Most viruses infect only a limited range of hosts. Adaptation of a virus to a novel host is a rare event, but when it does occur, it can result in the emergence of a new virus. A newly emerged virus often loses fitness in the original host. Such fitness trade-offs prevent continuous expansion of host ranges and explain why host ranges of viruses are limited. Even though viral mutations critical for adaptation to new hosts have been identified in many instances, little is known about how these mutations lead to the trade-offs (2, 4).

Tobacco mild green mosaic virus (TMGMV), a member of the genus Tobamovirus, infects a number of Nicotiana species but not tomato (20). The intracellular multiplication of TMGMV in tomato is prevented by the tm-1 protein, which binds to TMGMV replication proteins and inhibits RNA replication (11). We previously isolated a TMGMV mutant (TMGMV-T894M,F970Y) whose replication proteins have two amino acid substitutions (T894M and F970Y) and do not bind to tm-1 (11). TMGMV-T894M,F970Y was able to multiply in tomato protoplasts and caused systemic necrosis in tomato plants, although virus accumulation in systemic tissues was low (11) (Fig. 1A). Thus, by carrying the amino acid substitutions in the replication proteins, TMGMV-T894M,F970Y was able to escape from an inhibitory effect of tm-1, TMGMV expanded its host range.

Here, we examine whether TMGMV-T894M,F970Y lost fitness in its original host, Nicotiana benthamiana. In vitro transcripts from the infectious clone of wild-type (WT) TMGMV cDNA (J strain) (18), which was provided by Yasufumi Hikichi (Kochi University, Japan), and TMGMV-T894M,F970Y cDNA (11) were used for mechanical inoculation. Remarkably, TMGMV-T894M,F970Y did not produce obvious symptoms in N. benthamiana, in contrast to WT TMGMV (Fig. 1B). We then explored how TMGMV-T894M,F970Y lost its virulence in N. benthamiana. The accumulations of coat protein (CP) in inoculated leaves of N. benthamiana were comparable between WT TMGMV and TMGMV-T894M,F970Y (Fig. 2A), indicating that both RNA replication and cell-to-cell spread occurred normally. However, in upper uninoculated leaves, TMGMV-T894M,F970Y CP accumulated to lower levels than in WT TMGMV (to approximately 25% of the level in the WT at 7 days postinoculation [dpi]) (Fig. 2A). To further verify the spread of TMGMV-T894M,F970Y, we constructed WT and T894M,F970Y TMGMV derivatives in which the CP gene was replaced by the green fluorescent protein (GFP) gene. The GFP-coding region of pTL.G3 (14) was amplified by PCR using the primers 5'-CCCTATACATCTTTCTCGGTGTTGGTGTTAGTGTAG-3' and 5'-TGGGCCCACAACCCGGGGTTCGCG-3' and fused by overlap PCR with a TMGMV cDNA fragment that had been amplified using the primers 5'-CGCTGGGTGCATATCACGCCCCTGCGGGTGTTGGTATGAGT-3' and 5'-ACCAACAAATGATTGATAGAAGCATATTGACTAAAAAC-3'. The resulting fragment was cloned between the BspEI and BstEII sites of the full-length WT and T894M,F970Y TMGMV cDNA plasmids. When transcripts from these plasmids were inoculated onto N. benthamiana leaves, TMGMV-T894M,F970Y–GFP produced ring-shaped patterns of fluorescence, whereas WT TMGMV-GFP yielded a more uniform fluorescent signal (Fig. 2B).

The phenotype of TMGMV-T894M,F970Y in N. benthamiana plants (i.e., mild symptoms, reduced accumulation of CP in systemic tissues, and the formation of ring-shaped GFP foci) is similar to that of attenuated strains of plant viruses that are defective in suppression of RNA silencing, an antiviral host defense mechanism. The replication proteins of tobamoviruses are multifunctional proteins that play roles not only in viral RNA replication but also in the suppression of several kinds of host defense systems (12), including RNA silencing, and the attenuated strains of tobamoviruses carry mutations in these replication proteins (7, 9, 16, 19). Thus, we speculated that TMGMV-T894M,F970Y might be ineffective at suppressing RNA silencing. To test this possibility, we examined the ability of the 130K protein (one of two replication proteins of tobamoviruses ranging in mass from 122 to over 130 kDa) of WT TMGMV or TMGMV-T894M,F970Y to suppress RNA silencing by agroinfiltration in N. benthamiana leaves. Plasmids...
to express the 130K protein were constructed as follows: the open reading frames of the WT TMGMV and TMGMV-T894M,F970Y 130K proteins were amplified by PCR using the primers 5'-CCCGGTCAGAGATTTTACATATTTTC GACAAAC-3' and 5'-CCCGGAGCTTTACTATCTACCA CCGCTCTACC-3', digested with XbaI and SacI (restriction sites in the primers are underlined), and cloned into the binary vector pBI121. The resulting plasmids were named pBI-G130-WT and pBI-G130-T894MF970Y, respectively. A plasmid to express an inverted-repeat GFP RNA was also constructed. GAL4 cDNA was amplified by PCR using the primers 5'-ACCCGGGCTGCAGAAGCTACTGTCTTCTAT T-3' and 5'-ACCCGGGCTGCAGGTAGCGACACTCCCGGTCTAGAGTATGTTTTAATAGTTTGACACAAC-3', digested with PstI (restriction sites in the primers are underlined), and cloned into a pBluescript II SK+ derivative carrying GFP cDNA. The GFP-GAL4 fragment was inserted into the SacI site of pBI-erG3 (14), and the resulting plasmid was named pBI-erG3-IR. Agroinfiltration was performed essentially as described previously (14). Agrobacterium tumefaciens strains (C58C1/pGV2260) carrying pBI-erG3 (optical density at 600 nm [OD600] = 0.2) (14), pBI-erG3-IR (OD600 = 0.1), and any one of the pBI-G130-WT, pBI-G130-T894MF970Y, pBI-L130NRT (expressing the ToMV-L 130K protein as a positive control [14]), and pBI121 (expressing β-glucuronidase [GUS] as a negative control) (OD600 = 1)

**FIG. 1.** The TMGMV-T894M,F970Y mutant is scarcely virulent in *N. benthamiana*. Symptoms of WT TMGMV-inoculated and TMGMV-T894M,F970Y-inoculated tomato at 13 dpi (A) and *N. benthamiana* at 12 dpi (B).

**FIG. 2.** TMGMV-T894M,F970Y mutant replication proteins are unable to efficiently suppress RNA silencing. (A) Accumulation of WT TMGMV and TMGMV-T894M,F970Y CP in inoculated leaves and upper leaves (the second leaves above the inoculated leaves) of *N. benthamiana* were analyzed at 4 and 7 dpi, respectively, by SDS-PAGE and Coomassie brilliant blue (CBB) staining. Each lane represents an individual plant. (B) GFP-expressing TMGMV derivatives with WT- or TMGMV-T894M,F970Y-type replication proteins were inoculated onto an *N. benthamiana* leaf. GFP fluorescence of the inoculated leaf was observed at 5 dpi. Bar = 1 cm. (C) *A. tumefaciens* strains harboring plasmids that express GFP, GFP-inverted-repeat RNA, and the indicated proteins were coinfiltrated into an *N. benthamiana* leaf. GFP fluorescence was observed at 3 dpi. GUS and tomato mosaic virus (ToMV) 130K protein were used as negative and positive controls, respectively. (D) Protein and RNA accumulations in the *A. tumefaciens*-infiltrated *N. benthamiana* leaves at 3 dpi were analyzed by Western and Northern blotting, respectively. Anti-TMGMV replication protein rabbit antiserum was raised against an *Escherichia coli*-expressed fragment of the TMGMV 130K protein (amino acids 613 to 1111, reading from the N terminus). Anti-GFP antibody was purchased from Assay Designs, Inc. CBB-stained protein bands are shown as a loading control. 32P-labeled in vitro-transcribed RNA complementary to the GFP mRNA and an oligodeoxyribonucleotide complementary to the antisense strand of the GFP mRNA were used as probes for Northern blotting to detect GFP mRNA and siRNA, respectively. Methylene blue-stained rRNA bands are shown as a loading control. IR-GFP, inverted-repeat GFP. (E) Electrophoretic mobility shift assay of a 32P-labeled 22-nt RNA duplex incubated with an in vitro translation mixture of WT TMGMV or TMGMV-T894M,F970Y RNA (upper panel). The accumulation of replication proteins was analyzed by Western blotting (lower panel). ds, double-stranded.
plasmids were mixed and infiltrated into *N. benthamiana* leaves. Plants were grown at 25°C, and GFP fluorescence was observed at 3 dpi. Strikingly, GFP fluorescence was weaker in the area where the 130K protein of TMGMV-T894M,F970Y-expressing agrobacterium was infiltrated than in the area expressing the 130K protein of WT TMGMV (Fig. 2C). In TMGMV-T894M,F970Y 130K protein-expressing leaves, the levels of GFP mRNA and protein accumulation were lower than those in leaves expressing the WT TMGMV 130K protein (Fig. 2D). The accumulation level of the TMGMV-T894M,F970Y 130K protein was also lower than that for the WT 130K protein (Fig. 2D), probably because the expression of the 130K protein itself was also affected by RNA silencing. These results indicate that the 130K protein of TMGMV-T894M,F970Y is a weaker suppressor of RNA silencing than the WT counterpart.

The replication proteins of toboramiviruses bind small RNA duplexes, and this property is assumed to be important in RNA silencing suppression (1, 8, 15, 17). In toboramivirus-infected plants, some microRNAs (miRNAs) overaccumulate, possibly tk;4Because the replication proteins bind to the miRNAs and protect them from degradation (1, 15). The accumulation level of GFP small interfering RNA (siRNA) in WT TMGMV 130K protein-expressing *N. benthamiana* leaves was higher than those in GUS-expressing leaves (Fig. 2D), which might result from the protection of siRNAs by the 130K protein. We then examined the ability of WT and T894M,F970Y replication proteins to bind small RNA duplexes by electrophoretic mobility shift assay, essentially as described previously (10). Genomic RNAs of WT TMGMV and TMGMV-T894M,F970Y (3.25 μg) synthesized by *in vitro* transcription from the infectious cDNA clones were separately translated in 65 μl of the reaction mixture containing the extract of membrane-depleted evacuated BY-2 protoplasts (13) at 23°C for 1 h. Mock translation was performed as a control. The resulting mixtures were centrifuged at 100,000 × g for 30 min in a Beckman TLA-100.3 rotor, and supernatants were collected (S100). The S100 fraction (4 μl) was mixed with 0.5 μl of 125 nM[^32]P-labeled double-stranded small RNA (22 nucleotides [nt] with 2-nt overhangs), incubated at 23°C for 30 min, and analyzed by native PAGE followed by autoradiography. The replication proteins of TMGMV-T894M,F970Y bound small RNA duplexes less efficiently than did those of WT TMGMV (Fig. 2E). It was confirmed by Western blotting that the accumulation levels of the replication proteins in the S100 fractions were similar between the WT and mutant TMGMV (Fig. 2E, lower panel).

In this study, we found that TMGMV relinquished the ability to suppress RNA silencing through the binding of its replication proteins to small RNAs, which gave it the ability to replicate in tomato cells by escaping the inhibitory effect of tm-1. The observed fitness trade-off associated with host range expansion of TMGMV is attributable to the nonrobust nature of the genome encoding the multifunctional replication proteins. This indicates that the multifunctionality of the replication proteins can be an evolutionary constraint of toboramiviruses, which restricts their host ranges and explains in part the extremely low genetic diversity of toboramiviruses (6). Since the genomes of RNA viruses are small and often encode multifunctional proteins, similar scenarios may also apply to other viruses (3, 5).

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