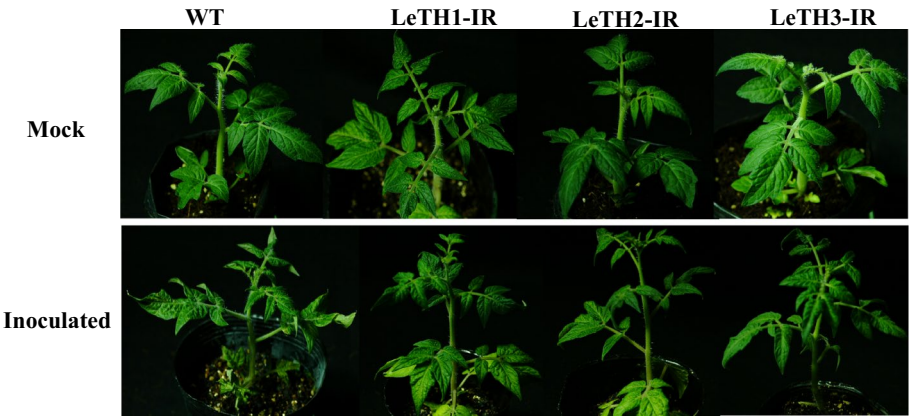

Fig. 2 Plants of transgenic tomato lines LeTH1-IR, LeTH2-IR and LeTH3-IR 10 days after ToMV or mock inoculation

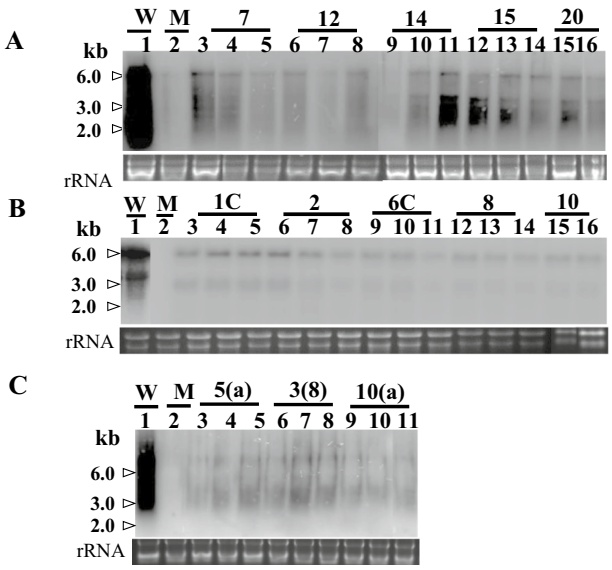

Fig. 3 Northern blot analysis of virus inoculated leaves of transgenic plants. **A.** LeTH1-IR. Lane 1, WT; lanes 2–4, line 7; lanes 5–7, line 12; lanes 8–10, line 14; lanes 11–13, line 15; lanes 14–15, line 20. **B.** LeTH2-IR. Lane 1, WT; lanes 2–4, line 1C; lanes 5–7, line 2; lanes 8–10, line 6C; lanes 11–13, line 8; lanes 14–15, line 10. **C.** LeTH3-IR. Lane 1, WT; lanes 2–4, line 5a; lanes 5–7, line 3(8); lanes 8–10, line 10(3). Detached leaves were inoculated with a suspension of ToMV-L at a concentration of 10 μg/ml. Northern blot analysis was performed 15 days after inoculation

obtained by double or triple crossing of these transgenic lines.

Only limited methods are available to control tobamovirus diseases, including protection by mild/attenuated viruses [28]. Transgenic approaches using viral genes are a matter of concern, in terms of potential risks [29]. Virus resistant plants using host-derived resistance related genes would be preferable, especially with an RNA silencing approach, because neither protein nor RNA from a transgene are produced. Thus our results could contribute to develop safer, virus-resistant plants.

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Compliance with ethical standards

Conflict of interest The authors have declared that no competing interests exist.

Ethical approval This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

References

1. Roossinck MJ (2011) The good viruses: viral mutualistic symbioses. Nature Rev Microbiol 9:99–108
2. Ishibashi K, Ishikawa M (2016) Replication of tobamovirus RNA. Annu Rev Phytopathol 54:55–78
3. Buck KW (1996) Comparison of the replication of positive-stranded RNA viruses of plants and animals. Adv Virus Res 47:159–251
4. Ohno T, Aoyagi M, Yamanashi Y, Saito H, Ikawa S, Meshi T, Okada Y (1984) Nucleotide sequence of the tobacco mosaic virus (tomato strain) genome and comparison with the common strain genome. J Biochem 96:1915–1923
5. Lai MMC (1998) Cellular factors in the transcription and replication of viral RNA genomes: a parallel to DNA-dependent RNA transcription. Virology 244:1–12
6. Lee WM, Ishikawa M, Ahlquist P (2001) Mutation of host delta Δ9 fatty acid desaturase inhibits brome mosaic virus RNA replication between template recognition and RNA synthesis. J Virol 75(5):2097–2106
7. Noueiry AO, Chen JB, Ahlquist P (2000) A mutant allele of essential, general translation initiation factor DED1 selectively inhibits translation of a viral mRNA. Proc Natl Acad Sci 97(24):12985–12990
8. Ishikawa M, Obata F, Kumagai T, Ohno T (1991) Isolation of mutants of *Arabidopsis thaliana* in which accumulation of tobacco mosaic virus coat protein is reduced to low levels. Mol Gen Genet 230(1–2):33–38
9. Yamanaka T, Imai T, Satoh R, Kawashima A, Takahashi M, Tomita K, Kubota K, Meshi T, Naito S, Ishikawa M (2002) Complete inhibition of tobamovirus multiplication by simultaneous mutations in two homologous host genes. J Virol 76:2491–2497
10. Tsujimoto Y, Numaga T, Ohshima K, Yano M, Ohsawa R, Derek BG, Naito S, Ishikawa M (2003) *Arabidopsis TOBAMOVIRUS MULTIPLICATION(TOM) 2* locus encodes a transmembrane protein that interacts with TOM1. EMBO 22:335–343
11. Asano M, Satoh R, Mochizuki A, Tsuda S, Yamanaka T, Nishiguchi M, Hirai K, Meshi T, Naito S, Ishikawa M (2005) Tobamovirus-resistant tobacco generated by RNA interference directed against host genes. FEBS Lett 579:4479–4484
12. Sunil K, Ashvini KD, Ruma K, Kukkundoor RK, Mathew KM, Harischandra SP (2012) Inhibition of TMV multiplication by siRNA constructs against *TOM1* and *TOM3* genes of *Capsicum annuum*. J Virol Meth 186(1–2):78–85
13. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391:806–811
14. Bernstein E, Caudy AA, Hammond SM, Hannon GJ (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 409:363–366
15. Hammond SM, Bernstein E, Beach D, Hannon GJ (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. Nature 404:293–296
16. Baulcombe DC (2004) RNA silencing in plants. Nature 431:356–363

17. Hamilton AJ, Baulcombe DC (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 286:950–952
18. Voinnet O (2001) RNA silencing as a plant immune system against viruses. Trends Genet 17:449–459
19. Waterhouse PM, Wang MB, Lough T (2001) Gene silencing as an adaptive defence against viruses. Nature 411:834–842
20. Sun HJ, Uchii S, Watanabe S, Ezura H (2006) A highly efficient transformation protocol for micro-tom, a model cultivar for tomato functional genomics. Plant Cell Physiol 47(3):426–431
21. Chetty VJ, Ceballos N, Garcia D, Narváez-Vásquez J, Lopez W, Orozco-Cárdenas ML (2013) Evaluation of four *Agrobacterium tumefaciens* strains for the genetic transformation of tomato (*Solanum lycopersicum* L.) cultivar Micro-Tom. Plant Cell Rep 32:239–247
22. Ali ME, Tabei Y, Kobayashi K, Yamaoka N, Nishiguchi M (2012) Molecular analysis of transgenic melon plants showing virus resistance conferred by direct repeat of movement gene of *Cucumber green mottle mosaic virus*. Plant Cell Rep 31(8):1371–1377
23. Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bull 19:11–15
24. Ali ME, Kobayashi K, Yamaoka N, Ishikawa M, Nishiguchi M (2013) Graft transmission of RNA silencing to non-transgenic scions for conferring virus resistance in tobacco. PLoS ONE 8(5):e63257
25. Haque AKMN, Tanaka Y, Nishiguchi M (2007) Analysis of transitive RNA silencing after grafting in transgenic plants with the coat protein gene of *Sweet potato feathery mottle virus*. Plant Mol Biol 63:35–47
26. Chen B, Jiang JH, Zhou XP (2007) A *TOM1* homologue is required for multiplication of Tobacco mosaic virus in *Nicotiana benthamiana*. J Zhejiang Univ Science B 8:256–259
27. Meyer P, Saedler H (1996) Homology dependent gene silencing in plants. Ann Rev Plant Physiol Plant Mol Biol 47:23–48
28. Kenyon L, Kumar S, Tsai WS, Hughes JďA (2014) Virus diseases of peppers (Capsicum spp.) and their control. Adv Virus Res 90:297
29. Cruz-Reyes R, vila-Sakar GÁ, Sánchez-Montoya G, Quesada M (2015) Experimental assessment of gene flow between transgenic squash and a wild relative in the center of origin of cucurbits. Ecosphere 6(12):248. https://doi.org/10.1890/ES15-00304.1