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Isolation of a Mutant of *Arabidopsis thaliana* Carrying Two Simultaneous Mutations Affecting Tobacco Mosaic Virus Multiplication within a Single Cell

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Tobacco mosaic virus strain Cg (TMV-Cg) infects *A. thaliana* systemically. In order to identify host factors involved in the multiplication of TMV-Cg, we isolated a mutant of *A. thaliana* from an M2 population mutagenized by fast neutron irradiation, in which the accumulation of the coat protein in upper systemic leaves was reduced to low levels. The phenotype of the mutant, YS241, was controlled primarily by a single nuclear recessive mutation named *tom2-1*, which was distinct from *tom1*, a separate mutation which also affects TMV-Cg multiplication. The *tom2-1* mutation affected the accumulation of TMV-related RNAs in protoplasts in a tobamovirus-specific manner, suggesting that the wild-type *TOM2* gene product is necessary for efficient amplification of TMV-related RNAs within a single cell, through specific interaction with virus-coded factors. Furthermore, we found that YS241 contained a single dominant modifier named *ttm1*, which increased the efficiency of multiplication of TMV-Cg and a tomato strain of TMV in a *tom2-1* genetic background, both in plants and in protoplasts. We propose that the *ttm1* element might be a translocated form of the *TOM2* gene.

INTRODUCTION

Plant viruses multiply in their systemic hosts through three distinct processes i.e., multiplication within a single cell, cell-to-cell movement, and long-distance movement. Each of these processes is thought to occur through the concerted action of both virus- and host-coded factors. Therefore, to elucidate the molecular mechanisms of plant virus multiplication, it is important to reveal the function of such host factors, as well as the virus-coded factors. At present, however, little is known about the host factors necessary for plant virus multiplication, excepting the case of a few examples (e.g., Quadt *et al.,* 1993; Osman and Buck, 1997).

Tobacco mosaic virus (TMV) is a member of the alpha-like viruses of higher eukaryotes (Goldbach *et al.*, 1991; Dolja and Carrington, 1992). The TMV virion contains a nonsegmented single-stranded genomic RNA of approximately 6.4 kb. The genomic RNA is messenger-sense and encodes at least four proteins: 130k/180k replication proteins, a 30k movement protein, and the coat protein (CP) (for review, see Meshi *et al.,* 1992; Dawson, 1992). The 130k/180k replication proteins are both involved in genomic RNA replication and subgenomic RNA transcription *via* genome-length minus-strand RNA (Ishikawa *et al.,* 1986, 1991b), and have extensive amino acid sequence similarity with replication proteins encoded by the genomes of alpha-like viruses including cucumber mosaic virus (CMV) and turnip yellow mosaic virus (TYMV) (Goldbach *et al.*, 1991; Dolja and Carrington, 1992). The 30k movement protein is involved in viral cell-to-cell and long-distance movement, and the CP is involved in viral long-distance movement (Deom *et al.,* 1987; Meshi *et al.*, 1987; Saito *et al.*, 1989; Arce-Johnson *et al.*, 1997). The simple genome organization, the high efficiency of multiplication, and the abundant knowledge available concerning the functions associated with the TMV genome make it an excellent model for investigation of the mechanisms of plant RNA virus multiplication.

We have been focusing on the identification of host factors involved in TMV multiplication, using a genetic approach with *Arabidopsis thaliana,* a model plant having advantages for molecular genetic studies (Meyerowits, 1989). The Cg strain of TMV (TMV-Cg) (Yamanaka *et al.,* 1998) systemically multiplies to high levels in the wild-type ecotype Columbia (Col-0) of *A. thaliana,* without inducing any detectable hypersensitive reactions (Ishikawa *et al.,* 1991c). Therefore, the complete set of host factors necessary for the efficient multiplication of TMV-Cg is present in the plant. If a mutation hits the host factor gene, causing it to lose its function in supporting TMV multiplication, TMV multiplication in the plant will be reduced. Previously, we screened ethyl methanesulfonate (EMS)-mutagenized *A. thaliana* (ecotype Col-0) and isolated two mutants, PD114 and PD378, in which the accumulation of TMV CP was reduced to low levels (Ishikawa *et al.,* 1991c). The phenotype of these two mutants was controlled by a single, recessive nuclear mutation in the same locus, named *tom1* (Ishikawa *et al.,*

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1991c). Inoculation of *tom1* protoplasts with TMV-Cg RNA by electroporation showed that the *tom1* mutation affected TMV multiplication within a single cell (Ishikawa *et al.,* 1993). The most simple interpretation of these observations is that the wild-type *TOM1* gene product is necessary for efficient TMV multiplication within a single cell.

In order to identify mutations other than *tom1* that cause reduced TMV multiplication, we screened *A. thaliana* plants mutagenized using a different mutagenic method, fast neutron irradiation, since EMS treatment and fast neutron irradiation are known to induce mutations at different rates between individual loci (Koorneef *et al.*, 1982). Furthermore, most mutants obtained by fast neutron irradiation contain chromosomal deletions or rearrangements (Shirley *et al.,* 1992; Nambara *et al.,* 1994; Bruggemann *et al.,* 1996), which are advantageous in the identification of the mutated genes (Sun *et al.*, 1992; Lisitsyn *et al.,* 1993). Here, we report the isolation of an *A. thaliana* mutant generated by fast neutron irradiation, in which TMV-Cg multiplication in a single cell is abrogated, and the genetic identification of the major recessive causal mutation named *tom2-1* and a dominant modifier named *ttm1*. We propose a hypothesis that *ttm1* is a translocated form of the *TOM2* gene.

RESULTS

Isolation of YS241, a mutant of *A. thaliana* in which the accumulation of TMV coat protein is reduced to low levels in a fast neutron-mutagenized M2 population

In order to identify host factors involved in the multiplication of TMV, we screened fast neutron-mutagenized M2 plants of *A. thaliana* for mutants in which the accumulation of TMV-Cg CP in uninoculated leaves of mechanically inoculated plants was reduced to low levels. The parental strain used for mutagenesis was ecotype Columbia with a trichomeless *gl1* mutation (we designate this strain simply as "gl1" hereafter). The *gl1* mutation did not detectably affect TMV CP accumulation. Like Col-0 plants, TMV-Cg-infected gl1 plants showed disease symptoms, such as mild stunting, earlier development of senescence in rosette leaves, curving of flower stalks, and reduced fertility compared to mock-inoculated plants (data not shown).

Screening was carried out as described previously (Ishikawa *et al.,* 1991c). Briefly, an upper uninoculated rosette leaf of each TMV-Cg-inoculated M2 plant was harvested at 8–11 days postinoculation (d.p.i.) and the accumulation of TMV-Cg CP was examined by dot ELISA (Ishikawa *et al.,* 1991c). M2 plants in which the accumulation of the CP was low or undetectable were allowed to self-pollinate, and the phenotype was checked again in the M3 generation. Among approximately 5000 fast neutron-mutagenized M2 plants examined, one line designated YS241 consistently showed reduced TMV-Cg CP accumulation in the M3 generation. YS241 bred true with respect to the phenotype until at least the M6 generation.

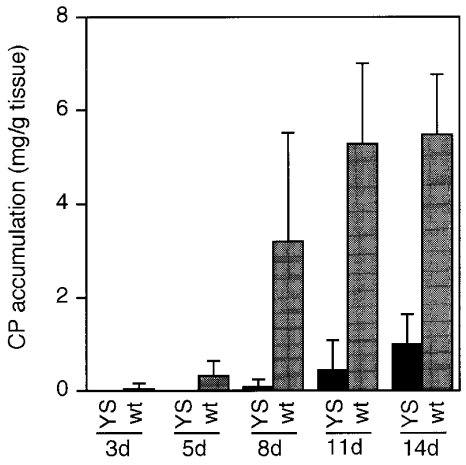

FIG. 1. Time course of TMV-Cg coat protein accumulation in YS241 and its parental gl1 plants. YS241 (M4 generation; indicated by "YS") and gl1 (wild type; indicated by "wt") plants were inoculated with TMV-Cg, upper uninoculated rosette leaves were harvested at 3, 5, 8, 11, and 14 d.p.i., and the accumulation levels of the CP for each plant were determined as described under Materials and Methods. Boxes and error bars show averages and standard deviations, respectively, of 14 measurements of TMV-Cg CP accumulation.

The time course of TMV-Cg CP accumulation in gl1 plants and direct descendant plants of YS241 (M4 generation) is shown in Fig. 1. In gl1 plants, the accumulation of TMV-Cg CP was detectable at 3 d.p.i. and reached a level of approximately 5.5 mg per g tissue around 14 d.p.i. In YS241 plants, the accumulation of TMV-Cg CP was delayed compared to that in the wild-type gl1 plants, and at 14 d.p.i., the concentration of TMV-Cg CP in upper uninoculated leaves of YS241 plants was approximately one-fifth of that in gl1 plants. In TMV-Cg-infected YS241 plants, disease symptoms were milder than those shown by the gl1 plants or undetectable (data not shown).

The phenotype of reduced accumulation of TMV-Cg coat protein in YS241 is controlled primarily by a single, recessive nuclear mutation named *tom2*

In order to determine the genetic basis of the phenotype of reduced accumulation of TMV-Cg CP, crosses were made between YS241 and the wild-type gl1 plants, and TMV-Cg CP accumulation in F1 plants was examined. The F1 plants inoculated with TMV-Cg showed disease symptoms similar to those shown by gl1 plants infected with the virus (data not shown), and the levels of accumulation of TMV-Cg CP were similar to those observed in gl1 plants (Fig. 2; compare YS × gl1 and gl1 × YS with YS and gl1). Among the F2 plants that were generated by self-pollination of the F1 plants, about one-fourth of the total plants exhibited the phenotype of reduced TMV-Cg CP accumulation similar to or even lower than in YS241 itself (see below); and the others showed wild-type levels of CP accumulation (Table 1).

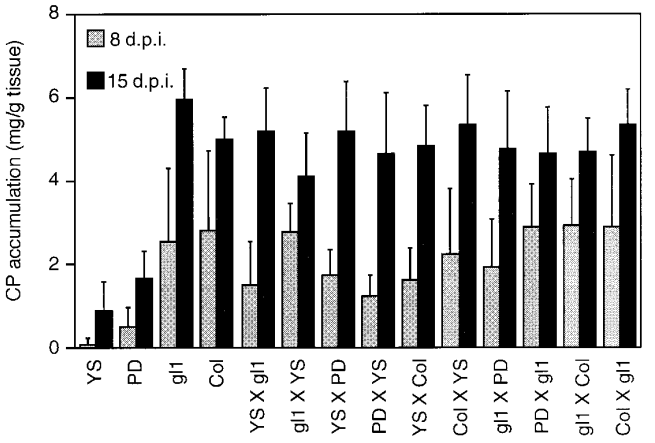

FIG. 2. Accumulation of TMV-Cg coat protein in F1 plants. F1 plants derived from the crosses (female × male) of [YS241 (M5 generation; indicated by "YS") × gl1], [gl1 × YS241 (M5)], [YS241 (M5) × PD114 (indicated by "PD")], [PD114 × YS241 (M5)], [YS241 (M5) × Col-0 (indicated by "Col")], [Col-0 × YS241 (M5)], [gl1 × PD114], [PD114 × gl1], [gl1 × Col-0], and [Col-0 × gl1] and plants obtained by self-pollination of YS241 (M5), PD114, gl1, and Col-0 were inoculated with TMV-Cg. Upper uninoculated leaves of the inoculated plants were harvested at 8 or 15 d.p.i. The accumulation levels of the CP were determined for each plant as described under Materials and Methods. Boxes and error bars show averages and standard deviations, respectively, of 10 measurements of TMV-Cg CP accumulation. Note that YS241 was derived from gl1 and PD114 from Col-0.

Next, TMV-Cg CP accumulation was examined in test cross plants, generated by a cross between F1 plants of [gl1 (female) × YS241 (male)] (female) and YS241 plants (male). Among them, half showed reduced accumulation of TMV-Cg CP, and the other half showed CP accumulation similar to that observed in the wild-type gl1 plants (Table 1). These results suggest that the phenotype of reduced accumulation of TMV-Cg CP is controlled by a single, recessive, nuclear mutation.

To examine allelism of the mutation controlling the phenotype of reduced TMV-Cg CP accumulation in YS241 with the previously identified *tom1* mutation, crosses were made between YS241 and PD114 (*tom1-1*) plants. The F1 plants showed TMV-Cg CP accumulation greater than that observed in YS241 and PD114 plants and similar to that in the wild-type gl1 (the parent of YS241), Col-0 (the parent of PD114), and F1 plants generated by the crosses between YS241 and Col-0, PD114 and gl1, or gl1 and Col-0 (Fig. 2). Because both *tom1-1* and the causal mutation of YS241 are recessive, this result suggests that the causal mutation of YS241 is distinct from *tom1*. We named the mutation controlling the phenotype of YS241 *tom2-1*. Direct descendants of YS241 (to the M6 generation) grew more slowly and had narrow and distorted leaves compared to the wild-type gl1 plants (data not shown). However, these phenotypes segregated independently from the phenotype of reduced accumulation of TMV-Cg CP.

To determine the map location of the *tom1-1* and *tom2-1* loci, crosses were made between wild-ype ecotype La-0 plants (in which TMV-Cg multiplies as vigorously as in Col-0 and gl1 plants) and PD114 (*tom1-1*, derived from Col-0; Ishikawa *et al.,* 1991c) or between La-0 and YS241 (*tom2-1*, derived from gl1), and F2 lines were established (An F2 line is a batch of F3 seeds or plants obtained by self-pollination of a single F2 individual). Among the F2 lines derived from either the *tom1-1* or the *tom2-1* mutant, approximately one-fourth consistently showed reduced TMV-Cg CP accumulation in the F3 generation. Using these F2 lines, linkage between *tom1-1* or *tom2-1* loci and restriction fragment length polymorphisms (RFLP) markers was examined. The *tom1-1* locus showed linkage only with the markers located in the bottom region of chromosome 4 and is suggested to be located between m557 (Chang *et al.,* 1988; 13 recombinants / 130 chromatids) and m600 (Hauge *et al.,* 1993; 7 recombinants / 130 chromatids). The *tom2-1* locus showed linkage only with the markers

TABLE 1

Genetic Segregation of the Phenotype of Low-Level Accumulation of TMV-Cg Coat Protein

Cross (female × male)	Type	Total	Number of plants in which TMV-Cg CP accumulated to		χ^{2b}
			High level[a]	Low level[a]	
(YS241[c] × gl1)/self	F2	103	75	28	0.26[d]
(gl1 × YS241[c])/self	F2	118	88	30	0.01[d]
(gl1 × YS241[c]) × YS241[c]	Test cross	104	52	52	0.00[d]

[a] The phenotype was determined as follows: Plants were inoculated with TMV-Cg, and upper uninoculated leaves were harvested 11 days after inoculation. The accumulation of the CP was examined by SDS–PAGE and staining with Coomassie brilliant blue dye. Plants in which TMV-Cg CP accumulated to levels similar to those in the wild-type gl1 plants were counted as "high-level," and plants in which the CP accumulated to levels similar to or lower than those in YS241 plants were counted as "low level."

[b] The χ^2 values were calculated on the basis of the expected ratio of high-level: low-level 3:1 and high-level:low-level 1:1 for the F2 and test cross plants, respectively.

[c] YS241 (M3) plants were used for the crosses.

[d] $P > 0.05$.

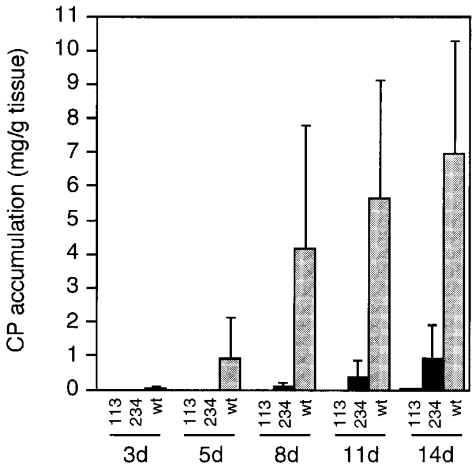

FIG. 3. Time course of TMV-Cg coat protein accumulation in B1-113, B1-234, and gl1 plants. B1-113 (indicated by "113"), B1-234 (indicated by "234"), and gl1 (wild type; indicated by "wt") plants were inoculated with TMV-Cg, upper uninoculated rosette leaves were harvested at 3, 5, 8, 11, and 14 d.p.i., and the accumulation levels of the CP for each plant were determined as described under Materials and Methods. Boxes and error bars show averages and standard deviations, respectively, of 10 measurements of TMV-Cg CP accumulation.

located in the central region of chromosome 1 and is suggested to be located between m253 (Chang *et al.,* 1988; 1 recombinant / 76 chromatids) and m299 (Chang *et al.,* 1988; 6 recombinants / 76 chromatids).

YS241 contains a dominant modifier, which increases the accumulation of TMV coat protein in a *tom2-1* genetic background

We found that among the F2 lines that were generated by the crosses between YS241 (M3) and the wild-type gl1 plants and which were homozygous for the *tom2-1* mutation, most lines showed similar levels of TMV-Cg CP accumulation (approximately 0.5 mg/g tissue at 11 d.p.i. in more than half of the inoculated F3 plants), but some lines (3 lines / 21 *tom2-1* lines) consistently showed lower apparent levels of TMV-Cg CP accumulation in the F3 generation. The F2 lines B1-234 and B1-113 were representatives of the former and the latter, respectively. Each line bred true with respect to the phenotype of TMV-Cg CP accumulation, looked normal in size of plants and shape of leaves, and did not segregate progenies with abnormal morphology. The time course of TMV-Cg CP accumulation in these two lines is shown in Fig. 3. In B1-234 descendants, TMV-Cg CP was detectable at 8 d.p.i., and 7 of 10 inoculated plants accumulated detectable levels of TMV-Cg CP at 14 d.p.i. The average TMV-Cg CP accumulation in these 10 B1-234 descendants at 14 d.p.i. was approximately 1.0 mg / g tissue: similar to the level of accumulation in YS241 (M4 generation) plants (Fig. 1). In B1-113 descendants, only 2 of 10 inoculated plants showed detectable levels of TMV-Cg CP accumulation at 14 d.p.i., and TMV-Cg CP accumulation was less than 0.1 mg / g tissue in these two plants.

When TMV-L, a tomato strain of TMV (Ohno *et al.,* 1984) which multiplies less efficiently in *A. thaliana* plants than TMV-Cg (Ishikawa *et al.* 1991c), was used as inoculum, the accumulation of TMV-L CP in B1-234 descendants and YS241 direct descendants was similar to that in the wild-type gl1 plants (Fig. 4; and data not shown); TMV-L CP was not detected in B1-113 descendants even at 21 d.p.i. (Fig. 4). YS241 direct descendants B1-234 and B1-113 bred true with respect to the phenotype for TMV-L CP accumulation. Among the 21 *tom2-1* F2 lines derived from the crosses between YS241 (M3) and gl1, all 3 lines showing undetectable TMV-L CP accumulation also consistently showed undetectable or very low levels of TMV-Cg CP accumulation consistently, and vice versa, suggesting that these two phenotypes are controlled by the same genetic element.

To explore the nature of the difference in TMV-L CP accumulation between B1-113 and B1-234, crosses were made between these two lines. In the F1 plants, the levels of accumulation of TMV-L CP examined by dot ELISA and SDS–PAGE at 21 d.p.i. were similar to those seen in the wild-type gl1 and B1-234 plants (Fig. 4, and data not shown). Among the F2 plants, approximately one-fourth showed low levels of TMV-L CP accumulation similar to those in B1-113, and the other plants accumulated TMV-L CP to wild-type levels (Table 2). Considering the 3:1 segregation, together with the result of the F1 analysis, it is suggested that the increase in TMV-L CP accumulation in the *tom2-1* genetic background found in B1-234 was controlled by a single, dominant nuclear

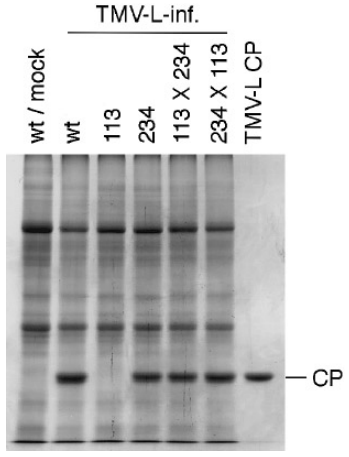

FIG. 4. Accumulation of TMV-L coat protein in B1-113, B1-234, and their F1 plants. B1-113 (indicated by "113"), B1-234 (indicated by "234"), and gl1 (wild-type; indicated by "wt") plants and F1 plants derived from the crosses (indicated as [female × male]) between B1-113 and B1-234 (five individuals for each kind of plant line) were inoculated with TMV-L, and upper uninoculated rosette leaves were harvested at 21 d.p.i. Total protein extract was separately prepared from each plant, equal volumes of the extracts were mixed, and the samples were analyzed by SDS–11% (containing 0.55% bisacrylamide) PAGE. The gel was stained with Coomassie brilliant blue dye, destained, and photographed. A protein sample from a mock-inoculated gl1 plant and purified TMV-L CP were concurrently analyzed. The position of the TMV-L CP (17.5 kDa) is indicated at the right.

TABLE 2

Genetic Segregation of the Phenotype of Low-Level Accumulation of TMV-L Coat Protein in the F2 Generation

Cross (female × male)	Total	Number of plants in which TMV-L CP accumulated to		χ^{2b}
		High level[a]	Low level[a]	
(B1-113 × B1-234)/self	49	34	15	0.82[c]
(B1-234 × B1-113)/self	49	38	11	0.17[c]

[a] The phenotype was determined as follows: Plants were inoculated with TMV-L, and upper uninoculated leaves were harvested 21 days after inoculation. The accumulation of the CP was examined by dot ELISA. Plants in which TMV-L CP accumulated to levels similar to those in B1-234 plants were counted as "high-level," and plants in which the CP accumulated to levels similar to those in B1-113 plants were counted as "low-level."

[b] The χ^2 values were calculated on the basis of the expected ratio of high-level:low-level 3:1.

[c] $P > 0.05$.

genetic element, originally carried by YS241. There was also a minor possibility that the reduction in TMV-L CP accumulation in the *tom2-1* genetic background found in B1-113 was controlled by a single, recessive nuclear genetic element, originally carried by the specific gl1 plant used for the cross with YS241.

To test these two possibilities, *tom2-1* plants (identified by TMV-Cg inoculation) were selected from the F2 plants derived from the crosses between gl1 and B1-113 or gl1 and YS241 direct descendants (M5 genetation), seeds were harvested separately from each F2 individual, and TMV-L CP accumulation was examined in several F3 plants for each F2 line. All 12 *tom2-1* lines generated by the cross between gl1 and B1-113 showed undetectable TMV-L CP accumulation in all F3 plants, similar to B1-113 itself. In contrast, among 31 *tom2-1* F2 lines generated by the crosses between gl1 and YS241 direct descendants (M5), 2 lines consistently showed low levels of TMV-L CP accumulation, similar to B1-113, and the remaining lines showed wild-type levels of TMV-L CP accumulation. These results are consistent with the hypothesis that the increase in TMV-L CP accumulation in the *tom2-1* genetic background found in B1-234 and direct descendants of YS241 compared to the accumulation in B1-113 is controlled by a single, dominant nuclear genetic element. We named this genetic element *ttm1* (tom-two mutation modifier). As described above, among 21 *tom2-1* F2 lines generated by the cross between YS241 direct descendants (M3) and gl1, 3 lines showed phenotype similar to B1-113. Therefore, in total, among 52 *tom2-1* F2 lines generated by the cross between YS241 direct descendants and gl1, 5 lines showed a phenotype similar to B1-113 (χ^2 [3:1] = 6.56, $P < 2.5$ %). Thus, *ttm1* is likely to be linked to *tom2-1*.

Because direct descendants of YS241 are supposed to carry the *ttm1* element homozygously in addition to the *tom2-1* mutation, we examined TMV-Cg multiplication in F1 plants generated by the crosses between B1-113 and gl1 or PD114 plants in order to determine the effect of the *tom2-1* mutation in the absence of the *ttm1* element. In F1 plants from both crosses, the accumulation of TMV-Cg CP was similar to or slightly lower than that observed in the wild-type gl1 plants (data not shown). These results confirm the previous conclusion that the *tom2-1* mutation is recessive and distinct from the *tom1* mutation.

Effects of the *tom2-1* mutation on the multiplication of viruses other than TMV

We examined whether the *tom2-1* mutation affects multiplication of turnip crinkle virus (TCV), turnip yellow mosaic virus (TYMV), or cucumber mosaic virus (CMV), which all belong to taxonomic groups other than tobamovirus. These three viruses systemically multiply in wild-type *A. thaliana* ecotype Columbia with or without the *gl1* mutation (Ishikawa *et al*., 1991c; and our unpublished results). B1-113 (*tom2-1*) plants were inoculated with these viruses, and respective CP accumulation in upper systemic leaves was examined by SDS–PAGE at 14 d.p.i. for TCV, 21 d.p.i. for TYMV, or 8 d.p.i. for CMV. In all cases, CP accumulation was similar to that observed in gl1 (*TOM2*) plants (data not shown). Similar results were obtained with the direct descendants of YS241 or B1-234 (*tom2-1 ttm1*).

Effects of the *tom2-1* mutation on the multiplication of TMV in protoplasts

In order to determine whether the *tom2-1* mutation affects the multiplication of TMV and other viruses within a single cell, B1-113 (*tom2-1*) and gl1 (*TOM2*) protoplasts were inoculated with viral RNAs by electroporation, and the percentage of infected (CP-positive) cells, as well as the accumulation of CPs and virus-related RNAs, was measured. To assess the quality of protoplasts, we first examined infection with TCV and CMV RNAs, which are expected to multiply at similar efficiencies in B1-113 and gl1 protoplasts. For both viruses, the percentage of infected (CP-positive) protoplasts and the accumulation of CPs and virus-related RNAs in B1-113 (*tom2-1*) protoplasts were similar to those observed in gl1 (*TOM2*) protoplasts (seven and four independent experiments for CMV and TCV, respectively). An example for CMV infection is shown in Fig. 5e (in the figure, the accumulation of CMV-related RNAs in B1-113 protoplasts was slightly less than that in gl1 protoplasts, but the difference was not consistent throughout repeated experiments).

B1-113 (*tom2-1*) and gl1 (*TOM2*) protoplasts (1×10^6 protoplasts each) were inoculated with 20 μg of TMV-Cg or TMV-L virion RNA and cultured for 42 h, and the respective CP accumulation was examined *in situ* by indirect immunofluorescence staining, using anti-TMV-Cg or anti-TMV-L antibodies (seven and three inde-

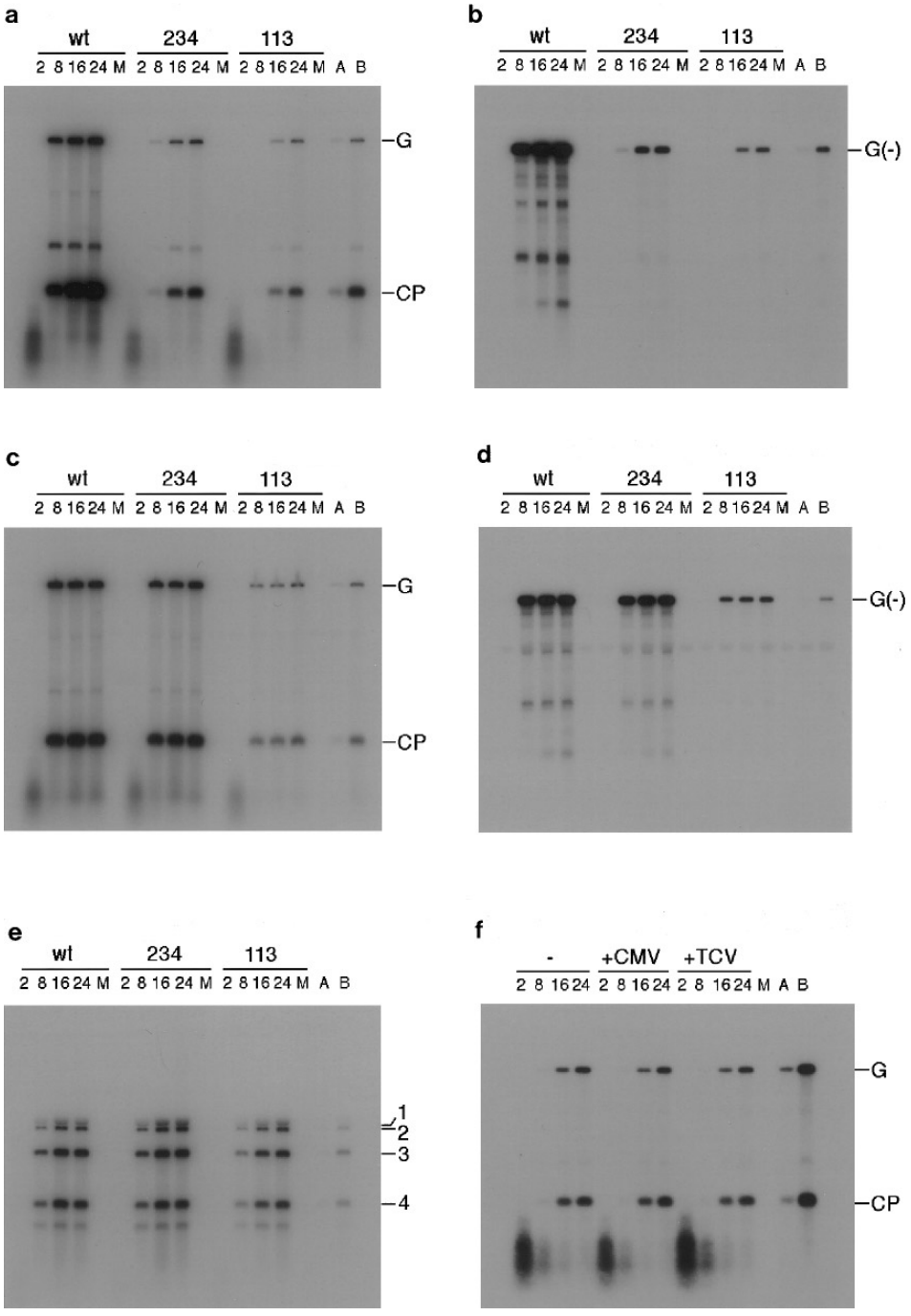

FIG. 5. Accumulation of virus-related RNAs in protoplasts derived from gl1 (*TOM2*; indicated by "wt"), B1-234 (*tom2-1 ttm1*; indicated by "234"), and B1-113 (*tom2-1*; indicated by "113"). Protoplasts were prepared from asceptic calli and inoculated with TMV-Cg virion RNA (a and b), TMV-L virion RNA (c and d), CMV virion RNA (e), or TMV-Cg virion RNA with or without CMV or TCV virion RNAs (f) by electroporation. Total RNA was extracted at 2, 8, 16, or 24 h.p.i., purified, and analyzed by Northern blot hybridization. Cell types and sampling times (h.p.i.) are indicated above the panels. (f) B1-113 protoplasts were used except for lanes indicated by "A" and "B." In lanes indicated by "A" and "B," RNA samples (24 h.p.i.) from gl1 protoplasts inoculated with TMV-Cg virion RNA (a, b and f), with TMV-L virion RNA (c and d), or with CMV virion RNA (e), which were diluted 1:100 and 1:10, respectively, with RNA from mock-inoculated gl1 protoplasts (24 h.p.i.), were applied. In lanes indicated by "M", RNA from mock-inoculated protoplasts (24 h.p.i.) was applied. To detect plus-strand RNAs of TMV-Cg in (a) and (f), a ^{32}P-labeled RNA probe synthesized by SP6 RNA polymerase from *Eco*RI-digested pCgP2 (Ishikawa *et al.,* 1993) was used. To detect minus-strand RNAs of TMV-Cg in (b), a ^{32}P-labeled RNA probe synthesized by SP6 RNA polymerase from *Eco*RI-digested pCgP1 (Ishikawa *et al.,* 1993) was used. To detect plus-strand RNAs of TMV-L in (c), a ^{32}P-labeled RNA probe synthesized by T3 RNA polymerase from *Hin*dIII-digested pT732 (Ishikawa *et al.,* 1991a) was used. To detect minus-strand RNAs of TMV-L in (d), a ^{32}P-labeled RNA probe synthesized by SP6 RNA polymerase from *Eco*RI-digested pLP2P (Ishikawa *et al.,* 1991b) was used. To detect plus-strand RNAs of CMV in (e), a ^{32}P-labeled RNA probe synthesized by T7 RNA polymerase from *Xho*I-digested pCYU3 (Ishikawa *et al.,* 1993) was used. The positions of bands corresponding to the genomic (indicated by "G") and CP subgenomic (indicated by "CP") RNAs and genome-sized minus-strand RNA (indicated by "G(−)") of TMV, and CMV RNAs 1, 2, 3, and 4 (indicated by "1", "2", "3," and "4," respectively) are shown at the right of each panel.

pendent experiments for TMV-Cg and TMV-L, respectively). In gl1 (*TOM2*) protoplasts, the intensity of fluorescence in TMV-CP-positive protoplasts was strong and uniform between protoplasts for each virus. In contrast, in B1-113 (*tom2-1*) protoplasts, the intensity of fluorescence in TMV-CP-positive protoplasts was weak and some signals were close to background levels. These results suggest that B1-113 (*tom2-1*) protoplasts that were successfully infected with TMV-Cg accumulated less TMV-Cg CP than gl1 (*TOM2*) protoplasts, even at 42 h postinoculation (h.p.i.).

As a more detailed analysis, we examined the time course of accumulation of TMV-related RNAs in protoplasts (eight and three independent experiments for TMV-Cg and TMV-L, respectively). Figure 5 shows the accumulation of the TMV-Cg-related plus-strand (a) and minus-strand (b) RNAs, and TMV-L-related plus-strand (c) and minus-strand (d) RNAs in gl1 (*TOM2*) and B1-113 (*tom2-1*) protoplasts. The accumulation of TMV-Cg-related RNAs in B1-113 (*tom2-1*) protoplasts was approximately one-tenth (or less, in most experiments) of that in gl1 (*TOM2*) protoplasts at 24 h.p.i. [Compare the lane for 24 h.p.i. B1-113 RNA with that for 24 h.p.i. gl1 RNA diluted 1:10 (the lanes marked by "B") in Figs. 5a and 5b]. The relative signal strength of subgenomic mRNA for the CP and genome-sized minus-strand RNA compared to that of genomic RNA of TMV-Cg in B1-113 (*tom2-1*) protoplasts was similar to that in gl1 (*TOM2*) protoplasts. In protoplasts inoculated with TMV-L virion RNA, the accumulation of plus-strand (genomic and CP subgenomic) RNAs at 24 h.p.i. was 5- to 10-fold lower in B1-113 (*tom2-1*) protoplasts than in gl1 (*TOM2*) protoplasts (Fig. 5c). The accumulation of genome-sized minus-strand RNA of TMV-L in B1-113 (*tom2-1*) protoplasts was only 3-fold lower than that in gl1 (*TOM2*) protoplasts (Fig. 5d).

When B1-113 (*tom2-1*) and gl1 (*TOM2*) protoplasts were inoculated with a mixture of TMV-Cg and TCV RNAs, or a mixture of TMV-Cg and CMV RNAs, the accumulation of RNAs related to each virus was similar to that found for single inoculations with each virus (Fig. 5f and data not shown; two and three independent experiments for CMV and TCV, respectively). This refutes the possibility that the observed differences in accumulation of TMV-Cg-related RNAs and CP were due to technical error during protoplast preparation or electroporation. From these observations, it was confirmed that the *tom2-1* mutation affected the accumulation of TMV-Cg-related RNAs within a single cell.

Next, we examined the effect of the *ttm1* element on the multiplication of TMV-Cg and TMV-L in protoplasts. The accumulation of CMV-related RNAs in B1-234 (*tom2-1 ttm1*) protoplasts was similar to that seen in gl1 (*TOM2*) and B1-113 (*tom2-1*) protoplasts (see Fig. 5e), confirming that the quality of the B1-234 protoplasts was as good as that of gl1 or B1-113. When inoculated with TMV-Cg virion RNA, the accumulation of TMV-Cg-related plus- and minus-strand RNAs in B1-234 (*tom2-1 ttm1*) protoplasts was significantly lower than in gl1 (*TOM2*) protoplasts, but slightly higher than in B1-113 (*tom2-1*) protoplasts (Figs. 5a and 5b). On the other hand, the accumulation of TMV-L-related plus- and minus-strand RNAs in B1-234 (*tom2-1 ttm1*) protoplasts was similar to or only slightly lower than in gl1 (*TOM2*) protoplasts and significantly higher than in B1-113 (*tom2-1*) protoplasts. Consistent results were obtained by immunofluorescence staining of TMV-Cg- or TMV-L-inoculated protoplasts. These observations paralleled the plant inoculation results.

DISCUSSION

In order to identify host factors involved in the multiplication of TMV, we screened for mutants of *A. thaliana* in which TMV multiplication was affected. In this study, a mutant named YS241, in which the accumulation of TMV-Cg CP in uninoculated leaves of infected plants was reduced to low levels, was isolated from a fast neutron-mutagenized M2 population. It carried one major recessive nuclear mutation named *tom2-1*, which was distinct from a previously identified *tom1* mutation (Ishikawa *et al.,* 1991c). The recessive nature of the *tom2-1* mutation suggests that the wild-type *TOM2* gene product is necessary for the efficient multiplication of TMV, and that the *tom2-1* allele has lost its function in supporting efficient TMV multiplication. The *tom2-1* mutation affected TMV-Cg and TMV-L multiplication, but did not detectably affect the multiplication of TCV, CMV and TYMV. These observations imply that there are specific interactions between the *TOM2* gene product and one or more of the TMV-encoded molecules involved in the multiplication of the virus, although it is not known how directly they interact with each other.

In *tom2-1* protoplasts, the accumulation levels of TMV-related RNAs and the CP were lower than those in wild-type protoplasts. This observation suggests that the *tom2-1* mutation affects TMV multiplication within a single cell, including expression of the viral replication proteins, replication of the viral RNA, and the stability of TMV-related RNAs or proteins. The accumulation of genomic, CP subgenomic, and minus-strand RNAs of TMV-Cg was reduced to a similar extent in *tom2-1* protoplasts compared to that in wild-type protoplasts. For TMV-L, the accumulation of minus-strand RNA was less affected by the *tom2-1* mutation than accumulation of the plus-strand RNAs. It is difficult, however, to ascertain whether this difference in the pattern of TMV-L RNA accumulation is sufficiently large as to suggest that the *TOM2* gene product is involved mainly in plus-strand accumulation of TMV-L, since we do not know what determines the extent of accumulation of the plus- and minus-strand RNAs. In *tom2-1* protoplasts, the level of TMV-Cg-related RNA accumulation was approximately one-tenth of that in wild-type protoplasts at 24 h.p.i. (Fig. 5a and b), while in *tom2-1* plants, the level of TMV-Cg CP

accumulation was much less than one-tenth of that in wild-type plants at 14 d.p.i. (Fig. 3). In whole plants, the accumulation of viral CP is influenced by many factors, such as the efficiency of virus multiplication within a single cell, cell-to-cell and long-distance movement of the virus, and plant defense functions which may be activated in the course of viral multiplication. At present, the exact factor(s) responsible for this difference in TMV-Cg accumulation between *tom2-1* protoplasts and plants remains unclear.

A previously isolated *tom1-1* mutant has characteristics similar to the *tom2-1* mutant: *tom1-1* is a recessive mutation affecting TMV multiplication within a single cell and is specific to TMV multiplication. However, the two mutants differ as follows: (i) With respect to the effect on TMV multiplication, the *tom2-1* mutation is more severe than the *tom1-1* mutation in plants (compare the B1-113 data in Fig. 3 of this paper with the PD114 data in Fig. 1 of Ishikawa *et al.,* 1991c). (ii) In *tom2-1* protoplasts, the signal strength of immunofluorescence of TMV CP was weaker than that in wild-type protoplasts even at 42 h.p.i. In contrast, in *tom1-1* protoplasts, the signal strength of immunofluorescence of TMV-Cg CP was similar to that in wild-type protoplasts at 42 h.p.i., although the percentage of CP-positive cells was lower in the *tom1-1* protoplasts (Ishikawa *et al.,* 1993). (iii) When *tom2-1* protoplasts were inoculated with a mixture of TMV-Cg and CMV RNAs, both viruses multiplied to a similar extent to independent inoculation with each viral RNA. In *tom1-1* protoplasts, TMV-Cg multiplication was further reduced by co-inoculation with CMV RNA (Ishikawa *et al.,* 1993). The present results with *tom2-1* protoplasts suggest that the previously observed reduction in the accumulation of TMV-related molecules, caused by the co-inoculation of CMV in *tom1-1* protoplasts, was not caused by nonspecific effects of the vigorous multiplication of CMV, but by some specific effects of CMV multiplication, e.g., competition of *TOM1*-like factors that support TMV multiplication between TMV and CMV replication machineries in *tom1-1* mutant cells, as discussed by Ishikawa *et al.* (1993).

Many other genetic traits that confer virus resistance to *A. thaliana* plants have been reported (Callaway *et al.,* 1996;Dempsey *et al.,* 1997; Ishikawa *et al.,* 1991c; Lee *et al.,* 1996; Lee *et al.,* 1994; Takahashi *et al.,* 1994; Yoshii *et al*., 1998; for reviews, see Simon, 1994, and Kunkel, 1996). Among them, *tom1-1* and *tom2-1* are the only mutations that are recessive and affect RNA virus multiplication within a single cell. Characterization of the *TOM1* and *TOM2* genes will provide us with unique and important information about the multiplication of RNA viruses.

The origin of the *ttm1* element

The mutant YS241 carried two mutations simultaneously: one major recessive mutation, *tom2-1*, which reduces the efficiency of TMV multiplication within a single cell, and one dominant modifier, *ttm1*, which increases the efficiency of TMV-L multiplication within a single cell in the presence of the *tom2-1* mutation. Furthermore, these two mutations were weakly linked to each other. The probability of two independent mutations, both affecting TMV multiplication, occurring in a single M2 individual must be extremely low, as mutants with abnormal TMV multiplication have been isolated only rarely. By screening approximately 5000 fast neutron-mutagenized M2 plants, we isolated only one such mutant, YS241, and by screening approximately 8000 EMS-mutagenized M2 plants, we isolated two such mutants. Therefore, it seems unlikely that the *tom2-1* and *ttm1* mutations occurred independently.

Fast neutron irradiation is reported to induce chromosomal deletions at high frequency (Shirley *et al.,* 1992; Sun *et al.,* 1992; Nambara *et al.,* 1994; Bruggemann *et al.,* 1996), and in some cases, inversions and translocations (Shirley *et al.,* 1992; Bruggemann *et al.,* 1996). Shirley *et al.* (1992) analyzed the *tt5* mutant (allele 40.443) that was produced by fast neutron irradiation and found that it contained an inversion of a 1420-bp region in the chalcone flavanone isomerase (*TT5*) gene and translocation of a 272-bp fragment from 38 cM away on the same chromosome to one of the breakpoints of the inversion. Therefore, it is plausible that in YS241, a DNA fragment containing the *TOM2* gene translocated to a nearby, but not very close, locus on the same chromosome, and following translocation, the translocated *TOM2* region (i.e., *ttm1* element) expressed a quantitatively and/or qualitatively altered gene product which is able to support TMV-L multiplication at a similar efficiency and which only supports TMV-Cg multiplication at a lower efficiency compared with that supported by the *TOM2* gene product. This hypothesis can explain the following points: (i) why two mutations, *tom2-1* and *ttm1*, which have opposing characteristics with regard to TMV multiplication, occurred in a single plant; (ii) why a dominant mutation was caused by fast neutron irradiation; and (iii) why the *tom2-1* mutation and the *ttm1* element are linked to each other.

Because the efficiency of multiplication of TMV-L and -Cg is reduced in *tom2-1* plants without *ttm1* (e.g., line B1-113), it is apparent that the TOM2 factor is involved in the multiplication process of both viruses, and possibly at the same step, considering the similarity of these viruses. However, the enhancing effect of the *ttm1* element on the multiplication of TMV-L and TMV-Cg differed in the *tom2-1* background: The *ttm1* element increased TMV-L multiplication to levels as high as those observed in wild-type plants, whereas its enhancing effect on TMV-Cg multiplication was limited. According to the above hypothesis, the alteration (whether qualitative or quantitative is not known at present) from the *TOM2* gene to the *ttm1* element affects the multiplication of TMV-L and TMV-Cg differently. The differential sensitivity of TMV multiplication to the alteration from *TOM2* to *ttm1*

might reflect structural differences in the replication proteins encoded by the viruses or in the structure of the genomic RNAs. Alternatively, the rate-limiting step during TMV multiplication in wild-type *A. thaliana* plants might be different between TMV-Cg and TMV-L. For example, the TOM2-related step might be rate-limiting for TMV-Cg multiplication in *tom2-1 ttm1* cells, but not rate-limiting for TMV-L multiplication in *tom2-1 ttm1* cells.

MATERIALS AND METHODS

Viruses and antisera

TMV-Cg (Yamanaka *et al.,* 1998), TMV-L (Ohno *et al.,* 1984; Meshi *et al.,* 1986), CMV-Y (Suzuki *et al.,*1991), TCV-B (Heaton *et al.,*1989), and TYMV (Morch *et al.,*1988) were propagated, and their virus particles and virion RNAs were purified, as described by Ishikawa *et al.* (1991c, 1993). Rabbit antisera against TMV-Cg, TCV, or CMV were obtained, as described by Ishikawa *et al.* (1993). Rabbit antiserum against TMV-L was obtained from Y. Okada.

Plant materials and growth conditions

A. thaliana (L.) Heynh. ecotypes Columbia (Col-0), a Columbia derivative with the *gl1* mutation (referred to as "gl1" in this paper), and Landsberg (La-0) were used as wild-type strains. M2 seeds of fast neutron-mutagenized gl1 were purchased from Lehle Seeds (Round Rock, TX). *A. thaliana* plants were cultivated as described by Ishikawa *et al.* (1991c). PD114 (*tom1-1*) is a mutant of *A. thaliana* in which TMV-Cg multiplication within a single cell is reduced, and which was isolated from an M2 population derived from EMS-mutagenized Col-0 seeds (Ishikawa *et al.,* 1991c, 1993).

Virus inoculation of plants and *Arabidopsis* mutant screening

A. thaliana plants were inoculated with viruses as described by Ishikawa *et al.* (1991c). Mutant screening was carried out as described by Ishikawa *et al.* (1991c). Of 5000 M2 plants screened, 417 M2 plants showed low levels of TMV-Cg CP accumulation, and among these 417 candidates, only one line, YS241, consistently showed reduced TMV-Cg CP accumulation in the M3 generation.

Genetic analysis

The F2 generations of progeny from crosses indicated in the text were scored for the phenotype of high or low levels of TMV-Cg and TMV-L CP accumulation by SDS–PAGE analysis of total proteins and dot ELISA (Ishikawa *et al.,* 1991c), respectively. F2 plants that showed low-level CP accumulation were allowed to self-pollinate, and the phenotype was checked in the F3 generation to refute the possibility that the F2 plants had failed to be inoculated.

Mapping of the *tom1* and *tom2* loci was carried out as follows: From the F2 lines derived from the crosses between PD114 (*tom1-1*; derived from Col-0) and La-0 or YS241 (*tom2-1*; derived from gl1) and La-0, 66 and 38 lines in which TMV-Cg CP accumulation was reduced were selected for *tom1* and *tom2*, respectively. Total DNA was extracted (Pruitt and Meyerowits, 1996) from pooled F3 individuals (more than 50 F3 plants per F2 line) for each mutant line. Linkage between the mutation and RFLP markers was examined by determining the genotype of the selected F2 lines with the phenotype of low-level accumulation of TMV-Cg CP for each RFLP marker: For *tom1*, linkage with RFLP markers m219, m215, m213, m252, m251, m429, m583, m433, m249, m339, m506, m557, m600, m447, m247, and m435 (Chang *et al.,* 1988; Hauge *et al.,* 1993) was examined. For *tom2*, linkage with RFLP markers m241, m235, m215, m335, m254, m253, m299, m213, m252, m251, m429, m583, m249, m506, m600, and m247 (Chang *et al.,* 1988; Hauge *et al.,* 1993) was examined. Standard procedures were used for DNA manipulation (Ausubel *et al.,* 1989).

Protoplast inoculation

A. thaliana protoplasts were prepared and inoculated with virion RNAs as described by Ishikawa *et al.* (1993).

Analysis of viral proteins and RNAs

Extraction and analysis of total protein from leaf materials by SDS–PAGE and quantitation of viral CPs were carried out as described by Ishikawa *et al.* (1991c). *In situ* indirect immunofluorescence staining of protoplasts using anti-viral CP antibodies was carried out as described by Ishikawa *et al.* (1993). Total nucleic acids were extracted from protoplasts, purified, denatured with glyoxal, separated in 1% agarose gels, transferred onto GeneScreen membranes (DuPont-NEN), and hybridized with ^{32}P-labeled probes, as described by Ishikawa *et al.* (1993). Probes used in this study are indicated in the legends to Fig. 5. Alternatively, to detect TCV sequence, pT7TCVms (Oh *et al.,* 1995) was labeled with ^{32}P by a Multiprime DNA Labeling System (Amersham) and used as a probe.

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