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Effects of the *tom1* Mutation of *Arabidopsis thaliana* on the Multiplication of Tobacco Mosaic Virus RNA in Protoplasts

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For the multiplication of RNA viruses, specific host factors are considered essential, but as of yet little is known about this aspect of virus multiplication. To identify such host factors, we previously isolated PD114, a mutant of *Arabidopsis thaliana*, in which the accumulation of the coat protein of tobacco mosaic virus (TMV) in uninoculated leaves of an infected plant was reduced to low levels. The causal mutation, designated *tom1*, was single, nuclear, and recessive. Here, we demonstrate that the *tom1* mutation affects the amplification of TMV-related RNAs in a single cell. When protoplasts were inoculated with TMV RNA by electroporation, the percentage of TMV-positive protoplasts (detected by indirect immunofluorescence staining with anti-TMV antibodies) was lower (about 1/5 to 1/10) among PD114 protoplasts than among wild-type protoplasts. In TMV-positive PD114 protoplasts, the amounts of the positive-strand RNAs (the genomic RNA and subgenomic mRNAs) and coat protein reached levels similar to, or slightly lower than, those reached in TMV-positive wild-type protoplasts, but the accumulation of the positive-strand RNAs and coat protein occurred more slowly than with the wild-type protoplasts. The parallel decrease in the amounts of the coat protein and its mRNA suggests that the coat protein is translated from its mRNA with normal efficiency. These observations support the idea that the *TOM1* gene encodes a host factor necessary for the efficient amplification of TMV RNA in an infected cell. Furthermore, we show that TMV multiplication in PD114 protoplasts is severely affected by the coinoculation of cucumber mosaic virus (CMV) RNA. When PD114 protoplasts were inoculated with a mixture of TMV and CMV RNAs by electroporation, the accumulation of TMV-related molecules was approximately one-fifth of that in PD114 protoplasts inoculated with TMV RNA alone. No such reduction in the accumulation of TMV-related molecules was observed when wild-type protoplasts were inoculated with a mixture of TMV and CMV RNAs or when wild-type and PD114 protoplasts were inoculated with a mixture of TMV and turnip crinkle virus RNAs. These observations are compatible with a hypothetical model in which a gene(s) that is distinct from the *TOM1* gene is involved in both TMV and CMV multiplication.

Tobacco mosaic virus (TMV) is one of the most simple and most well-characterized viruses of plants (24, 43). Its genome is a single-stranded, messenger-sense RNA encoding at least three nonstructural (130,000-molecular-weight [130K], 180K, and 30K) proteins and the coat protein (CP) (9, 31). The 130K protein and its readthrough product, the 180K protein, are encoded near the 5′ terminus of the genomic RNA and are translated directly from the genomic RNA (4, 36). The 30K protein and CP are encoded downstream (3′) of the 180K protein open reading frame and translated from respective subgenomic mRNAs which are 3′ coterminal to the genomic RNA and are produced during replication (8, 10, 14, 46). After TMV invades a cell of a systemic host plant, it multiplies through three distinct processes. First, virions uncoat to release the genomic RNA, and the genomic and subgenomic RNAs are amplified via genome-length negative-strand RNA. Second, TMV spreads from the infected cell to neighboring cells through plasmodesmata. This process is called cell-to-cell movement. Third, TMV spreads from the infection center to distant parts of the plant through the phloem. This process is called long-distance transport. The 130K and 180K proteins are involved in viral RNA amplification in a cell (16), the 30K protein is required for cell-to-cell movement (5, 25), and the CP is necessary for long-distance transport (38, 41).

Not only virus-encoded factors but also specific host-encoded factors other than those necessary for basic translation of virus-encoded polypeptides have been thought to play essential roles during the multiplication of positive-strand RNA viruses (for examples, see references 13, 18, 34, and 37). However, only a few such host factors, e.g., those in the replicase of bacteriophage Qβ (2, 44), have been identified. In order to understand the molecular mechanisms of the multiplication process of positive-strand RNA viruses, it will be necessary to identify and characterize host factors as well as virus-encoded factors involved in the process.

In a search for such host factors, one of the most powerful strategies is a genetic approach. The first step is to isolate host mutants in which the multiplication of a virus is abnormal (reduced) and to characterize the genetic properties of the mutations. The next step is to reveal the viral multiplication inhibition mechanisms in the mutants and to isolate and characterize the gene which carries the causal mutation. *Arabidopsis thaliana* is a cruciferous plant having many advantages for genetic and molecular biological studies (for reviews, see references 3, 6, 26, and 27). Several *A. thaliana* genes whose genetic properties are known but whose products have not been identified have been cloned (for examples, see references 7, 40, 48, and 49).

Previously, we found that *A. thaliana* is susceptible to infection with TMV-Cg, a crucifer strain of TMV: the CP of TMV-Cg accumulated to a high level in uninoculated rosette leaves several days after inoculation (18). Using this simple virus-host model system, we planned to identify host factors involved in the multiplication of TMV. To do this, of 6,000 M2 *A. thaliana* plants descended from ethyl methane-

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sulfonate (EMS)-treated seeds, two independent mutants (PD114 and PD378) in which the accumulation of TMV-Cg CP in the uninoculated leaves of infected plants was reduced to low levels were isolated (18). The causal mutations of the two mutants were single, nuclear, and recessive and belonged to the same complementation group (18). We named the causal mutation *tom1*. It was recently revealed that the *tom1* locus is mapped on chromosome 4 (48a). Turnip crinkle virus (TCV) and turnip yellow mosaic virus (TYMV), both of which belong to different taxonomic groups from tobamoviruses, multiplied equally in wild-type and PD114 plants, suggesting that the *tom1* mutation specifically affects TMV multiplication (18).

In this study, in order to determine whether the *tom1* mutation affects the process of TMV multiplication in a single cell or during spreading of the virus, we examined the production of TMV-Cg-related molecules in wild-type and PD114 protoplasts inoculated with TMV-Cg RNA, and we show that TMV multiplication is affected in PD114 protoplasts. Furthermore, we present experimental data suggesting the presence of a gene(s) that is distinct from the *TOM1* gene and that is involved in the multiplication of both TMV and cucumber mosaic virus (CMV).

(In this article, the words "infect" and "inoculate" have distinct meanings as indicated in the following examples. "Virus-infected protoplasts" indicate protoplasts in which the genomic RNA of the virus was substantially amplified and, as a result, the CP was produced. "Inoculated protoplasts" indicate protoplasts which were used for electroporation. Therefore, protoplasts which are inoculated but not infected with a virus can exist.)

MATERIALS AND METHODS

Plant materials. PD114 is a mutant of *A. thaliana* in which the accumulation of TMV-Cg CP in the uninoculated leaves of infected plants is reduced to low levels and was isolated from the wild-type ecotype Columbia (Col-0) by mutagenesis with EMS as a mutagen (18). For protoplast inoculation, we used a PD114 line which was descended directly from mutagenized seeds (M6 generation) and two independent PD114 lines which were established by backcrossings as follows: (i) backcross of the pollen of PD114 to Col-0, (ii) self-fertilization of the F1 plants to obtain F2 seeds, (iii) growth of F2 plants and select *tom1/tom1* homozygotes, (iv) repetition of the above steps once more. The results obtained with these lines were consistent with each other.

Viruses, cDNA clones, and antisera. TMV-Cg and TCV were obtained and propagated as described by Ishikawa et al. (18). CMV-Y was obtained from Y. Takanami and propagated in *Nicotiana tabacum* L. cv. Xanthi nc plants (42). Virions of TMV-Cg and TCV were purified by precipitation with polyethylene glycol and successive centrifugation (22, 33). Virions of CMV were purified by successive centrifugation (42). Viral RNAs of TMV-Cg, TCV, and CMV were purified from virions by phenol extraction and ethanol precipitation. pCg0-1, a cDNA clone of TMV-Cg RNA, was constructed by Meshi et al. (25a) by the method described by Ohno et al. (31), and its 1.2-kb double-stranded cDNA insert corresponds to a 3′ portion of the 30K-protein gene, the complete CP gene, and the 3′ noncoding region. pCgP1 was constructed by inserting the 0.52-kb *Nsp*7524V-*Bal*I fragment (corresponding to nucleotides 872 to 351 from the 3′ end of the TMV-Cg genomic RNA, i.e., a 3′ portion of the 30K-protein gene and a 5′ portion of the CP gene) of pCg0-1 between the *Sma*I and *Acc*I sites of pGEM-3Zf(+) (Promega). pCgP2 was constructed by inserting the 0.32-kb *Bal*I-*Nsp*7524V fragment (corresponding to nucleotides 351 to 33 from the 3′ end of the TMV-Cg genomic RNA, i.e., a 3′ portion of the CP gene and the 3′ noncoding region) of pCg0-1 between the *Sma*I and *Acc*I sites of pGEM-3Zf(+) (Promega). pT7TCV, a full-length cDNA clone of TCV RNA, was obtained from A. E. Simon and C. Zhang. Plasmid pCYU3 was constructed by inserting the full-length CMV-Y RNA3 double-stranded cDNA at the *Eco*RI site of pGEM-3Zf(+) by using an *Eco*RI linker. Rabbit antiserum against TMV-Cg was obtained from E. Shikata, rabbit antiserum against TCV was from A. E. Simon and C. Zhang, and rabbit antiserum against CMV (D strain) was from the American Type Culture Collection.

Preparation of protoplasts and electroporation. Protoplasts of *A. thaliana* were prepared by the method described by Guzman and Ecker (11), with the following modifications. *Arabidopsis* calli derived from 100 to 150 seedlings were cultured in RM28 medium as described by Guzman and Ecker (11). Half of the suspension-cultured calli were transferred to 30 ml of 0.6 M mannitol and incubated at room temperature for 10 min. Then the supernatant was discarded and replaced with 30 ml of filter-sterilized modified PIM. Modified PIM consisted of RM28 medium with 1% Cellulase Onozuka RS (Yakult Pharmaceutical Industry Co., Ltd., Nishinomiya, Japan), 0.2% Pectolyase Y23 (Seishin Pharmaceutical Co., Ltd., Tokyo, Japan), 5 mM $CaCl_2$, and 0.5 M mannitol, and its pH was adjusted to 5.8 with KOH. The suspension of calli in modified PIM was incubated at 25°C with gentle shaking in a 200-ml Erlenmeyer flask. After 3 h of incubation, the suspension which became turbid with protoplasts was filtrated through a stainless steel sieve (aperture, 63 μm) to remove undigested calli. The filtrate containing protoplasts was incubated (settled) for an additional 1 h in a plastic dish at 25°C. Then, protoplasts were washed by repeating six times the cycle of pelleting the protoplasts by centrifugation, discarding the supernatant, and resuspending the protoplasts in 0.6 M mannitol. Protoplasts were finally suspended in electroporation buffer (5 mM morpholineethanesulfonic acid [MES], 70 mM KCl, 0.5 M mannitol, pH 5.8), at a concentration of 6.7×10^6 protoplasts per ml, and used for electroporation.

Electroporation was essentially carried out as described by Watanabe et al. (47). Briefly, 600 μl of protoplast suspension (4×10^6 protoplasts) was mixed with 200 μl of inoculum RNA in electroporation buffer and electroporated once (the capacity of the condenser was 47 μF, the condenser was charged at 300 V, and the distance between electrodes was 4 mm). The electroporated protoplast suspension was left on ice for 30 min, incubated at 25°C for 5 min, collected by centrifugation, and suspended in 4 ml of protoplast culture medium which consisted of RM28 medium with 0.4 M mannitol. The protoplasts were cultured at 23°C in the dark. Time zero after infection was defined as the time when electroporated protoplasts were transferred to 25°C.

Indirect immunofluorescence staining. Protoplasts were fixed on glass slides (32). Fixed protoplasts were stored in 95% ethanol at −20°C if necessary. After fixation, the slides were washed four times for 10 min each in cold PBS (10 mM NaPi buffer, pH 7.2; 150 mM NaCl), and primary antibodies (anti-TMV-Cg, anti-TCV, and anti-CMV-D rabbit sera diluted 1:100 in PBS containing 1 mg of normal goat immunoglobulin G [IgG] [Cappel, West Chester, Pa.]) per ml were applied for 1.5 h at 37°C. The slides were then washed as described above, and secondary antibodies (fluorescein isothiocyanate-conjugated anti-rabbit IgG goat IgG [Seika-

gaku Kogyo Co., Ltd., Tokyo, Japan] diluted 1:50 in PBS containing 1 mg of normal goat IgG per ml) were applied for 1.5 h at 37°C. The slides were washed as described above, mounted in 10% glycerol in PBS, and observed.

Protein analyses. Proteins of *Arabidopsis* protoplasts were prepared (45) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (21). The CPs of TCV and CMV-Y were detected by staining with Coomassie brilliant blue dye. The CP of TMV-Cg was detected by immunoblotting. Immunoblotting was carried out with Biodyne A membranes (Pall) and a blotting detection kit (RPN23; Amersham) according to the manufacturers' directions. Anti-TMV-Cg serum was used as a primary antibody at a 1:50,000 dilution.

RNA analysis. Total nucleic acids were extracted from protoplasts (45), denatured with 1 M glyoxal, and separated by 1% agarose gel electrophoresis (39). Northern (RNA) blotting and hybridization with GeneScreen membranes (Du Pont-NEN) were carried out according to the manufacturer's directions. Probes for hybridization were as follows. For the detection of TCV-related RNAs, pT7TCV labeled with ^{32}P by nick translation (39) was used. For the detection of TMV-Cg positive-strand RNAs, ^{32}P-labeled in vitro transcript synthesized from *Eco*RI-digested pCgP2 by using SP6 RNA polymerase (23) was used. For the detection of TMV-Cg negative-strand RNAs, a mixture of ^{32}P-labeled in vitro transcript synthesized from *Eco*RI-digested pCgP1 by using SP6 RNA polymerase and ^{32}P-labeled in vitro transcript synthesized from *Hin*dIII-digested pCgP2 by using T7 RNA polymerase was used. For the detection of CMV-Y positive-strand RNAs, ^{32}P-labeled in vitro transcript synthesized from *Xho*I-digested pCYU3 by using T7 RNA polymerase was used. Note that the *Xho*I site is located at nucleotide 1839 from the 5′ end of CMV-Y RNA3 (29). After hybridization and washing, the blots were exposed to preflashed X-ray films (RX; Fuji Photo Film) with intensifying screens at −80°C. The signal intensity of bands on X-ray films was determined by densitometry at a wavelength of 600 nm with a Shimadzu dual-wavelength flying spot scanner (model cs-9000).

RESULTS

Preparation of protoplasts of *A. thaliana* and inoculation of TMV RNA. To reveal the molecular mechanisms by which the accumulation of the CP of TMV is reduced to low levels in *A. thaliana* plants bearing the *tom1* mutation, we examined whether the reduction was also observed in PD114 protoplasts. Protoplasts were prepared from liquid-cultured calli generated from aseptic seedlings as described in Materials and Methods. By this method, we could consistently obtain approximately 2×10^8 protoplasts from about 100 seedlings for both the wild-type (Col-0) and PD114 lines. No visible differences were observed between protoplasts isolated from the Col-0 and PD114 lines. We inoculated 4×10^6 Col-0 protoplasts with 20 μg of TMV-Cg RNA by electroporation under conditions in which either 0.4 or 0.5 M mannitol in electroporation buffer (see Materials and Methods) and condensers with a capacity of 47, 100, or 330 μF (condensers were charged at 300 V in all cases) were used. Among these conditions, the highest level of TMV-Cg CP accumulation was obtained when 0.5 M mannitol in electroporation buffer and a 47-μF condenser were used. Under this condition, approximately 20% of the total inoculated protoplasts were consistently TMV-Cg positive (detected by indirect immunofluorescence staining with anti-TMV-Cg antibodies). We used this condition of electroporation in the following experiments.

TABLE 1. Percentage of TMV-positive protoplasts, determined by indirect immunofluorescence staining

Cell type	Inoculum RNA	% Protoplasts TMV positive at time after electroporation[a]			
		6 h	18 h	30 h	42 h
Col-0	Mock[b]	0	0	0	0
	TMV[c]	0	21	19	21
PD114	Mock[b]	0	0	0	0
	TMV[c]	0	0.2	3	3

[a] Percentages were determined by examining approximately 500 protoplasts. For electroporation, 4×10^6 protoplasts were used.
[b] Electroporation buffer did not contain TMV-Cg RNA.
[c] Electroporation buffer contained 20 μg of TMV-Cg RNA.

Accumulation of the CP in wild-type (Col-0) and PD114 protoplasts inoculated with TMV RNA. We inoculated Col-0 and PD114 protoplasts (4×10^6 protoplasts each) with 20 μg of TMV-Cg RNA by electroporation. At 6, 18, 30, and 42 h after electroporation, the protoplasts were processed for indirect immunofluorescence staining with anti-TMV-Cg antibodies. As shown in Table 1, the percentage of TMV-Cg-positive protoplasts reached a plateau at 18 h postinoculation (p.i.) for Col-0 (final level was approximately 20%) and at 30 h p.i. for PD114 (final level was approximately 3%). This result suggests that all the protoplasts which were infected with TMV-Cg showed positive signals after 18 h p.i. for Col-0 and after 30 h p.i. for PD114 and that the percentage of protoplasts infected was approximately 20% for Col-0 and approximately 3% for PD114. The strengths of immunofluorescence of the TMV-Cg-positive Col-0 protoplasts at 18, 30, and 42 h p.i. and the TMV-Cg-positive PD114 protoplasts at 30 and 42 h p.i. appeared to be similar. The immunofluorescence of the TMV-Cg-positive PD114 protoplasts at 18 h p.i. was, on average, weaker than that of the TMV-Cg-positive PD114 protoplasts at 30 and 42 h p.i. (data not shown). These observations suggest that with PD114 protoplasts, the efficiency of infection is lower and that it takes a longer time for TMV-Cg CP to accumulate to a detectable level by indirect immunofluorescence staining than with Col-0 protoplasts.

Figure 1B shows the accumulation of TMV-Cg CP in PD114 and Col-0 protoplasts inoculated with TMV-Cg RNA and harvested at 42 h p.i. The signal intensity of the TMV-Cg CP band in the total protein from inoculated PD114 protoplasts was nearly the same as that in the total protein from inoculated Col-0 protoplasts, which was diluted 1:10 with the total protein from mock-inoculated Col-0 protoplasts (Fig. 1B). This result indicates that the amount of TMV-Cg CP in the total inoculated PD114 protoplasts (4×10^6) was about 1/10 of that in the total inoculated Col-0 protoplasts (4×10^6) at 42 h p.i. Taking the difference in the percentage of infected (TMV-Cg-positive) protoplasts in PD114 and Col-0 into consideration, these observations suggest that the amount of CP in infected (TMV-Cg-positive) PD114 protoplasts is nearly the same as, or slightly less than, that in infected (TMV-Cg-positive) Col-0 protoplasts at 42 h p.i.

Accumulation of TMV-related RNAs in wild-type (Col-0) and PD114 protoplasts inoculated with TMV RNA. To elucidate the cause of the abnormality in the accumulation of TMV-Cg CP in PD114 protoplasts, we examined the accumulation of TMV-related RNAs by Northern blot analysis. For the detection of positive-strand RNAs of TMV-Cg, ^{32}P-labeled RNA carrying the anti-sense sequence of a part

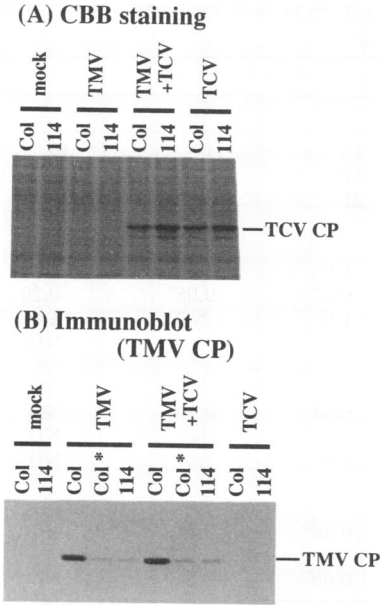

FIG. 1. Accumulation of the CP of TCV and TMV-Cg in PD114 and wild-type (Col-0) protoplasts. Protoplasts (4×10^6 protoplasts of each strain) were mock inoculated or inoculated with TMV-Cg (20 μg) and/or TCV (5 μg) RNA by electroporation, cultured, and harvested at 42 h p.i. Total protein from 4×10^4 (A) or 1×10^5 (B) protoplasts were separated in a sodium dodecyl sulfate–9% (containing 0.27% bisacrylamide) (A) or 11% (containing 0.55% bisacrylamide) (B) polyacrylamide gel and stained with Coomassie brilliant blue dye (A) or processed for immunoblot analysis with TMV-Cg antibodies (B). The cell type and inoculum are shown at the tops of the panels. In panel B, the asterisks indicate that the protein samples were diluted 1:10 with protein sample of mock-inoculated Col-0 protoplasts, and the same volume of samples as in the other lanes was applied. The positions of the CPs of TCV (A) and TMV-Cg (B) are shown to the right of the panels. In panel A, TMV-Cg CP migrated to the front, and, therefore, TMV-Cg CP could not be detected.

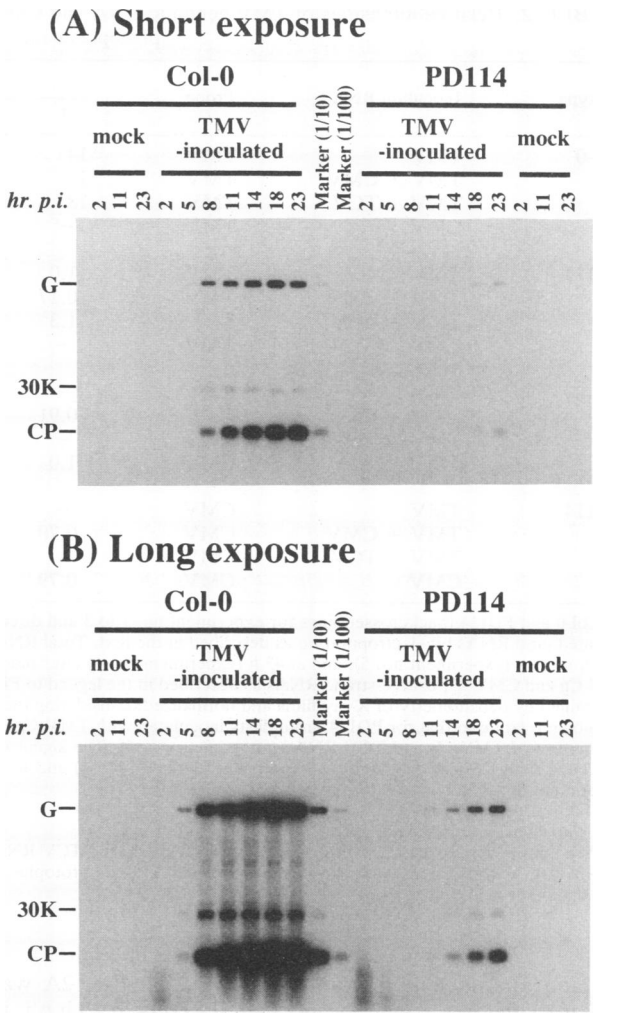

FIG. 2. Time course of accumulation of the TMV-Cg-related positive-strand RNAs in PD114 and wild-type (Col-0) protoplasts. Protoplasts (4×10^6 protoplasts each) were mock inoculated or inoculated with TMV-Cg RNA (20 μg) by electroporation, cultured, and harvested at 2, 5, 8, 11, 14, 18, and 23 h p.i. Total RNA was extracted from the protoplasts, denatured with 1 M glyoxal, separated by 1% agarose gel electrophoresis, blotted onto a nylon membrane, and hybridized with a ^{32}P-labeled RNA probe complementary to a 3′ portion of the genomic RNA of TMV-Cg. Total RNA from 2.8×10^4 protoplasts was applied in each lane. The cell type, inoculum, and the time when the protoplasts were harvested are indicated at the tops of the panels. For the lanes indicated by "Marker (1/10) and (1/100)," total RNA extracted from Col-0 protoplasts inoculated with TMV-Cg RNA at 23 h p.i., which was diluted 1:10 and 1:100, respectively, with total RNA from mock-inoculated Col-0 protoplasts, was applied. The positions of the genomic RNA (G), 30K-protein (30K), and CP (CP) subgenomic mRNAs of TMV-Cg are indicated to the left of the panels. In panel B, the X-ray film was exposed to the blot approximately 10 times longer than in panel A.

of TMV-Cg RNA corresponding to a 3′ portion of the CP gene and the 3′ noncoding region was used as a probe. Because the two subgenomic mRNAs (30K protein and CP) are 3′ coterminal to the genomic RNA, the genomic RNA, 30K-protein, and CP subgenomic mRNAs of TMV-Cg were detected with this probe (Fig. 2).

Figure 2 shows the accumulation of TMV-Cg-related positive-strand RNAs (the genomic RNA, 30K-protein, and CP subgenomic mRNAs) in Col-0 and PD114 protoplasts. The TMV-Cg genomic RNA band in the total RNA extracted from PD114 protoplasts inoculated with TMV-Cg RNA at 23 h p.i. was as intense as that in the total RNA extracted from Col-0 protoplasts inoculated with TMV-Cg RNA at 23 h p.i., which was diluted 1:10 with the total RNA from mock-inoculated Col-0 protoplasts (Fig. 2). This indicates that the amount of TMV-Cg genomic RNA in the total inoculated PD114 protoplasts (4×10^6) was about 1/10 of that in the total inoculated Col-0 protoplasts (4×10^6) at 23 h p.i. In other experiments, the amount of TMV-Cg genomic RNA in the total inoculated PD114 protoplasts (4×10^6) was estimated to be 1/10 to 1/20 of that in the total inoculated Col-0 protoplasts (4×10^6) at 25 or 26 h p.i. and approximately 1/10 of that in the total inoculated Col-0 protoplasts (4×10^6) at 46 h p.i. (Table 2). These results suggest that, in infected (TMV-Cg-positive) PD114 protoplasts, the amount of TMV-Cg genomic RNA finally reaches levels nearly the same as, or slightly lower than, those in infected (TMV-Cg-positive) Col-0 protoplasts, because 20 and 3% of the total inoculated protoplasts were infected (TMV-Cg-positive) for Col-0 and PD114, respectively.

The signal intensity of the TMV-Cg genomic RNA band in

TABLE 2. Relative intensities of TMV genomic RNA and CMV RNA3 bands on Northern blots of total RNAs from Col-0 and PD114 protoplasts inoculated with viral RNAs[a]

Cell type	Inoculum RNA	Probe	Band intensity in expt no.:					
			1	2	3	4	5	6
Col-0	TMV	TMV	14	22	13	11	10	ND[b]
	TMV + CMV	TMV	15	19	19	7.2	ND	ND
	TMV + TCV	TMV	18	39	21	ND	14	ND
	CMV	TMV	–[c]	–[d]	–	–	ND	ND
PD114	TMV	TMV	1.0	1.0	1.0	1.0	1.0	ND
	TMV + CMV	TMV	0.17	0.16	0.27	0.057	0.20	0.15
	TMV + TCV	TMV	1.3	1.4	1.0	ND	1.0	1.0
	CMV	TMV	–	–[d]	–	–	ND	ND
Col-0	TMV	CMV	–	–	–	–	ND	ND
	TMV + CMV	CMV	0.91	1.0	0.84	0.80	ND	ND
	TMV + TCV	CMV	–	–	–	ND	ND	ND
	CMV	CMV	1.0	1.0[d]	1.0	1.0	ND	ND
PD114	TMV	CMV	–	–	–	–	–	ND
	TMV + CMV	CMV	0.70	1.3	0.58	0.85	+[e]	+[e]
	TMV + TCV	CMV	–	–	–	ND	–	–
	CMV	CMV	0.79	1.0[d]	0.66	0.87	ND	ND

[a] Col-0 and PD114 (backcrossed lines for experiment no. 1 to 3 and directly descending M6 lines for experiment no. 4 to 6) protoplasts were inoculated with indicated viral RNAs by electroporation as described in the text. Total RNA was extracted from protoplasts at 25 h (experiment no. 1 to 3), at 46 h (experiment no. 4), at 26 h (experiment no. 5), and at 43 h (experiment no. 6) after inoculation and processed for Northern blot analysis with ^{32}P-labeled probes specific to TMV-Cg and CMV-Y positive-strand RNAs as described in the legend to Fig. 5. The signal intensities of TMV-Cg genomic RNA and CMV-Y RNA3 bands were determined by densitometry of X-ray films and normalized, considering the concentration of total RNA. The signal intensities of TMV-Cg genomic RNA bands are shown relative to that for PD114 protoplasts inoculated with TMV-Cg RNA alone (experiment no. 1 to 5) and to that for PD114 protoplasts inoculated with the mixture of TMV-Cg and TCV RNAs (experiment no. 6). The signal intensities of CMV-Y RNA3 bands are shown relative to that for Col-0 protoplasts inoculated with CMV-Y RNA alone (experiment no. 1, 3, and 4) and to that for Col-0 protoplasts inoculated with the mixture of CMV-Y and TCV RNAs (experiment no. 2).

[b] ND, not determined.

[c] –, no signal was detected.

[d] Protoplasts were inoculated with the mixture of CMV-Y and TCV RNAs instead of CMV-Y RNA alone.

[e] +, CMV-Y RNA3 signals as strong as those for other PD114 protoplasts inoculated with the mixture of TMV-Cg and CMV-Y RNAs (experiment no. 1 to 4) were detected.

the lane for inoculated Col-0 at 23 h p.i. in Fig. 2A was similar to that in the lane for inoculated PD114 at 23 h p.i. in Fig. 2B. In contrast, the signal intensities of TMV-Cg genomic RNA bands in the lanes for inoculated Col-0 at 8, 11, and 14 h p.i. in Fig. 2A were stronger than those in the lanes in Fig. 2B for inoculated PD114 at 8, 11, and 14 h p.i., respectively. These observations indicate that the TMV-Cg-related RNAs accumulated more slowly in infected (TMV-Cg-positive) PD114 protoplasts than in infected (TMV-Cg-positive) Col-0 protoplasts.

The ratios of the signal intensities of the CP mRNA and 30K-protein mRNA bands to that of the genomic RNA band in PD114 protoplasts were similar to those for Col-0 protoplasts (Fig. 2), suggesting that the *tom1* mutation affects the accumulation of the genomic RNA and the CP and 30K-protein mRNAs almost equally. The observed patterns of the accumulation of the CP mRNA in PD114 and Col-0 protoplasts are consistent with the delay of the appearance of TMV-Cg CP-positive protoplasts in PD114 (Table 1) and the difference in TMV-Cg CP accumulation between PD114 and Col-0 protoplasts at 42 h p.i. (Fig. 1). Thus, it is likely that the accumulation of TMV-related positive-strand RNAs is affected, and, as a result, the accumulation of TMV-Cg CP is affected in PD114 protoplasts (i.e., the CP is translated from its mRNA with normal efficiency).

In Fig. 2B (see also panels A and C of Fig. 4 and 5), low-molecular-weight smear-like bands were detected in the lanes for both PD114 and Col-0 protoplasts inoculated with TMV-Cg and harvested at 2 h p.i. The smear-like bands are presumably derived from the degradation products of inoculum TMV-Cg RNA which was stuck to the outside of the protoplasts or which was introduced into the protoplasts.

Figure 3 shows the accumulation of negative-strand RNAs of TMV-Cg. For the detection of negative-strand RNAs of TMV-Cg, ^{32}P-labeled RNAs carrying a part of TMV-Cg RNA sequence corresponding to a 3′ portion of the 30K-protein gene, the complete CP gene, and the 3′ noncoding region were used as a probe. This probe detected genome-length negative-strand RNA of TMV-Cg and did not detectably cross-hybridize with TMV-Cg virion RNA (Fig. 3). The intensity of the genome-length negative-strand RNA band in the total RNA extracted from PD114 protoplasts inoculated with TMV-Cg RNA at 26 h p.i. was slightly less than that in the total RNA extracted from Col-0 protoplasts inoculated with TMV-Cg RNA at 26 h p.i., which was diluted 1:10 with the total RNA from mock-inoculated Col-0 protoplasts. Densitometric determination of the intensities of the bands on Northern blots suggested that, at 26 h p.i., per total inoculated protoplasts (4×10^6), the amount of the genome-length negative-strand RNA in PD114 was 6.1% of that in Col-0 (from Fig. 3) and that the amount of the genomic RNA in PD114 was 9.7% of that in Col-0 (data not shown). Therefore, the relative amount of genomic RNA to the genome-length negative-strand RNA in infected PD114 protoplasts is approximately 1.6-fold (in another experiment, 2.6-fold at 25 h p.i.) higher than that in infected Col-0 protoplasts at 26 h p.i.

Inoculation of TCV RNA into wild-type (Col-0) and PD114

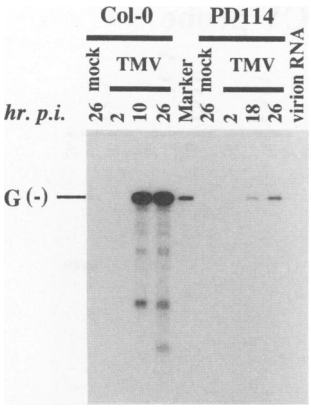

FIG. 3. Accumulation of TMV-Cg-related negative-strand RNAs in PD114 and wild-type (Col-0) protoplasts. Protoplasts (4×10^6 protoplasts of each strain) were mock inoculated or inoculated with TMV-Cg RNA (20 μg) by electroporation, cultured, and harvested at 2, 10, and 26 h p.i. for Col-0 and at 2, 18, and 26 h p.i. for PD114. Total RNA was extracted from the protoplasts, denatured with 1 M glyoxal, separated by 1% agarose gel electrophoresis, blotted onto a nylon membrane, and hybridized with ^{32}P-labeled RNA probes carrying a genomic RNA sequence of TMV-Cg corresponding to a 3′ portion of the 30K-protein gene, the complete CP gene, and the 3′ noncoding region. Total RNA from 2.8×10^4 protoplasts was applied in each lane. The cell type, inoculum, and the time when the protoplasts were harvested are indicated at the top of the panel. For the lane indicated by "Marker," total RNA extracted from Col-0 protoplasts inoculated with TMV-Cg RNA at 26 h p.i., which was diluted 1:10 with total RNA from mock-inoculated Col-0 protoplasts, was applied. For the lane indicated by "virion RNA," 9 ng of TMV-Cg virion RNA mixed with total RNA from 2.1×10^4 mock-inoculated Col-0 protoplasts was applied. Note that, in total RNA extracted from 2.8×10^4 Col-0 protoplasts inoculated with TMV-Cg RNA and harvested at 26 h p.i., approximately 1 ng of TMV-Cg genomic RNA exists. Therefore, the signals in this figure appear not to be derived from TMV-Cg positive-strand RNAs. The position of the genome-length negative-strand RNA [G (−)] is indicated to the left of the panel.

protoplasts. In PD114 plants, the accumulation of TMV-Cg CP is reduced to low levels, but the CP of TCV, which belongs to a taxonomic group different from that of TMV, accumulates with the same efficiency as in Col-0 plants (18). In order to test whether the specificity is also observed at the protoplast level, we inoculated 5 μg of TCV RNA into Col-0 and PD114 protoplasts (4×10^6 protoplasts each). Indirect immunofluorescence staining with anti-TCV antibodies showed that approximately 70% of the total inoculated protoplasts were TCV positive for both PD114 and Col-0 at 42 h p.i. (data not shown). The patterns of the accumulation of TCV CP (Fig. 1A) and TCV-related RNAs (Fig. 4B and D) in PD114 protoplasts inoculated with TCV RNA were similar to those in Col-0 protoplasts inoculated with TCV RNA. These observations indicate that the specificity of action of the *tom1* mutation toward TMV multiplication is also observed at the protoplast level.

When PD114 protoplasts (4×10^6) were inoculated with a mixture of TCV (5 μg) and TMV-Cg (20 μg) RNAs, the percentage of TCV-positive protoplasts and the patterns of the accumulation of TCV CP and TCV-related RNAs were similar to those in Col-0 protoplasts (4×10^6) inoculated with the mixture of TCV and TMV-Cg RNAs and those in Col-0 and PD114 protoplasts (4×10^6 protoplasts each) inoculated with TCV RNA (5 μg) alone (Fig. 1A and 4B and D). The percentage of TMV-Cg-positive protoplasts and the patterns of the accumulation of TMV-Cg CP and TMV-Cg-related positive-strand RNAs in protoplasts (4×10^6) inoculated with the mixture of TCV and TMV-Cg RNAs were similar to those in protoplasts (4×10^6) inoculated with TMV-Cg RNA (20 μg) alone for either PD114 or Col-0 (Fig. 1B and 4A and C). These observations were further confirmed in other experiments (Table 2). These data suggest that the cause of the reduction in TMV-Cg-related molecules in inoculated PD114 protoplasts is neither an unexpected failure of electroporation nor an inefficient introduction of viral RNAs into PD114 protoplasts in electroporation, since TCV-related molecules accumulated normally in PD114 protoplasts inoculated with the mixture of TCV and TMV-Cg RNAs.

Inhibition of TMV multiplication in PD114 protoplasts by coinoculation of CMV RNA. To examine the generality of the results described in the previous section, we performed similar experiments with CMV instead of TCV. CMV is a positive-strand RNA virus whose genome is divided into three pieces (3.4-kb RNA1, 3.1-kb RNA2, and 2.2-kb RNA3) (35). The 1a and 2a proteins, which are encoded by CMV RNAs 1 and 2, respectively, are necessary for the replication of CMV RNA (30) and have amino acid sequence similarity to the replication proteins of TMV (the 130K and 180K proteins) (1, 12). Furthermore, the genomic RNAs of TMV and CMV have similar 5′ cap and 3′ tRNA-like structures. Therefore, it is suggested that TMV and CMV RNAs replicate by similar molecular mechanisms (1, 12).

We inoculated wild-type (Col-0) and PD114 protoplasts (4×10^6 protoplasts each) with TMV-Cg RNA (20 μg) and/or CMV (Y strain) RNA (7 μg) and examined the accumulation of TMV-Cg-related and CMV-Y-related RNAs in the protoplasts by Northern blot analysis (Fig. 5). In Fig. 5B and D, a ^{32}P-labeled in vitro transcript which is complementary to the 3′ portion of CMV-Y RNA3 corresponding to nucleotides 1839 to 2217 from the 5′ end (29) was used as a probe. CMV-Y RNA3 and its subgenomic RNA4 comprise the entire sequence complementary to the probe. CMV-Y RNAs 1 and 2 have 3′-terminal sequences which are highly conserved among RNA1, -2, and -3 (19, 20) and therefore cross-hybridize to the probe.

When PD114 protoplasts were inoculated with the mixture of TMV-Cg and CMV-Y RNAs, the accumulation of TMV-Cg-related RNAs was significantly reduced compared with that in PD114 protoplasts inoculated with TMV-Cg RNA alone or the mixture of TMV-Cg and TCV RNAs (Fig. 5C). Densitometric quantitation of TMV-Cg genomic RNA bands on Northern blots suggested that the accumulation of TMV-Cg genomic RNA in total RNA from 2.8×10^4 PD114 protoplasts inoculated with the mixture of TMV-Cg and CMV-Y RNAs was less than 30% of that in total RNA from 2.8×10^4 PD114 protoplasts inoculated with TMV-Cg RNA alone (Table 2). Indirect immunofluorescence staining with anti-TMV-Cg antibodies showed that, among PD114 protoplasts inoculated with the mixture of TMV-Cg and CMV-Y RNAs, less than 0.5% of total inoculated protoplasts were TMV-Cg positive at 42 h p.i. (note that approximately 2 to 3% of total inoculated PD114 protoplasts were TMV-Cg positive among PD114 protoplasts inoculated with TMV-Cg RNA with or without TCV RNA at 42 h p.i.), and the immunofluorescence of TMV-Cg-positive protoplasts was weaker than that of TMV-Cg-positive protoplasts among PD114 protoplasts inoculated with TMV-Cg RNA with or without TCV RNA (data not shown). These results were reproduced in repeated experiments (Table 2).

No drastic difference in the accumulation of CMV-Y-

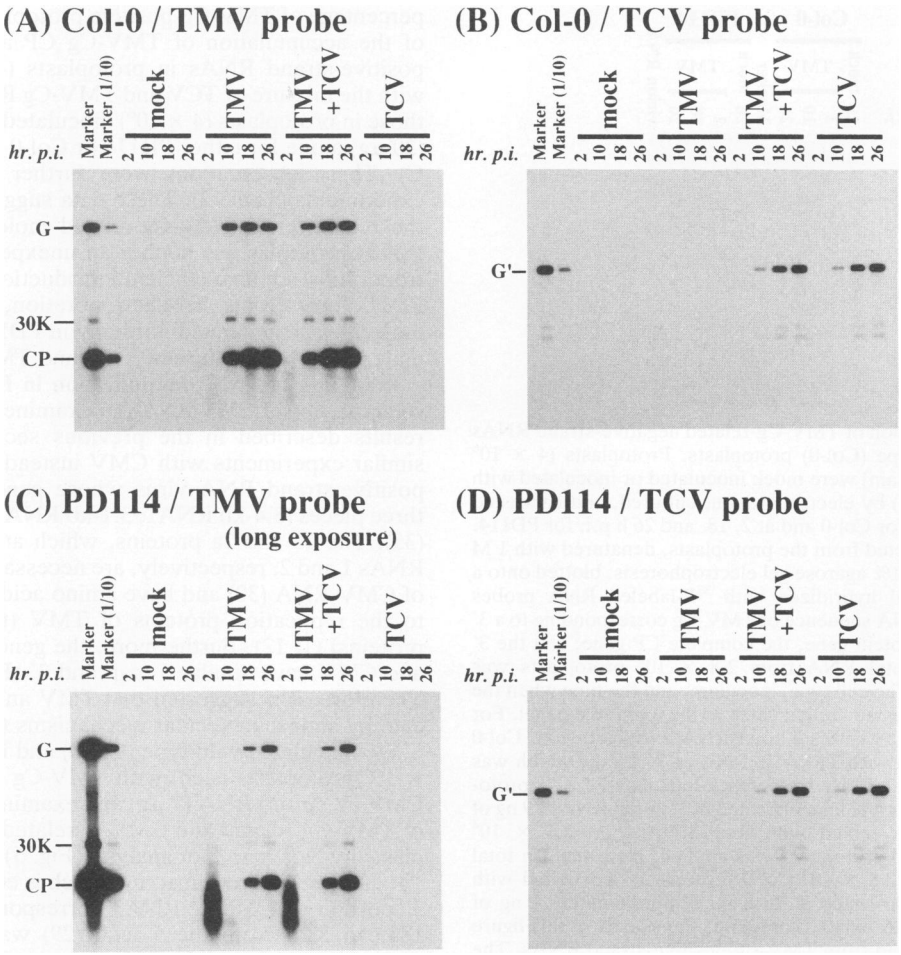

FIG. 4. Accumulation of TMV-Cg-related positive-strand and TCV-related RNAs in PD114 and wild-type (Col-0) protoplasts. Col-0 (A and B) and PD114 (C and D) protoplasts (4×10^6 protoplasts each) were mock inoculated or inoculated with TMV-Cg (20 μg) and/or TCV (5 μg) RNAs by electroporation. The protoplasts were cultured and harvested at 2, 10, 18, and 26 h p.i. Total RNA was extracted from the protoplasts, denatured with 1 M glyoxal, separated by 1% agarose gel electrophoresis, blotted onto nylon membranes, and hybridized with the same TMV-Cg-specific probe as in Fig. 2 (A and C) or ^{32}P-labeled probe made by nick translation of a full-length TCV cDNA plasmid DNA (B and D). Total RNA from 2.8×10^4 protoplasts was applied in each lane. The inoculum and the time when protoplasts were harvested are indicated at the tops of the panels. In each panel, the same samples were loaded for the two lanes at the left. For the lanes indicated by "Marker," total RNA extracted from Col-0 protoplasts inoculated with the mixture of TMV-Cg and TCV RNAs at 26 h p.i. was applied. "(1/10)" means that the RNA sample was diluted 1:10 with total RNA from mock-inoculated Col-0 protoplasts. The positions of the genomic RNA (G), 30K-protein (30K), and CP (CP) subgenomic mRNAs of TMV-Cg and the genomic RNA of TCV (G') are indicated at the left of the panels. Note that, in panel C, the X-ray film was exposed to the blot approximately 10 times longer than in panel A. The exposure times for panels B and D were the same.

related RNAs was observed among wild-type (Col-0) and PD114 protoplasts inoculated with CMV-Y RNA alone and wild-type (Col-0) and PD114 protoplasts inoculated with the mixture of CMV-Y and TMV-Cg RNAs (Fig. 5B and D). The percentage of CMV-positive protoplasts was 40 to 50% when Col-0 and PD114 protoplasts were inoculated with CMV-Y RNA with or without TMV-Cg RNA.

As seen in Table 2, minor differences in the signal strengths of TMV-Cg genomic RNA and CMV-Y RNA3 bands were observed. (i) TMV-Cg genomic RNA bands in total RNAs from Col-0 and PD114 protoplasts inoculated with the mixture of TMV-Cg and TCV RNAs were, on average, slightly more intense than those in total RNAs from Col-0 and PD114 protoplasts inoculated with TMV-Cg RNA alone, respectively. (ii) CMV-Y RNA3 bands in total RNAs from PD114 protoplasts inoculated with CMV-Y RNA with or without TMV-Cg RNA and from Col-0 protoplasts inoculated with the mixture of TMV-Cg and CMV-Y RNAs were, on average, slightly less intense than those from Col-0 protoplasts inoculated with CMV-Y RNA alone. These differences are small, and, for the present, we cannot definitely say whether the differences are significant. A significance of the differences in CMV-Y RNA3 bands between Col-0 and PD114 protoplasts inoculated with CMV-Y RNA would suggest that the *tom1* mutation affects CMV multiplication slightly.

DISCUSSION

As the first step to identify the host-encoded factors involved in the multiplication process of plant RNA viruses, we have previously isolated two independent mutants of *A.*

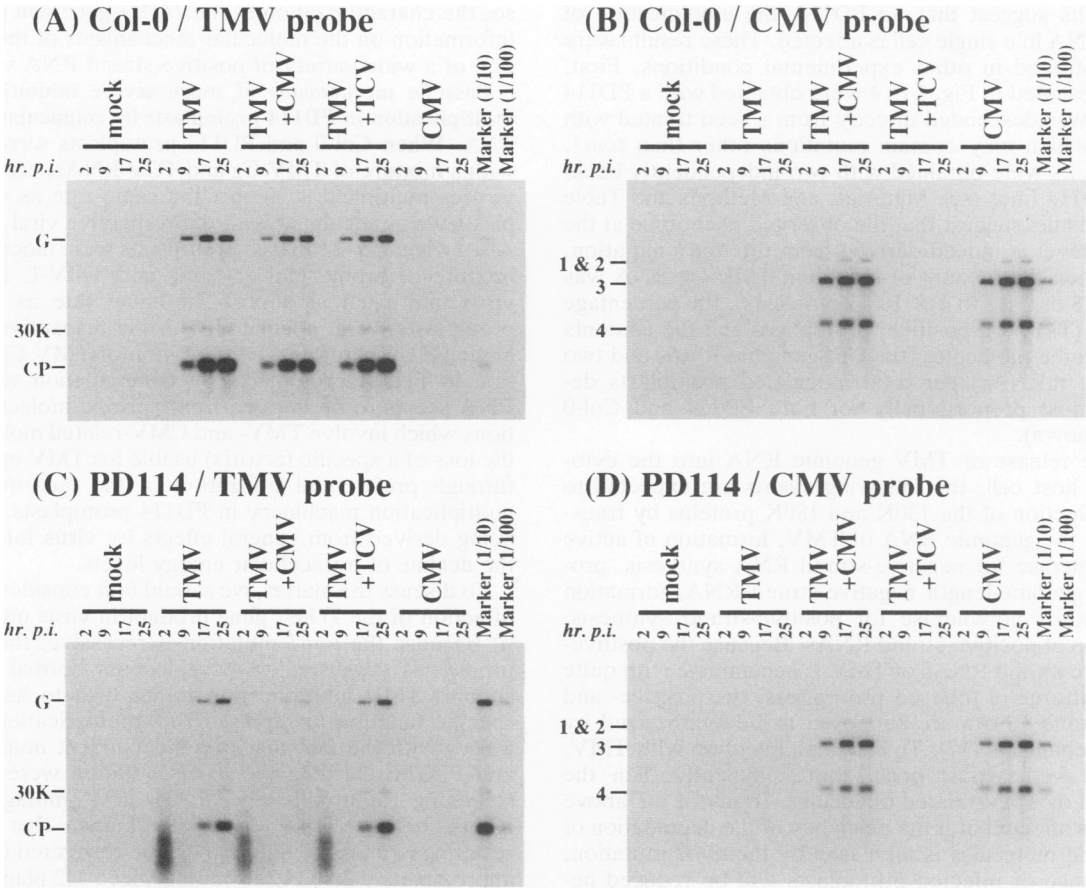

FIG. 5. Accumulation of TMV-Cg-related and CMV-Y-related positive-strand RNAs in PD114 and wild-type (Col-0) protoplasts. Col-0 (A and B) and PD114 (C and D) protoplasts (4×10^6 protoplasts each) were mock inoculated or inoculated with various combinations of TMV-Cg (20 μg), CMV-Y (7 μg), and TCV (5 μg) RNAs, as indicated, by electroporation. The protoplasts were cultured and harvested at 2, 9, 17, and 25 h p.i. Total RNA was extracted from the protoplasts, denatured with 1 M glyoxal, separated by 1% agarose gel electrophoresis, blotted onto nylon membranes, and hybridized with the same TMV-Cg-specific probe as in Fig. 2 (A and C) or with ^{32}P-labeled RNA probe complementary to a 3′ portion of CMV-Y RNA3 (B and D). Total RNA from 2.8×10^4 protoplasts was applied in each lane. The inoculum and the time when protoplasts were harvested are indicated at the tops of the panels. In each panel, the same samples were loaded in the two lanes at the right. For the lanes indicated by "Marker (1/10)" and "Marker (1/100)," total RNAs extracted at 25 h p.i. from Col-0 protoplasts inoculated with the mixture of TMV-Cg and CMV-Y RNAs and diluted 1:10 and 1:100, respectively, with total RNA from mock-inoculated Col-0 protoplasts were loaded. The positions of the genomic RNA (G), 30K-protein (30K), and CP (CP) subgenomic mRNAs of TMV-Cg and RNA1 (1), RNA2 (2), RNA3 (3), and RNA4 (4) of CMV-Y are indicated at the left of the panels. Note that, in panel C, the X-ray film was exposed to the blot approximately 10 times longer than that shown in panel A. The exposure times for panels B and D were the same.

thaliana in which the accumulation of TMV-Cg CP in upper uninoculated leaves of infected plants is reduced to low levels (18). The causal mutations of the two mutants were shown to be single, nuclear, and recessive and to belong to the same complementation group (18). We named the mutation *tom1*. The recessiveness of the *tom1* mutation implies that the *TOM1* gene product assists the multiplication of TMV or inhibits the activities that prevent the multiplication of TMV in plants. For full accumulation of viral CP in upper uninoculated leaves, all the following processes must proceed with normal efficiencies: invasion of virus particles into a cell, uncoating of virus particles to release bare genomic RNA molecules, amplification of TMV-related RNAs in a cell, cell-to-cell movement, long-distance transport, accumulation of TMV-related molecules in uninoculated leaves. In this study, in order to define which step is abnormal in plants homozygous for the *tom1* mutation, we introduced TMV-Cg RNA into wild-type and PD114 protoplasts by electroporation and examined the production of TMV-related molecules.

The *tom1* mutation affects TMV multiplication in a single cell. By the electroporation method, TCV RNA is supposed to be introduced equally well into PD114 and wild-type (Col-0) protoplasts because TCV-related molecules accumulated to nearly the same level in inoculated PD114 and Col-0 protoplasts. If TMV-Cg RNA were introduced into PD114 and Col-0 protoplasts with equal efficiency, as was the case with TCV RNA (although the sizes of the genomic RNA molecules of TMV-Cg [6.4 kb] and TCV [4.0 kb] are different), the same amount of TMV-Cg RNA would be expected to be introduced into PD114 and Col-0 protoplasts.

In PD114 protoplasts inoculated with TMV-Cg RNA, the percentage of infected (TMV-Cg positive by immunofluorescence staining) cells was low and the accumulation of positive-strand RNAs and CP of TMV was delayed compared with those in inoculated wild-type (Col-0) protoplasts.

These results suggest that, in PD114, the amplification of TMV-Cg RNA in a single cell is affected. These results were further confirmed in other experimental conditions. First, the data presented in Fig. 1 to 4 were obtained with a PD114 line which was descended directly from a seed treated with EMS and which may contain mutations other than *tom1*. Similar results were obtained with two independently backcrossed PD114 lines (see Materials and Methods and Table 2). These results suggest that the observed phenotype at the protoplast level is indeed derived from the *tom1* mutation. Second, when the amount of inoculum TMV-Cg RNA was decreased (8 or 2 μg to 4×10^6 protoplasts), the percentage of infected (TMV-Cg-positive) protoplasts and the amounts of TMV-related molecules (the CP, genomic RNA, and two subgenomic mRNAs) per total inoculated protoplasts decreased almost proportionally for both PD114 and Col-0 (data not shown).

After the release of TMV genomic RNA into the cytoplasm of a host cell, the following events are expected to occur: production of the 130K and 180K proteins by translation from the genomic RNA of TMV, formation of active RNA polymerase for negative-strand RNA synthesis, production of genome-length negative-strand RNA, formation of active RNA polymerase for positive-strand synthesis, regeneration of positive-strand RNAs. Because the positive- and negative-strand RNAs of TMV-L accumulate with quite different patterns in tobacco protoplasts, the positive- and negative-strand RNAs are supposed to be synthesized by distinct mechanisms (17). To establish infection with TMV, the above events must occur more efficiently than the degradation of TMV-related molecules. If any of the above processes is affected or if the efficiency of the degradation of TMV-related molecules is increased by the *tom1* mutation, the percentage of infected protoplasts will be reduced because the possibility of abortive infection will increase, and the production of TMV-related molecules will be delayed, as was observed in our experiments. Our results suggest that, at 26 h p.i., in TMV-infected PD114 protoplasts, the amount of genomic RNA relative to that of genome-length negative-strand RNA is slightly higher (1.6-fold; in another experiment, 2.6-fold at 25 h p.i.) than that in TMV-infected Col-0 protoplasts. However, at present, it is difficult to define whether negative-strand RNA synthesis is more severely affected by the *tom1* mutation than positive-strand RNA synthesis, because we do not know the relation between the activities of positive- and negative-strand RNA syntheses and the amount of genomic RNA relative to that of genome-length negative-strand RNA at 26 h p.i. Which process(es) (production or degradation of the 130K and 180K proteins, negative-strand RNA, or positive-strand RNAs is directly affected by the *tom1* mutation remains to be determined in future experiments.

For the multiplication of TMV RNA in a single cell, the 180K protein is necessary and the 30K protein and CP are dispensable (16, 17, 25, 41). The 130K and 180K proteins of TMV have a similarity in amino acid sequence to the proteins involved in viral RNA replication which are encoded on the genomes of not only a wide variety of plant viruses (including brome mosaic virus, CMV, and TYMV) but also animal alphaviruses (1, 12, 28). Therefore, similar molecular mechanisms may underlie the replication of these viruses. The observation that the *tom1* mutation does not affect the multiplication of TYMV and CMV at least greatly (18 and this report) might suggest that factors similar but not identical to the *TOM1* gene product are involved in the multiplication of TYMV, CMV, and other related viruses. If so, the characterization of the *TOM1* gene will provide new information on the molecular mechanisms of the multiplication of a wide variety of positive-strand RNA viruses.

Possible mechanisms of more severe inhibition of TMV multiplication in PD114 protoplasts by coinoculation of CMV RNA. When Col-0 and PD114 protoplasts were inoculated with a mixture of TMV-Cg and TCV RNAs, each of the two viruses multiplied at almost the same rate as when protoplasts were inoculated with the respective viral RNA alone. Also, when BY-2 tobacco protoplasts were inoculated with a mixture of brome mosaic virus and TMV-L RNAs, each virus multiplied at almost the same rate as when BY-2 protoplasts were inoculated with the respective viral RNA alone (15). Therefore, the inhibition of TMV-Cg multiplication in PD114 protoplasts by coinoculation with CMV-Y RNA seems to be derived from specific molecular interactions which involve TMV- and CMV-related molecules, e.g., the loss of a specific factor(s) usable for TMV multiplication through preferential utilization of the factor(s) by CMV multiplication machinery in PD114 protoplasts, rather than being derived from general effects by virus infection, e.g., the decline of intracellular energy levels.

To discuss the matter, we should first consider the manner of action of the *TOM1* gene product in virus multiplication. (i) Because the *tom1* mutation is recessive, the *tom1* gene product is suggested to have lost its normal function to support TMV multiplication rather than to have gained a specific function to repress TMV multiplication. This idea agrees with the fact that two independent mutants (PD114 and PD378) carrying the *tom1* mutation were isolated by screening approximately 6,000 EMS-mutagenized M2 plants, because it is empirically known that a mutation resulting in a loss of function can be recovered by screening approximately 2,000 EMS-mutagenized M2 plants (6). (ii) In the two independent mutants (PD114 and PD378) carrying the *tom1* mutation, the efficiency of TMV multiplication is reduced to similar levels but not to zero (18). One possible explanation for this is that the ability of the *tom1* gene products in PD114 and PD378 to support TMV multiplication is completely lost but that a factor(s) other than the *TOM1* gene product supports the multiplication of TMV with low efficiency; another possible explanation is that the *TOM1* gene product is only an enhancer of TMV multiplication and that TMV can multiply without the *TOM1* gene product. Both explanations are consistent with the fact that TMV multiplication is reduced to similar levels in the two independent mutants carrying the *tom1* mutation. Another possibility is that the abilities of the *tom1* gene products in PD114 and PD378 to support TMV multiplication are reduced to similar extents but not to zero (i.e., the *tom1* mutations are leaky). (iii) As mentioned earlier, TMV and CMV are suggested to have similar replication mechanisms (1, 12). If so, why is CMV multiplication not greatly affected by the *tom1* mutation which affects the multiplication of TMV severely? One possible explanation is that CMV utilizes a factor(s) which is similar but not identical to the *TOM1* gene product in place of the *TOM1* gene product. Another, and perhaps more minor, possibility is that the multiplication of CMV is supported by the *TOM1* gene product and that the *tom1* mutation specifically affects TMV multiplication and does not affect CMV multiplication.

From the above considerations, among several possible explanations for the results presented here, the following scenario seems to be the most likely. TMV multiplication is supported mainly by the *TOM1* gene product and secondarily (with low efficiency) by a hypothetical factor(s), and the

hypothetical factor(s) is utilized during CMV multiplication. In this scenario, in wild-type protoplasts, TMV multiplication is supported mainly by the *TOM1* gene product and secondarily by the hypothetical factor(s) and the multiplication of TMV is not affected severely even if CMV multiplication machinery preferentially utilizes the hypothetical factor(s). In PD114 cells (in the absence of the *TOM1* gene product), TMV multiplication is supported only by the hypothetical factor(s) and the preferential utilization of the hypothetical factor(s) by CMV multiplication machinery will result in more severe repression than when PD114 protoplasts were inoculated with TMV RNA alone.

Thus, the above model is compatible with our results. The isolation of a mutant with reduced CMV multiplication rates, and construction and characterization of a double mutant with *tom1*, would elucidate this model. Currently, we are trying to isolate such mutants.

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