Increased Viral Yield and Symptom Severity Result from a Single Amino Acid Substitution in the Turnip Yellow Mosaic Virus Movement Protein

Ching-Hsiu Tsai[1] and Theo W. Dreher[1,2]

[1]Department of Agricultural Chemistry, Program in Genetics, and [2]Center for Gene Research and Biotechnology, Oregon State University, Corvallis 97331 U.S.A.

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Turnip yellow mosaic virus is a positive-strand RNA virus that produces light green or yellow-green mosaic symptoms in Chinese cabbage plants. We have characterized a strain that produces nearly uniform yellow-green chlorosis in systemically infected Chinese cabbage leaves. The increased symptom severity is due to the single nucleotide substitution U1888→C, which results in a tyrosine to histidine substitution in the movement protein encoded by ORF-69. Coding by the overlapping ORF-206 is not affected. The mutation results in fourfold higher accumulations of viral products in systemically infected Chinese cabbage leaves but does not affect viral replication in isolated protoplasts. These results suggest that the increased viral yield and symptom severity result from improved viral spread in the host plant. These effects were specific to Chinese cabbage, since neither viral yield nor symptoms in turnips were affected by the U1888→C mutation.

Additional keyword: ribonuclease protection analysis.

Turnip yellow mosaic virus (TYMV), the type member of the tymovirus group of monopartite positive-strand RNA viruses, was first described by Markham and Smith in 1946. Strains that differ in symptom severity, ranging in Chinese cabbage from mild mosaic involving pale green islands to brilliant white mottling, have been recognized (Matthews 1980), but no investigations of the viral genetic element(s) responsible for symptom variations have been reported to date.

In the course of our studies with TYMV, we observed the spontaneous generation of a strain that produced more severe, almost uniformly chlorotic symptoms in the systemically infected leaves of Chinese cabbage plants. The severe symptoms accompanied increased yields of virus in infected tissue. Since the use of a virus strain that produced higher accumulations of viral products would improve the sensitivity of our studies on TYMV gene expression and replication, we determined the genetic alteration responsible for the severe chlorosis. We report that the severe symptoms result from a single nucleotide substitution in the 6.3-kb genome of TYMC, the Corvallis isolate of TYMV type that was cloned from a turnip-adapted type strain of TYMV obtained from Strasbourg, France (Weiland and Dreher 1989). The substitution alters one amino acid in the protein encoded by ORF-69, which is dispensable for RNA replication in single cells, but is required for virus spread in the plant (Bozarth *et al.* 1992).

Corresponding author: T. Dreher.

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RESULTS

Characterization of a TYMV isolate that causes severe symptoms on Chinese cabbage plants.

During a series of studies on TYMV replication in Chinese cabbage, one plant inoculated with TYMC RNA (i.e., RNA derived from pTYMC; referred to as wild type) and another inoculated with TYMC+A1460 RNA (which has a wild-type phenotype; Tsai and Dreher 1992) developed unusually severe symptoms on systemically infected leaves (Fig. 1). Infections in Chinese cabbage derived either from a cloned source (TYMC transcripts) or from our original TYMV-type stock (Strasbourg, France) consistently presented vein clearing, followed by the development of a pale green/dark green mosaic on systemically infected leaves. By contrast, the two plants with severe symptoms displayed an almost uniform yellow-green chlorosis on systemically infected leaves, although dark green flecks appeared on later emerging leaves. There were no distinct differences compared with wild-type infections in the appearance of chlorotic lesions on the inoculated leaves, nor in the timing or appearance of the systemic symptoms. Virus from the plants with severe symptoms were passaged onto healthy plants, which developed the same severe symptoms, indicating that the novel symptoms resulted from an alteration of the viral genome, rather than host plant variability. The new isolates are referred to as TYMC(s) and TYMC+A1460(s), reflecting the original T7 transcript from which the severe symptoms arose.

To characterize the genetic alterations in the severe TYMC(s) and TYMC+A1460(s) isolates, we probed their genomes by ribonuclease (RNase) protection analysis. Three minus-sense probes derived from pTYMC (Fig. 2A) were hybridized to virion RNA of each isolate, and to TYMC as control, and the hybrids were treated with a mixture of RNases T1 and A. The full-length 6.3-kb-long

*Hin*dIII$^{3'}$-*Eco*RI$^{5'}$ probe fully protected a portion of the TYMC RNA from RNase cleavage, but three bands were produced when the TYMC(s) genome was probed (Fig. 2B). Two bands of 5 and 2 kb represented complementary fragments resulting from a single cleavage of the hybridized duplex RNA, while the 7 kb fully protected band indicated that RNase cleavage did not go to completion. This result suggests the presence of a mismatch between the TYMC and TYMC(s) sequences about 2 kb from one end of the genome.

The 3′, 3-kb-long *Hin*dIII$^{3'}$-*Sst*I^{3286} probe protected both TYMC(s) and TYMC genomes fully (Fig. 2B), indicating that the mutation in TYMC(s) was located 2 kb from the 5′ end of the genome. Probing with the 4.5-kb probe corresponding to sequences between *Bam*HI^{1755} and the 3′ end (*Hin*dIII) verified this conclusion, and mapped the mutation more accurately to a position about 130 nucleotides downstream of the *Bam*HI^{1755} site, i.e., about 1,900 nucleotides from the 5′ end of the genome. This was indicated by a 130-bp fragment arising from RNase protection analysis of the TYMC(s), but not the TYMC, genome (Fig. 2C). No other differences between the digestion patterns of the TYMC(s) and TYMC genomes were observed.

RNase protection analysis of the TYMC+A1460(s) genome (not shown) indicated the presence of a similarly positioned mismatch, in addition to the expected mismatch at nucleotide +1460.

One nucleotide change in TYMC is responsible for causing severe symptoms in Chinese cabbage plants.

To determine the nucleotide change(s) in the severe-symptom isolates and to verify that the mutations indicated by the RNase protection assays were indeed responsible for the development of severe symptoms, we cloned the 1.2-kb cDNA fragment corresponding to nucleotides 1240 to 2440 of the TYMC(s) and TYMC+A1460(s) genomes. In the case of TYMC+A1460(s) RNA, this cloning was successful by PCR amplification, but with TYMC(s) RNA, amplification failed repeatedly, perhaps due to interference by nucleic acid secondary structure. Double-stranded cDNA corresponding to the 5′ half of TYMC(s) RNA was synthesized without amplification, and the *Bam*HI^{1755} to *Sst*I^{3286} fragment was cloned into pUC18 (Materials and Methods). The sequences between *Bam*HI^{1755} and *Kpn*I^{2366} were determined for the clones derived from both TYMC(s) and TYMC+A1460(s). The only mutation found relative to TYMC was U+1888→C, and this same mutation was present in both TYMC(s) and TYMC+A1460(s).

Because RNase protection analysis overlooks some mismatches (Myers *et al.* 1985), it was necessary to verify that the U+1888→C mutation alone was responsible for the severe-symptom phenotype. The 0.6-kbp *Bam*HI^{1755}-*Kpn*I^{2366} fragment derived from TYMC(s) was subcloned into pTYMC (see Materials and Methods), creating pTYMC+C1888. The entire subcloned fragment was resequenced to ensure the presence of only the desired mutation. Inoculation of Chinese cabbage plants with capped TYMC+C1888 transcripts yielded severe systemic symptoms identical to those of TYMC(s) developed on systemically infected leaves. Virion RNA extracted from the infected leaves was subjected to RNase protection analysis, revealing the same digestion pattern observed for TYMC(s) RNA (Fig. 2B,C). The partial sequence of the progeny RNA was also determined after reverse transcription and PCR amplification between nucleotides 1817 and 2324. The U+1888→C substitution was the only mutation found (not shown).

These experiments firmly establish that the severe symptoms observed in Chinese cabbage were due to the U+1888→C transition. This substitution alters the coding of ORF-69, resulting in a tyrosine to histidine substitution in the ORF-69 product, but does not alter the coding of ORF-206.

Severe symptoms are associated with more efficient viral movement in plants rather than enhanced replication in cells.

We have previously shown that ORF-69 expression is dispensable for viral replication in isolated protoplasts (Bozarth *et al.* 1992). It would thus be expected that the U+1888→C mutation, which affects only the coding of ORF-69, does not affect RNA replication in protoplasts. The replication of TYMC+C1888 was studied in Chinese

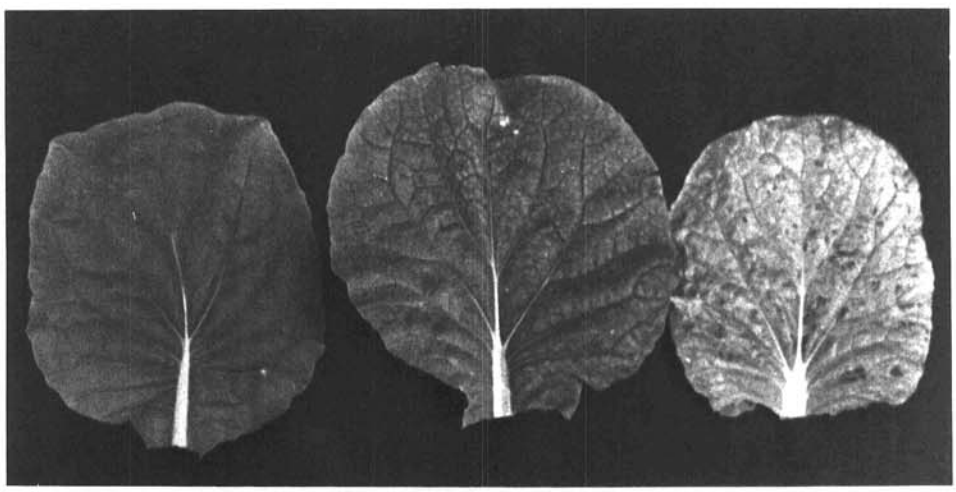

Fig. 1. Symptomatology of turnip yellow mosaic virus (TYMV) isolates on Chinese cabbage. Equivalent noninoculated leaves are shown from a mock-inoculated plant (left), and from systemically infected plants inoculated with TYMC (middle) and TYMC+C1888 (right).

cabbage protoplasts and in plants, and compared with that of other mutants, which from previous studies were shown (or were expected) to replicate more efficiently than TYMC. TYMC-U57 and TYMC+A92 have been studied previously (Tsai and Dreher 1991, 1992) and the combined mutants TYMC+A92-U57 and TYMC+A92+C1888-U57 were constructed by subcloning. The additional mutants were studied to screen for a variant that replicated to higher levels than TYMC, since such a variant would facilitate our further studies on the molecular biology of TYMV. Because virion RNAs have a higher specific infectivity than *in vitro* transcripts (Weiland and Dreher 1989), virion RNAs were freshly prepared from plants systemically infected with TYMC and each of the above mutants.

Chinese cabbage protoplasts (4×10^5) were inoculated with each of the virion RNAs (3 μg) and were harvested after incubating for 48 hr at 25° C. The accumulations of coat protein and of genomic and subgenomic RNAs were determined in Western and Northern blots, respectively (Fig. 3), and bands were quantified by laser scanning densitometry (Table 1). Two Chinese cabbage plants were inoculated with each virion RNA, and fully formed systemically infected leaves were harvested 3 wk after inoculation. Virions were prepared from each plant. Protoplasts infected with mutants TYMC+C1888 and TYMC+A92+C1888-U57 accumulated viral products to levels 1.2–1.6 relative to TYMC (Fig. 3; Table 1). However, the accumulations of mutant virus in plants were three- to fourfold higher than for TYMC (Table 1). The accumulations of viral products in protoplasts infected with the other mutants (TYMC+A92, TYMC-U57, and TYMC+A92-U57) ranged between 1.0 and 2.0 relative to TYMC, and the yields in plants were 1.2–1.4 relative to TYMC (Table 1). Thus, the mutants carrying the C+1888 mutation were distinguished by an increased accumulation relative to TYMC in plants that was not matched by increased replication in protoplasts. For the other mutants, the slightly enhanced accumulations were similar in protoplasts and plants. These results support the conclusion that improved virus spread due to the mutation in the ORF-69 product underlies the enhanced yield and more severe symptoms associated with the U+1888→C mutation. The increased area of chlorosis clearly indicates that the mutant infections spread to a higher proportion of leaf cells. It is uncertain whether the increased severity of chlorosis (yellow green cf. pale green) is a consequence of diminished amounts of healthy tissue surrounding chlorotic areas, or a direct effect of some interaction between the mutant ORF-69 product and the host cell. The data of Table 1 indicate, however, that the intensified symptoms are unlikely to result from increased levels of virus within infected cells.

The infection of TYMC+C1888 was compared with TYMC in turnip plants (cv. Just Right). We had previously

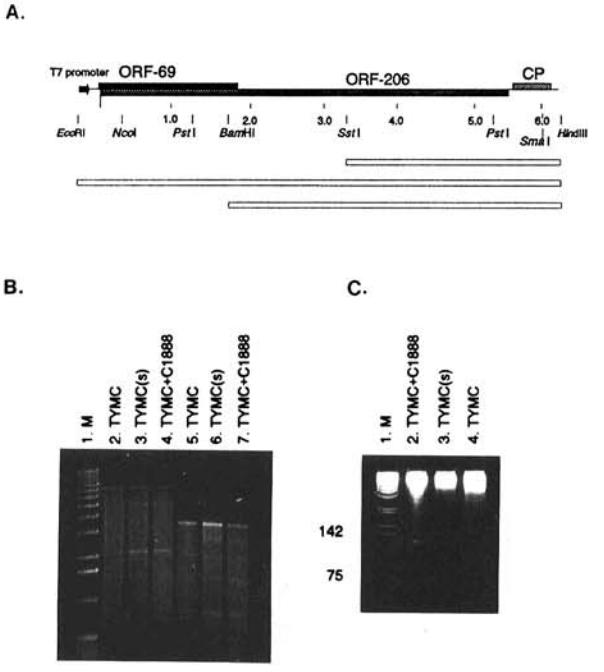

Fig. 2. Localization of the mutation responsible for severe symptoms by RNase protection analysis. **A,** Diagram of the insert of pTYMC (Weiland and Dreher 1989), the genomic cDNA clone from which infectious transcripts can be generated with T7 RNA polymerase. The major open reading frames (ORFs) and relevant restriction sites are indicated. The sequences represented in the three minus-sense RNA probes used for RNase protection analysis are indicated by the open boxes: *Hin*dIII$^{3'}$-*Sst*I^{3286}; full-length, *Hin*dIII$^{3'}$-*Eco*RI$^{5'}$; *Hin*dIII$^{3'}$-*Bam*HI^{1755}. **B,** Examples of ribonuclease protection analysis. The virion RNAs indicated were hybridized to the full-length probe (lanes 2–4) or to the *Hin*dIII$^{3'}$-*Sst*I^{3286} probe (lanes 5–7) and subjected to RNase digestion. Note that TYMC(s) is RNA derived from the original plant that had developed severe symptoms, while TYMC+C1888 is reconstructed mutant RNA derived from the mutated cDNA. The digestion products were separated on a 1% agarose gel and visualized by staining with ethidium bromide. M, 1-kb ladder DNA markers (Gibco-BRL). **C,** Example of RNase protection analysis. The indicated virion RNAs were probed with the *Hin*dIII$^{3'}$-*Bam*HI^{1755} probe and the digestion products were separated by electrophoresis on a 5% polyacrylamide gel.

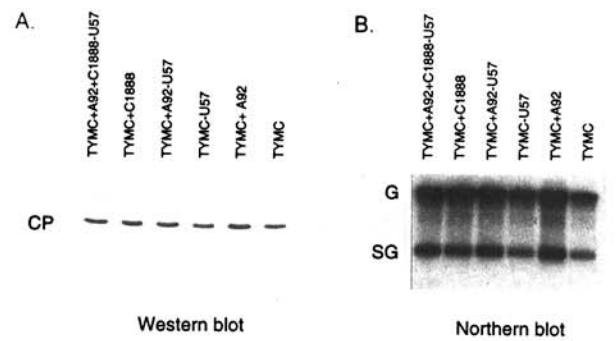

Fig. 3. Replication of TYMC and derivatives in Chinese cabbage protoplasts. Representative experiments related to Table 1 are shown. Protoplasts (4×10^5) were inoculated with 3 μg of TYMC or mutant virion RNAs, as indicated, and harvested 48 hpi. **A,** Detection of coat protein in a Western blot made after separation of proteins from 5×10^4 protoplasts by 14% SDS-PAGE. The blot was probed with anti-TYMV antiserum, and developed using horseradish peroxidase-linked second antibody and 4-chloro-1-naphthol color reagent. **B,** Detection of viral genomic (G, 6.3 kb) and subgenomic (SG, 0.7 kb) RNAs in a Northern blot. RNAs from 4×10^4 protoplasts were separated on a 1% agarose gel, blotted and probed with a ^{32}P-labeled transcript complementary to 0.9-kb at the 3′ end of the genomic RNA. TYMC mutants are named with a + to denote a nucleotide substitution at a position numbered conventionally from the 5′ end, and a − to denote a substitution in the 3′ noncoding region, numbered from the 3′ end.

found that TYMC replicated faster in turnip than in Chinese cabbage protoplasts (J. J. Weiland and T. W. Dreher, unpublished). We found both the symptoms and yield resulting from infection of turnip plants with TYMC and TYMC+C1888 to be similar. The yields were comparable to those produced by TYMC+C1888 in Chinese cabbage, while the symptoms were intermediate in severity between those caused by the two virus strains in Chinese cabbage: vein clearing, and distinct yellow-green mosaic, but no uniform yellow-green chlorosis.

DISCUSSION

Uniformly chlorotic phenotypes have been described for several viruses. For cucumber mosaic virus infecting tobacco, the chlorotic phenotype has been associated with a single amino acid substitution in the coat protein (Shintaku *et al.* 1992), while for cauliflower mosaic virus infecting turnip, chlorosis has been associated with uncharacterized sequences from gene VI (involved in the regulation of viral gene expression) and from an intergenic region (Stratford and Covey 1989). Our studies with TYMV infecting Chinese cabbage have now shown that chlorotic symptoms can result from a mutation in the movement protein encoded by ORF-69. The development of chlorosis can thus be influenced by a variety of viral genetic elements, presumably reflecting a variety of interactions between a replicating virus and the host that affect chloroplast health. The genetic makeup of the host clearly also influences the development of chlorosis (also noted by Shintaku *et al.* 1992), as demonstrated by the lack of differential symptoms between TYMC and TYMC+C1888 in turnips.

The severe symptoms induced by TYMC+C1888 in Chinese cabbage were found to correlate with a higher accumulation of virus in systemically infected leaves (Table 1). The L_{11} and $L_{11}A$ attenuated strains of TMV provide a similar example where both viral yield and symptom severity are decreased as a result of impaired cell-to-cell movement. The L_{11} and $L_{11}A$ strains produce mild symptoms and accumulate to yields about 10–20% those of the L strain. The altered phenotype is due to mutations in the essential nonstructural gene encoding p126/p183 (Nishiguchi *et al.* 1985) that have no influence on the accumulation of genomic and coat protein subgenomic RNAs in isolated protoplasts, but do decrease the levels of subgenomic RNA encoding the p30 movement protein (Watanabe *et al.* 1987). Symptom severity and virus accumulation in turnips infected with cauliflower mosaic virus were also found to be coordinately influenced by sequence variations in the movement protein (gene I), although gene VI sequences were additionally implicated (Anderson *et al.* 1991).

The movement protein encoded by TYMC+C1888 has a tyrosine to histidine substitution relative to TYMC (Dreher and Bransom 1992) at position 601 of the 628 amino acid long protein. Interestingly, histidine is present at this position in the reported sequences of both the Paris (France) holding of TYMV-type (also originally obtained from Strasbourg, France, but apparently maintained in Chinese cabbage; Morch *et al.* 1988) and an Australian isolate of TYMV (Keese *et al.* 1989). Our studies indicate that our cloned TYMV isolate, TYMC, produces relatively mild symptoms in Chinese cabbage, and that TYMC+C1888 is more representative on this host of the TYMV strains described by Markham and Smith (1946, 1949) and Matthews (1980). However, TYMC and TYMC+C1888 are indistingishable on turnip, perhaps reflecting the fact that TYMC was cloned from a turnip-adapted type strain of TYMV (Weiland and Dreher 1989).

Symptom development in viral infections is clearly a complex process that is influenced by various viral elements (Daubert 1988; Stratford and Covey 1989). Our studies with TYMV emphasize the importance of virus movement in symptom development and point to the potential for controlling viral infections by impairing cell-to-cell movement.

MATERIALS AND METHODS

Materials.

Chinese cabbage (*Brassica pekinensis* (Lour.) Rupr. 'Spring A-1') and turnip (*Brassica rapa* L. 'Just Right') plants were grown in a growth chamber under 16-hr day-length at 21° C. Plasmid pTYMC (Weiland and Dreher 1989), a cDNA clone from which infectious TYMC (TYMV-Corvallis strain) genomic RNA can be transcribed with T7 RNA polymerase, was the original source of all viral RNAs used. Enzymes and reagents were from the sources described in Tsai and Dreher (1992).

Virion RNA extraction and characterization.

Virus was isolated from infected plants by polyethylene glycol precipitation according to Lane (1986) and quanti-

Table 1. Replication of turnip yellow mosaic virus (TYMC) mutants in Chinese cabbage

	Protoplasts[a]			Plants[b]
Mutant	Relative coat protein	Relative genomic RNA	Relative subgenomic RNA	Relative virus yield
TYMC (wild type)	1.0	1.0	1.0	1.0
TYMC+A92	1.14	1.57	1.94	1.23
TYMC−U57	1.02	1.20	1.04	1.41
TYMC+A92−U57	1.24	1.60	1.95	1.19
TYMC+C1888	1.18	1.64	1.37	4.03
TYMC+A92+C1888−U57	1.20	1.26	1.40	3.32

[a] Protoplasts were harvested 48 hpi and analyzed as shown in Figure 3. The results were derived from four separate experiments and were quantified by laser densitometric scanning.
[b] Virions were purified from at least two systemically infected leaves harvested 3 wk after inoculation.

tated spectrophotometrically. Virion RNA was prepared from virus by two phenol/chloroform extractions and one chloroform/isoamyl alcohol extraction, followed by ethanol precipitation. The concentration of RNA was determined spectrophotometrically, and its quality was assessed by agarose gel electrophoresis. Ribonuclease protection experiments were performed as described (Tsai and Dreher 1992). Virion RNAs (2 μg) were probed with minus-strand RNAs representing TYMC sequences between the following restriction sites: *Hin*dIII$^{3'}$-*Eco*RI$^{5'}$ (full length), *Hin*dIII$^{3'}$-*Bam*HI^{1755}, and *Hin*dIII$^{3'}$-*Sst*I^{3286}.

The partial sequences of virion RNAs were derived after reverse transcription and amplification by polymerase chain reaction (PCR). This involved PCR amplification using d(GCGGAGAGAAGACAATTCAAGGTG) as 3′ oligomer to prime opposite nucleotide 2431, in combination with d(CCTGAGGCAACATTGG) to prime at nucleotide 1255 or d(CCTTTTACTCCCACGACTTC) to prime at nucleotide 1836. Amplified fragments were either cloned into pUC18 or sequenced directly (Winship 1989).

Cloning and transcription.

Since our previous studies with TYMV have focused on the 3′ noncoding region, we have adopted the convention of numbering nucleotides from the 5′ beginning with G+1, and those in the 3′ noncoding region beginning at the 3′ terminus with A-1 (Tsai and Dreher 1992).

The double mutant TYMC+A92-U57 was constructed by fusing the mutant sequences from two previously reported mutant clones, pTYMC-U57 (Tsai and Dreher 1991) and pTYMC+A92 (Tsai and Dreher 1992). Fragments between the unique *Eco*RI$^{5'}$ and *Bam*HI^{1755} sites were exchanged. In preparation of the mutant pTYMC+C1888, double-stranded cDNA was synthesized by using the (−) sense oligomer d(AAGGCGTCGGAAAACAT GGCGGAAGAGC) to prime at nucleotide 3738 for first-strand synthesis, and a 59-mer oligomer corresponding to a 5′ *Eco*RI site and the T7 promoter fused to nucleotides 1–35 of the TYMV genome for second-strand synthesis. The double-stranded cDNA was digested with *Bam*HI^{1755} and *Sst*I^{3286} to generate a 1.5-kb fragment that was cloned into pUC18. A 0.6-kbp *Bam*HI^{1755}-*Kpn*I^{2366} cDNA fragment containing the C+1888 mutation was then subcloned into pTYMC to create pTYMC+C1888. The triple mutant TYMC+A92+C1888-U57 was constructed by subcloning the 1.5-kb *Bam*HI^{1755} to *Sst*I^{3286} from pTYMC+C1888 into pTYMC+A92-U57.

Plasmid DNAs were prepared from 50-ml bacterial cultures, and the mutant sequences were confirmed by double-stranded DNA sequencing (Chen and Seeburg 1985). Capped genomic transcripts labeled with [α-^{32}P]UTP (0.1 Ci/mmol) were prepared with T7 RNA polymerase from DNA templates linearized with *Hin*dIII, and were analyzed as described previously (Weiland and Dreher 1989) prior to inoculation.

RNA inoculations and analysis of viral products.

Protoplasts were prepared from Chinese cabbage plants and inoculated as previously described (Tsai and Dreher 1992). Inoculated protoplasts were incubated under constant light at 25° C for 48 hr prior to harvest. Three-week-old Chinese cabbage plants with two true leaves were used for inoculations of whole plants (Tsai and Dreher 1992).

The levels of coat protein in harvested protoplasts were analyzed in Western blots using horseradish peroxidase-labeled secondary antibody and the chromogenic substrate 4-chloro-1-naphthol as described (Weiland and Dreher 1989). RNA was extracted from protoplasts, glyoxalated, electrophoresed through 1% agarose, and transferred to nylon membranes as described (Weiland and Dreher 1989). The hybridization probe was a ^{32}P-labeled RNA transcript complementary to 0.9 kb at the 3′ end of TYMV RNA (*Pst*I-*Hin*dIII fragment, see Fig. 2A), permitting the detection of both genomic and subgenomic RNAs (Tsai and Dreher 1991). The results of Western and Northern blots were quantitated by scanning laser densitometry (Tsai and Dreher 1991).

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