An inhibitor of viral RNA replication is encoded by a plant resistance gene

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The tomato *Tm-1* gene confers resistance to tomato mosaic virus (ToMV). Here, we report that the extracts of *Tm-1* tomato cells (GCR237) have properties that inhibit the *in vitro* RNA replication of WT ToMV more strongly than that of the *Tm-1*-resistance-breaking mutant of ToMV, LT1. We purified this inhibitory activity and identified a polypeptide of ≈ 80 kDa (p80^{GCR237}) using LC-tandem MS. The amino acid sequence of p80^{GCR237} had no similarity to any characterized proteins. The p80^{GCR237} gene cosegregated with *Tm-1*; transgenic expression of p80^{GCR237} conferred resistance to ToMV within tomato plants; and the knockdown of p80^{GCR237} sensitized *Tm-1* tomato plants to ToMV, indicating that *Tm-1* encodes p80^{GCR237} itself. We further show that *in vitro*-synthesized *Tm-1* (p80^{GCR237}) protein binds to the replication proteins of WT ToMV and inhibits their function at a step before, but not after, the viral replication complex is formed on the membrane surfaces. Such binding was not observed for the replication proteins of LT1. These results suggest that *Tm-1* (p80^{GCR237}) inhibits the replication of WT ToMV RNA through binding to the replication proteins.

Tm-1 | Tomato mosaic virus | inhibitory interaction

Genetic resistance has commonly been used to protect cultivated plants from viral infection. One major class of virus resistance genes is dominant, encodes proteins that contain nucleotide-binding sites and leucine-rich repeats, and elicits a hypersensitive reaction (HR) in response to infection (1). Another is recessive and encodes mutated host proteins that are primarily required for virus multiplication (1, 2).

The tomato *Tm-1* gene is a semidominant trait that inhibits tomato mosaic virus (ToMV) multiplication in protoplasts (3, 4). *Tm-1* was bred into cultivated tomato (*Solanum lycopersicum*) from the wild tomato *Solanum habrochaites* PI126445 (5) and has been used widely to protect tomato plants from ToMV infection. ToMV mutants that are able to multiply in *Tm-1* tomato plants have been isolated; the *Tm-1* resistance-breaking property of these mutants, including the LT1 strain, was correlated with mutations in the coding region of the replication proteins, i.e., the 130-kDa (130K) protein and its translational readthrough product, the 180-kDa (180K) protein (6–8).

Several dominant genes also inhibit virus multiplication in protoplasts (1). Examples include the potato *Rx* gene that confers resistance to potato virus X and encodes a protein that contains nucleotide-binding sites and leucine-rich repeats (9). In *Rx* protoplasts, the multiplication of *Rx*-insensitive virus is inhibited when coinoculated with the *Rx*-sensitive potato virus X strain, suggesting that the HR induced by *Rx*-sensitive virus inhibits even the multiplication of *Rx*-insensitive virus (10). In contrast, coinoculation with WT ToMV in *Tm-1* protoplasts has no effect on LT1 multiplication (11), suggesting that *Tm-1* does not mediate an inducible nonspecific resistance like an HR. Based on these characteristics, it was proposed that *Tm-1* cells constitutively express an inhibitor of ToMV RNA replication (6, 12).

In this paper, we show that *Tm-1* encodes a protein that binds to and inhibits the functioning of the replication proteins of ToMV. The findings indicate that *Tm-1* has characteristics

different from those of previously identified virus resistance genes in plants.

Results and Discussion

An Inhibitory Factor of ToMV RNA Replication Is Present in the *Tm-1* Tomato Cell Extract. Previously, we found that an extract isolated from evacuated tobacco BY-2 protoplasts (BYL) is capable of translating ToMV RNA into the replication proteins, and that, if ribonucleoside triphosphates are added to the mixture after translation, ToMV RNA is replicated (13). Taking into consideration the possibility that *Tm-1* cells express an inhibitor of ToMV RNA replication, we investigated whether *in vitro* ToMV RNA replication could be inhibited by the addition of an extract from evacuated *Tm-1* protoplasts. To this end, we established a suspension-cultured cell line from GCR237 tomato plants homozygous for *Tm-1*. Consistent with a previous report (14), protoplasts isolated from this cell line showed resistance to WT ToMV, whereas the *Tm-1* resistance-breaking ToMV mutant LT1 multiplied efficiently (Fig. 1A). In contrast, protoplasts prepared from tobacco BY-2 cells or a suspension-cultured cell line derived from a near-isogenic *Tm-1*-lacking tomato, GCR26 (a generous gift from Tsubasa Takahashi), allowed WT ToMV to multiply at a level comparable to LT1 (Fig. 1A).

We prepared evacuated protoplast extracts from each cell line, added them to BYL, and performed the translation-replication reaction for WT ToMV and LT1 RNA. The level of WT ToMV RNA replication, represented by the incorporation of ³²P into genomic RNA, was significantly reduced upon the addition of the GCR237 (*Tm-1*) cell extract, compared with the level of LT1 RNA replication (Fig. 1B). In contrast, the levels of *in vitro* RNA replication were similar between WT ToMV and LT1 in the absence of tomato cell extract or in the presence of GCR26 cell extract. Assuming the difference between the replication of WT ToMV and LT1 RNA represents *Tm-1* activity, we fractionated the GCR237 cell extracts (Fig. 1C). The sixth and seventh fractions from Mono Q column chromatography, which was the final purification step, showed high *Tm-1* activity (Fig. 1D) and contained several specific protein bands (Fig. 1E),

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Abbreviations: ToMV, Tomato mosaic virus; HR, hypersensitive reaction; BYL, evacuated BY-2 protoplast lysate; CMV, cucumber mosaic virus; TRV, tobacco rattle virus; mdBYL, membrane-depleted BYL; dpi, days postinoculation.

Data deposition: The nucleotide sequence data reported in this paper have been deposited in the DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank nucleotide sequence databases [accession nos. AB287296 (*Tm-1*^{GCR237}) and AB287297 (*tm-1*^{GCR26})].

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Fig. 1. Detection and purification of the activity to inhibit ToMV RNA replication *in vitro* from evacuated *Tm-1* tomato protoplast extracts. (A) ToMV multiplication in protoplasts. Protoplasts from suspension-cultured *Tm-1* tomato (GCR237), ToMV-susceptible tomato (GCR26), and tobacco (BY-2) cells were mock-inoculated or inoculated with WT (wt) ToMV (*Tm-1*-sensitive strain) RNA or LT1 (*Tm-1*-resistance-breaking strain) RNA by electroporation and cultured for 24 h. ToMV genomic RNA (G) and 28S rRNA (loading control) were detected by using Northern blot hybridization and methylene blue staining, respectively. (B) The effect of adding tomato cell extracts on ToMV RNA replication. The evacuated GCR237 or GCR26 protoplast extracts were added to the uncoupled translation and replication reactions for WT ToMV RNA or LT1 RNA, as described in *Materials and Methods*. 32 P-labeled RNA products were separated by PAGE followed by autoradiography. The positions of ToMV genomic RNA (G) and the replicative-form RNA (RF) are indicated. Asterisks represent background signals. (C) Purification of *Tm-1* activity. (D) Detection of *Tm-1* activity in the Mono Q fractions. The Mono Q fractions were added to the uncoupled translation and replication reactions for WT ToMV RNA or LT1 RNA as described in *Materials and Methods*. 32 P-labeled RNA products were analyzed as in B (see above for abbreviations). (E) Proteins contained in the Mono Q fractions. The Mono Q fractions were subjected to SDS/PAGE, followed by silver staining. Arrow indicates the p80 band.

from which we identified an ≈ 80 -kDa protein (p80) using LC-tandem MS (arrow in Fig. 1E).

We cloned the cDNA encoding p80 from GCR237 (p80^{GCR237}). The p80^{GCR237} polypeptide had an amino acid sequence similar to that of uncharacterized proteins found in plants, fungi, and bacteria, yet showed no resemblance to any protein with known functions, including the plant resistance genes cloned and characterized to date [see supporting information (SI) Fig. 5]. The *in vitro*-translated p80^{GCR237} protein inhibited the replication of WT ToMV RNA, but not LT1 RNA, suggesting that p80^{GCR237} is a specific inhibitor of WT ToMV RNA replication (Fig. 2). The p80^{GCR237} gene contains nine exons and eight introns and encodes a 754-aa polypeptide (SI Fig. 5). We also identified a splicing variant of p80^{GCR237} mRNA that lacked the second exon, which encodes amino acid residues 46–263. The translation product from this shorter splicing variant from GCR237 did not inhibit WT ToMV RNA replication *in vitro* (Fig. 2).

p80^{GCR237} Is the *Tm-1* Gene Product. We found a p80-like gene in GCR26 (p80^{GCR26}). The coding region of p80^{GCR26} differs from that of p80^{GCR237} by 49 nucleotides, 25 of which result in amino acid substitutions (SI Fig. 5), although GCR26 is near-isogenic to GCR237. The *in vitro* translation products of the p80^{GCR26}

mRNA did not affect WT ToMV replication (Fig. 2). The mRNA sequence of the p80-coding region from *S. habrochaites* PI126445 was identical to that from GCR237, suggesting that p80^{GCR237} was derived from *S. habrochaites* PI126445. To determine whether p80^{GCR237} cosegregates with *Tm-1*, we designed

Fig. 2. *In vitro*-synthesized p80^{GCR237} inhibits ToMV RNA replication. Messenger RNAs for p80^{GCR237}, p80^{sv} (the product of the splicing variant mRNA lacking the second exon), and p80^{GCR26} were translated by using mdBYL at 23°C for 1 h (0.5 pmol of mRNA/5 μ l of translation reaction mixture). The translation mixtures containing p80-related proteins (or a mock-translated mixture as a control) were added to the uncoupled translation and replication reactions for WT (wt) ToMV RNA or LT1 RNA, as described in *Materials and Methods*. 32 P-labeled RNA products were analyzed as in Fig. 1B; see Fig. 1B legend for abbreviations. In this condition, the p80^{GCR237} protein is produced ≈ 20 times as much as the 130K protein on a molar basis (data not shown).

Fig. 3. The $p80^{GCR237}$ gene confers ToMV resistance to tomato plants. (A) Inhibition of ToMV multiplication in GCR26 tomato plants expressing $p80^{GCR237}$. The T2 plants of transgenic line nos. 24 or 12, or nontransgenic GCR26 or GCR237 plants were mechanically inoculated with WT (wt) ToMV, LT1, or CMV. GCR26 plants were also mock-inoculated. Total RNA was prepared separately from the ToMV-inoculated leaves at 4 dpi, from the uninoculated upper leaves of CMV-inoculated plants at 10 dpi, and from healthy plants (for p80 mRNA detection). Equal amounts of RNA from three individuals were mixed for each plant genotype–virus combination, and the mixture was analyzed by Northern blotting and hybridization. (B) Multiplication of WT ToMV in $p80$ -silenced *Tm-1* tomato. GCR237 plants were inoculated with *A. tumefaciens* strains harboring the pTRV vector with (pTRV-p80) or without (pTRV) the fragment of the $p80^{GCR237}$ cDNA. After 3 weeks, WT ToMV or LT1 were challenge-inoculated within young leaves. Total RNA was extracted from the upper uninoculated leaves 10 days after challenge inoculation. ToMV RNA and 28S rRNA were detected as in Fig. 1A. Each lane represents a sample from one plant. Typically, the accumulation of WT ToMV genomic RNA was <1% of that of LT1 genomic RNA in pTRV-inoculated plants. High levels of WT ToMV RNA accumulation comparable to those for LT1 RNA were observed in 32 of 47 pTRV-p80-inoculated GCR237 plants, but were not observed in any of the 22 pTRV-inoculated GCR237 plants.

sequence-characterized amplified region (SCAR) markers to independently detect the $p80^{GCR237}$ and $p80^{GCR26}$ genes. All of the F_2 plants derived from the crosses between GCR237 and GCR26 carried either or both SCAR markers, suggesting that $p80^{GCR237}$ and $p80^{GCR26}$ sequences are allelic to each other. Furthermore, 25 of the 95 F_2 plants tested were homozygous for $p80^{GCR26}$, allowing for high-level multiplication of ToMV (SI Fig. 6). The remaining plants were carriers of the $p80^{GCR237}$ gene, with reduced levels of ToMV multiplication, indicating that this designed sequence-characterized amplified region marker is linked to the *Tm-1* locus (SI Fig. 6).

To determine whether the expression of $p80^{GCR237}$ confers resistance to ToMV *in vivo*, GCR26 tomato plants were transformed with a gene cassette containing the $p80^{GCR237}$ cDNA under the control of the cauliflower mosaic virus 35S RNA promoter. As expected from the semidominance of the *Tm-1* gene, the T2 plants that expressed $p80^{GCR237}$ were resistant to WT ToMV (Fig. 3A). In transgenic line no. 24, in which the levels of $p80^{GCR237}$ mRNA were comparable to endogenous $p80$ mRNA, the level of LT1 multiplication was similar to that in nontransgenic GCR26 or GCR237 plants (Fig. 3A). In transgenic line no. 12, which overexpressed $p80^{GCR237}$ mRNA by >20 times, LT1 multiplication was reduced to significantly low levels, whereas the multiplication of cucumber mosaic virus (CMV) was unaffected (Fig. 3A).

We next knocked down $p80^{GCR237}$ expression using the virus-induced gene silencing method with the tobacco rattle virus (TRV) vector (15), followed by challenge with WT ToMV.

Significant levels of WT ToMV RNA accumulated in the uninoculated upper leaves of GCR237 plants that had been inoculated with TRV carrying the $p80^{GCR237}$ sequence (Fig. 3B; also see SI Text). Thus, we conclude that *Tm-1* encodes $p80^{GCR237}$. Henceforth, we refer to $p80^{GCR237}$ and $p80^{GCR26}$ as *Tm-1^{GCR237}* and *Tm-1^{GCR26}*, respectively, based on their mode of inheritance. Because the knockdown of *Tm-1^{GCR237}* sensitized *Tm-1* tomato plants to WT ToMV, it is unlikely that *Tm-1^{GCR237}* is a dominant-negative form of an essential factor for ToMV multiplication.

Tm-1 Inhibits the *in Vitro* ToMV RNA Replication at a Step After Translation of ToMV RNA and Before the Formation of Replication Complexes on the Membranes. ToMV RNA replication occurs in membrane-bound complexes (16–18). When ToMV RNA is translated in membrane-depleted BYL (mdBYL), the 130K and 180K replication proteins are produced, but ToMV-related RNA is not synthesized. However, ToMV RNA replication occurs when the BYL membranes are introduced back into the ToMV RNA-translated mdBYL (19). To elucidate the mechanism by which *Tm-1^{GCR237}* inhibits ToMV RNA replication, we synthesized the *Tm-1^{GCR237}* protein by translation in mdBYL and added it to the uncoupled *in vitro* translation and replication reaction for WT ToMV or LT1 RNA at one of three times: (i) before viral RNA translation in mdBYL; (ii) after viral RNA translation in mdBYL and before the addition of BYL membranes; or (iii) after incubation with BYL membranes (Fig. 4A). There was no inhibition of WT ToMV RNA replication upon addition of the *Tm-1^{GCR237}* protein after incubation with BYL membranes, whereas WT ToMV-specific inhibition was observed in the other cases (Fig. 4A). The levels of the 130K protein accumulation were similar for WT ToMV and LT1 in all cases (Fig. 4A Lower). Thus, it appears that *Tm-1^{GCR237}* inhibits ToMV RNA replication by affecting the function of ToMV replication proteins before, but not after, they make contact with the membranes. It is unlikely that *Tm-1^{GCR237}* affects the synthesis or stability of the replication proteins.

Tm-1 Binds to ToMV Replication Proteins to Inhibit Their Functions. When the above results were considered in light of the fact that *Tm-1* resistance-breaking ToMV strains carry mutations in the 130K and 180K replication protein-coding region (6–8), we were prompted to determine whether *Tm-1^{GCR237}* interacts with these proteins. FLAG-tagged *Tm-1^{GCR237}* and *tm-1^{GCR26}* proteins were synthesized by *in vitro* translation in mdBYL. The *Tm-1^{GCR237}*-FLAG protein inhibited the *in vitro* replication of WT ToMV RNA, as did the nontagged *Tm-1^{GCR237}* protein (data not shown). These *Tm-1^{GCR237}*-FLAG or *tm-1^{GCR26}*-FLAG preparations were incubated with WT ToMV RNA- or LT1 RNA-translated mdBYL, after which affinity purification was performed by using anti-FLAG antibody-conjugated agarose beads. The replication proteins of WT ToMV were detected in the *Tm-1^{GCR237}*-FLAG-purified fraction, whereas those of LT1 were not (Fig. 4B). In contrast, *tm-1^{GCR26}*-FLAG did not coprecipitate the replication proteins of either WT ToMV or LT1 (Fig. 4B). These results suggest that *Tm-1^{GCR237}* inhibits WT ToMV RNA replication by binding, whether directly or indirectly, to the 130K and 180K proteins, and the LT1 mutation reduces the affinity of the replication proteins to *Tm-1^{GCR237}*. Although we were unable to detect the interaction *in vitro*, the replication proteins of LT1 should still have a weak affinity for the *Tm-1^{GCR237}* protein, because the overexpression of *Tm-1^{GCR237}* in GCR26 plants reduced the efficiency of LT1 multiplication (Fig. 3A, line no. 12).

Concluding Remarks. Previous attempts to identify the *Tm-1* gene using map-based cloning proved unsuccessful, because the *Tm-1* locus is located near the centromere of chromosome 2, where the

Fig. 4. Tm-1^{GCR237} (p80^{GCR237}) binds to ToMV replication proteins and inhibits ToMV RNA synthesis if added before the formation of the replication complex on the membrane surface. (A) The effect of the timing of the addition of Tm-1^{GCR237} on ToMV RNA replication. Tm-1^{GCR237} was synthesized in mdBYL and added to the uncoupled translation and replication reaction mixture for WT (wt) ToMV or LT1 RNA (i) before the translation in mdBYL, (ii) after termination of translation with puromycin and before the addition of membranes (BYL P30), or (iii) after incubation with membranes and before addition of rNTPs. As a control, mock-translated mdBYL was added. ³²P-labeled RNA products were analyzed as in Fig. 1B; see Fig. 1B legend for abbreviations. The 130K protein was detected by Western blotting after incubation with the membranes. (B) Interaction between Tm-1^{GCR237} and ToMV replication proteins. FLAG-tagged Tm-1^{GCR237} or FLAG-tagged tm-1^{GCR26} proteins and the replication proteins of WT ToMV or LT1 were synthesized by translation in mdBYL, mixed, and purified by using anti-FLAG antibody-conjugated agarose beads. Protein samples before (input) or after (FLAG-IP) FLAG purification were analyzed by Western blotting using anti-130K protein or anti-FLAG antibodies. Positions for the 130K, 180K, and FLAG-tagged Tm-1^{GCR237} (tm-1^{GCR26}) protein bands are indicated. The asterisk represents the background signal derived from a host (BYL) protein.

frequency of recombination is extremely low (20–22). We identified the *Tm-1* gene by purifying its inhibitory activity toward ToMV RNA replication *in vitro*. We also showed that Tm-1^{GCR237} binds directly or indirectly to ToMV replication proteins and inhibits their function only before they form active replication complexes on membranes. The Tm-1 protein has been predicted to have the TIM barrel structure (SI Fig. 5), which is found in a wide variety of enzymes (23). Currently, there are no clues to understanding the cellular functions of the Tm-1 protein.

Although most of the dominant traits that confer resistance against viruses elicit an HR (1), several others may encode inhibitory factors of viral functions like Tm-1^{GCR237}. The systemic spread of tobacco etch virus is inhibited in some ecotypes of *Arabidopsis thaliana* without elicitation of an HR (24); the *RTM1* and *RTM2* genes are required for this resistance. Positional cloning revealed that these genes encode a lectin-like protein (25) and a small heat-shock-like protein (26), respectively. Although the mechanisms of resistance are not well understood, one possibility is that the RTM system inhibits viral movement functions. In cowpea, a dominant trait that inhibits the multiplication of cowpea mosaic virus in protoplasts is suggested to be associated with the inhibition of viral polyprotein processing, although the corresponding resistance gene has not been identified (27).

Essentially, the key to successful viral adaptation within its host lies not just in the use of its host factors but in evading inhibition. In the natural hosts that permit the multiplication of a virus, the virus must have already evolved to escape the inhibitory interactions between viral and host factors. Considering that most studies of virus multiplication have been performed in the natural host, the ubiquitousness of the inhibitory interactions may have been underestimated. Recent reports have shown that the overexpression of natural host-derived factors that bind to viral molecules can inhibit virus multiplication (28–30). These examples suggest that, even in the natural hosts, the inhibitory interactions between host and viral factors occur, although the inhibition is weak enough to allow virus multiplication. Future studies should explore how widely such inhibitory interactions contribute to determine the host range and multiplication efficiency of viruses.

Materials and Methods

Viruses. WT (*Tm-1*-sensitive) ToMV (ToMV-L; ref. 31), the *Tm-1*-resistance-breaking mutant of ToMV (LT1, designated as T1 in ref. 6), and the Y strain of CMV (32) were propagated and purified, and their virion RNA was purified as described (33, 34). TL1le (35) was used in several experiments as the *Tm-1*-sensitive ToMV strain to assay for Tm-1 activity (Fig. 1D). Purified virions (100 µg/ml) were mechanically inoculated, and the viral RNA that accumulated in inoculated leaves at 4 days postinoculation (dpi; for ToMV in Fig. 3A) or in upper uninoculated leaves at 10 dpi (for CMV in Fig. 3A and for ToMV in Fig. 3B) were analyzed by Northern blot hybridization (34, 36).

Plants and Cells. GCR237 and GCR26 are near-isogenic lines of tomato (*Solanum lycopersicum* cv. Craigella), with the exception that GCR237 is homozygous for *Tm-1* and GCR26 lacks *Tm-1* (37). A suspension-cultured cell line was established from GCR237 seedlings in Murashige–Skoog medium supplemented with 2 mg/liter 1-naphthaleneacetic acid/0.2 mg/liter 2,4-dichlorophenoxy acetic acid/0.2 mg/liter zeatin. The suspension-cultured cell line of GCR26 was obtained from Tsubasa Takahashi and maintained in the same medium. *Nicotiana tabacum* BY-2 cell lines were maintained as described (38). Protoplasts were isolated from 3-day-cultured cell suspension and inoculated with ToMV RNA by electroporation (14). Transformation of GCR26 tomato plants using *Agrobacterium tumefaciens* was performed as described (39). *S. habrochaites* PI126445 was obtained from the Germplasm Resources Information Network.

Detection of Tm-1 Activity in GCR237 Cell Extracts. BYL (extract of evacuated BY-2 protoplasts) was prepared as described (38). To obtain mdBYL, BYL (135 µl) was centrifuged at 30,000 × *g* for 15 min, and the supernatant was recovered. The pellet was resuspended in 40 µl of TR buffer [30 mM Hepes-KOH (pH 7.4)/80 mM KOAc/1.8 mM Mg(OAc)₂/2 mM DTT/one tablet of Complete EDTA-free (Roche, Indianapolis, IN) per 50 ml] to obtain P30BYL membrane suspension. Evacuated tomato protoplast extracts were prepared by using the protocol for the tobacco BY-2 cells (38) with slight modifications. Twenty nanograms (10 fmol) of WT ToMV (or TL1le in Fig. 1D) or LT1 RNA was translated in 10 µl of mdBYL-based reaction mixture at 23°C for 1 h (38). The translation mixture (10 µl) was incubated at 23°C for 20 min with 5 µl of a test sample, followed by incubation at 15°C for 2 h with 5 µl of P30BYL and further incubation at 23°C for 1 h with 5 µl of a ribonucleoside triphosphate mixture [5 mM ATP/5 mM GTP/5 mM UTP/125 µM CTP/50 mM DTT/500 µg/ml actinomycin D/150 mM creatine phosphate/17 mM Mg(OAc)₂] containing 10 µCi [α -³²P]CTP (1 Ci = 37 GBq). The reaction was terminated by phenol extraction, and the RNA products were purified and analyzed by electrophoresis in 8 M urea–2.4% polyacrylamide gel and autoradiography. The origi-

1 h, followed by the addition of 0.5 μ l of 6 mM puromycin to terminate translation. Then, 5 μ l of Tm-1^{GCR237}- or mock-translated mdBYL mixture was added to the mixture for a total sample volume of 15.5 μ l. The mixture was incubated at 23°C for 5 min, followed by the addition of 5 μ l of P30BYL membrane suspension and further incubation at 15°C for 2 h. Tm-1^{GCR237}- or mock-translated mdBYL mixture (5 μ l) was added to each mixture for a total sample volume of 25.5 μ l and incubated at 15°C for 5 min. A portion of each mixture (20 μ l) was used for the RNA replication reaction (38) and the remainder for Western blotting. Tm-1^{GCR237}-translated mdBYL was added only once.

FLAG Immunoprecipitation Experiment (Fig. 4B). Tm-1^{GCR237}-FLAG or tm-1^{GCR26}-FLAG mRNA (6 μ g) or WT ToMV RNA or LT1 RNA (3 μ g) was translated in mdBYL-based reaction mixture (80 μ l) at 23°C for 1 h. Mock translation was also performed as a control. The Tm-1^{GCR237}-FLAG-, tm-1^{GCR26}-FLAG-, or mock-translated reaction mixtures (25 μ l) were mixed with WT ToMV RNA-, LT1 RNA-, or mock-translated reaction mixtures (25 μ l), incubated at 23°C for 20 min, and 1 μ l of each

mixture was analyzed as an input sample. The remainder of each sample was diluted by 10 times with TR buffer and incubated with 20 μ l of anti-FLAG antibody-conjugated agarose beads (Sigma, St. Louis, MO; 50% slurry) at 4°C for 1 h. The beads were washed with 1 ml of TR buffer four times and then eluted in 40 μ l of TR buffer containing 100 ng/ μ l FLAG peptide (Sigma). The eluted protein samples (10 μ l) were analyzed by Western blotting to detect FLAG epitope and ToMV replication proteins. Anti-FLAG antibody was purchased from Sigma and the anti-130K antibody has been described (17).

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