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A cDNA Clone of Tomato Mosaic Virus Is Infectious in Plants

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A cDNA clone of tomato mosaic virus (ToMV) genomic RNA was fused to the cauliflower mosaic virus 35S RNA promoter and the nopaline synthase gene polyadenylation signal. The transcriptional initiation site of the 35S RNA promoter was altered by in vitro mutagenesis so that the resulting transcripts start at the first nucleotide of the ToMV sequence. In addition, 12 nucleotides were inserted in the 5′ untranslated region of the ToMV genome. This plasmid, pSLN, was used to inoculate several host plants of ToMV. Among five plant species tested, only *Chenopodium quinoa* accumulated large amounts of viral particles. The infectivities and systemic movements of the resulting viruses were the same as those of virus preparations obtained from a ToMV infection of *C. quinoa*. Primer extension analyses revealed that the 5′ end of the viral genomic RNA was identical to those of RNAs isolated from virus progeny of an infection with T7 transcripts analogous to pSLN. Moreover, the insertion in the 5′ untranslated region of the viral genome was stably maintained through several systemic passages of the virus. Thus, inoculation of plants with a plasmid containing a cDNA clone of an RNA virus under the control of a eukaryotic promoter seems to be a convenient alternative to the generation of in vitro transcripts and should facilitate the analysis of viral mutants generated at the DNA level.

Tomato mosaic virus (ToMV) is a rod-shaped plant virus with a genome consisting of one molecule of plus-stranded RNA. ToMV is closely related to tobacco mosaic virus and, like tobacco mosaic virus, has been used for many fundamental studies concerning packaging, replication, movement and host-pathogen interactions of plant viruses (2, 4, 6, 12, 16). A major breakthrough was achieved when full-length cDNA clones of ToMV were obtained. The viral cDNA was fused to the lambda PM promoter, thus allowing the generation of in vitro transcripts by *Escherichia coli* RNA polymerase (8). These in vitro-generated RNAs proved to be infectious when inoculated onto host plants. Until now, this system has successfully been applied to many plant viruses with promoters and RNA polymerase enzymes from different sources (1). However, since the genomes of most RNA viruses are several kilobases in size, considerable effort has to be invested to optimize the in vitro transcription conditions to obtain a high yield of full-length and infectious RNA molecules. This is especially true when mutant viruses, which can readily be generated at the DNA level, are to be analyzed for the functions of their gene products. Thus, a less laborious and time-consuming method to obtain progeny virus from cDNA would be highly desirable.

For animal and bacterial RNA viruses, it has been demonstrated that cDNA is infectious by itself. For example, plasmids containing a full-length cDNA copy of polio virus were introduced into mammalian cells by DNA transfection. This procedure proved to be sufficient to obtain a replicating virus population (15). It has been speculated that cryptic promoter sequences in the bacterial plasmids lead to the formation of infectious transcripts in the nucleus. Accordingly, the yield of progeny virus could be improved substantially by fusing the viral cDNA to a eukaryotic promoter (18). Here, we investigate whether cDNA constructs of ToMV, a plant RNA virus, can similarly give rise to infectious viral RNAs when transiently expressed in plant tissue under the control of a strong promoter.

For the construction of an infectious cDNA clone containing the genome of a plant RNA virus, a full-length clone of ToMV was joined to the 35S RNA promoter from cauliflower mosaic virus (CaMV) and the polyadenylation signal of the nopaline synthase (NOS) gene in pUC18. The intermediate construct, pSP2, contained the CaMV 35S promoter from pDO432 (11) as a 390-bp *Acc*I-*Bam*HI fragment and the complete ToMV cDNA from pLFW3 (8) with three additional nucleotides between the transcriptional start site and the 5′ end of the ToMV sequence (Fig. 1A). To fuse the ToMV cDNA clone precisely to the CaMV 35S promoter, in vitro mutagenesis was performed. Single-stranded DNA was isolated from a subclone of pSP2 spanning the CaMV 35S promoter and the first 565 bp of the ToMV sequence. For the synthesis of the complementary DNA strand, the oligonucleotide 5′-CATTTCATTTGGAGAGCGTATTTTTACAAA CAACT-3′ was used. Sequence analysis of the mutagenesis products revealed that in many of the recombinants a second mutation had occurred in addition to the removal of the three nucleotides at the junction between the CaMV 35S RNA promoter and the ToMV 5′ end. Because of sequence homology of the first 5 and the last 6 nucleotides of the primer used for the in vitro mutagenesis experiment with the 5′ untranslated sequence of ToMV (Ω region), insertions of additional nucleotides took place between positions 54 and 62 in the ToMV sequence (Fig. 1B). One of these mutants, which contained 12 additional nucleotides in this region, was used for the generation of further constructs. We reasoned that such a mutant should facilitate the identification of progeny virus from cDNA infection experiments and the investigation of recombination frequencies of the resulting viruses. In addition, the unique structure of the ToMV Ω region, consisting of a consecutive stretch of CAA repeats, was interrupted by the insertion of the in vitro mutagenesis primer in this mutant (Fig. 1B). The mutagenized region was reinserted into pSP2 to reconstitute a full-length ToMV cDNA. To this construct the NOS polyadenylation signal was added, giving rise to the final plasmid pSLN (Fig. 2). For the construction of a plasmid containing the ToMV genomic sequence under the control of the T7 RNA polymerase

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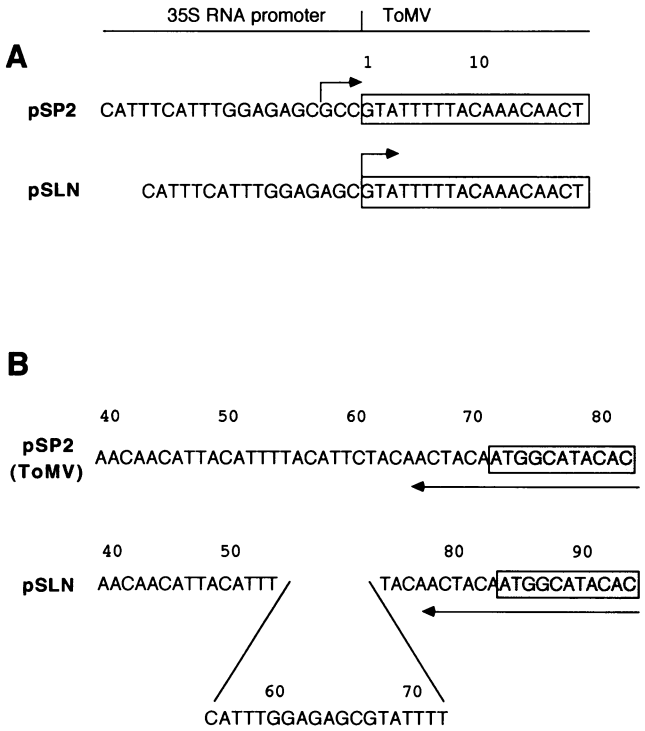

FIG. 1. Nucleotide sequence of the 5′ end of the ToMV cDNA expression construct pSLN used for DNA infection experiments. (A) Fusion of the CaMV 35S RNA promoter to the ToMV sequence (boxed). The transcription initiation site of the 35S RNA promoter is indicated by arrows. In pSLN, the three additional nucleotides at the junction between the promoter and the ToMV 5′ end have been removed by in vitro mutagenesis. The sequence of the oligonucleotide used for this experiment is identical to the sequence of pSLN shown here. (B) Nucleotide insertion between positions 54 and 62 of the ToMV sequence in pSLN, presumably due to partial homology of the oligonucleotide used for the in vitro mutagenesis reaction with this region of the ToMV cDNA. The binding site of the oligonucleotide used for primer extension analyses of viral progeny is indicated by arrows.

promoter, the 5′ end of ToMV was fused to the T7 promoter by the polymerase chain reaction (17). The resulting plasmid was designated pT7-ToMV. Like pSLN, pT7-ToMV contains an insertion of 12 nucleotides between positions 54 and 62 in the ToMV sequence.

To test whether the ToMV expression construct pSLN is infectious, different host plants of ToMV were inoculated

TABLE 1. Results of inoculating different host plants of ToMV with purified virus preparations, in vitro transcripts of pT7-ToMV, or the ToMV expression construct pSLN[a]

Host	Virus recovery from plants inoculated with:		
	pSLN	In vitro transcript of pT7-ToMV	ToMV
Nicotiana tabacum			
cv. Samsun nn	±[b]	+	+
cv. Samsun NN	−	ND[c]	+
Lycopersicon esculentum cv. Craigella	−	+	+
Datura stramonium	−	ND	+
Spinacea oleracea	−	ND	+
Chenopodium quinoa	+	ND	+

[a] Successful ToMV infection was determined by SDS-PAGE and by Western blotting.
[b] Virus could be obtained after inoculation with 100 μg of pSLN, 4 weeks of incubation, and two systemic passages.
[c] ND, not determined.

with 50 μg each of supercoiled plasmid DNA. After 7 days, the inoculated leaf was homogenized with an equal volume of 100 mM Tris buffer (pH 7) and the extracts were analyzed for the virus content by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7) and Western immunoblotting (13) with an antiserum directed against the ToMV coat protein and the enhanced chemiluminescence detection system (Amersham). As controls, infection experiments were performed with in vitro transcripts of pT7-ToMV (3) and with a preparation of the purified virus. The results from different infection experiments are presented in Table 1. All plants tested are known to be host plants of ToMV and thus were successfully infected by the purified virus as well as by the genomic viral RNA obtained from in vitro transcription reactions. In contrast, an accumulation of ToMV after inoculation with the DNA construct pSLN was only observed with *Chenopodium quinoa*, a systemic host plant for ToMV. The amount of virus formed was roughly proportional to the amount of pSLN DNA rubbed onto the leaves (Fig. 3). Even 1 μg of pSLN DNA applied to a middle-sized leaf of *C. quinoa* proved to be sufficient to yield an accumulation of about 20 ng of progeny virus per mg of leaf material after 1 week of incubation. To test whether the virus particles resulting from the cDNA application procedure were infectious, *Nicotiana tabacum* cv. Samsun NN plants, which constitute a local lesion host of ToMV, were infected with virus preparations isolated from *C. quinoa*

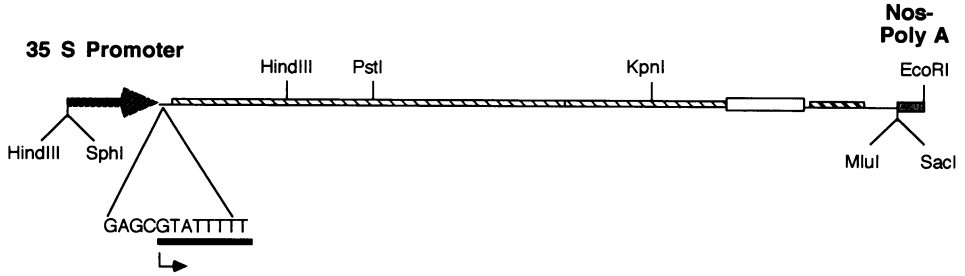

FIG. 2. Structure of the ToMV cDNA expression construct pSLN. Open reading frames are boxed (▨, 126- or 183-kDa protein; □, 30-kDa protein; ▩, coat protein). The 3′ end of the NOS gene and the CaMV 35S RNA promoter are indicated. The nucleotide sequence shown depicts the fusion of the 35S RNA promoter to the ToMV 5′ end. The transcriptional start site is indicated by an arrow.

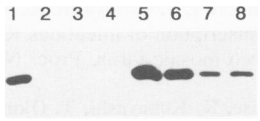

FIG. 3. Immunodetection of the ToMV coat protein in leaf extracts from *C. quinoa*. Soluble proteins were extracted from infected leaves. Equal amounts of protein per sample were separated by SDS-PAGE on a 15% polyacrylamide gel and analyzed for the ToMV coat protein by Western blotting. Lanes 1, 0.1 μg of ToMV; 2, leaf extract from an untreated plant; 3 through 8, leaf extracts from plants inoculated with buffer, 50 μg of pUC18, 0.1 μg of ToMV, 50 μg of pSLN, 10 μg of pSLN, and 1 μg of pSLN, respectively.

leaves inoculated with pSLN or ToMV. Equal amounts of virus progeny, as estimated from the Western blots, were rubbed onto half leaves of tobacco plants. A virus inoculum of 0.1 μg of ToMV per 50-μl volume yielded an average of about 155 local lesions for the virus preparation obtained from cDNA infection experiments and about 158 local lesions for the virus progeny of authentic ToMV. The morphologies and the sizes of the local lesions were identical (data not shown). In addition, the infectivity of the pSLN virus progeny was tested on a systemic host of ToMV. To this end, the lower leaves of 6-week-old tomato (*Lycopersicon esculentum* cv. Craigella) plants were infected with 20 μl of leaf extract from *C. quinoa* plants that had been inoculated with 10 μg of pSLN DNA. After 7 days, large amounts of ToMV (10 μg of virus per mg of leaf tissue) could be detected in the youngest leaves of the tomato plants by SDS-PAGE and by Western blotting (data not shown). Furthermore, the genomic RNA of virus resulting from the cDNA infection procedure was analyzed by primer extension experiments. To be able to distinguish between wild-type ToMV and the virus progeny derived from a cDNA infection, a modified ToMV cDNA sequence was used to construct pSLN plasmids containing additional nucleotides inserted between positions 54 and 62 in the ToMV sequence. This insertion should give rise to a viral genomic RNA that is 12 ribonucleotides longer than that of authentic ToMV at the 5′ end (Fig. 1B). As a positive control, T7 transcripts that were generated from a vector (pT7-ToMV) containing the same mutagenized ToMV sequence as pSLN were used. The lower leaves of tomato plants were infected with 20 μl of leaf extract from *C. quinoa* that had been previously infected with 10 μg of pSLN, with in vitro transcripts resulting from 1 μg of pT7-ToMV or with 1 μg of ToMV. After 2 weeks, ToMV was purified from the upper leaves of the infected plants (5). The viral genomic RNA was isolated, and 0.1 μg of each RNA was analyzed by primer extension reactions (14) with an oligonucleotide complementary to positions 64 to 82 in the ToMV sequence (5′-GTGTATGCCATTGT AGTT-3′; Fig. 1B). The progeny virus of pSLN and the viruses resulting from an infection with T7 transcripts of pT7-ToMV gave rise to extended products that were 12 nucleotides longer than wild-type ToMV RNA (Fig. 4). To make sure that no nucleotide exchanges had occurred in the progeny virus, the regions between positions 1 and 82 of the primer extension products, including the inserted sequence, were amplified by the polymerase chain reaction. The resulting fragments were cloned in pUC18 and analyzed by DNA sequencing. No differences in the nucleotide sequences could be observed between the mutagenized ToMV sequence and the polymerase chain reaction products (data not shown).

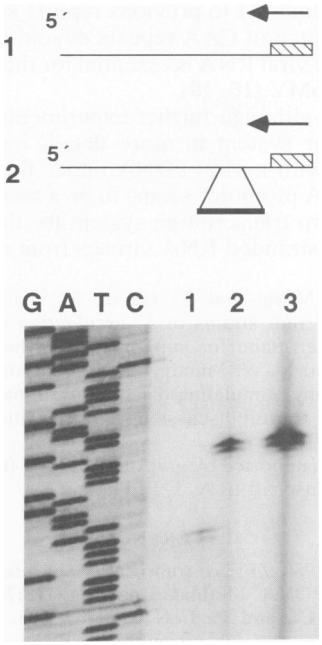

FIG. 4. Analysis of the viral progeny derived from a cDNA infection with pSLN by primer extension. *C. quinoa* plants were inoculated; after 7 days, the viral progeny was propagated in tomato plants. Primer extension reactions were performed with viral RNAs obtained from infections with authentic ToMV (lane 1), plasmid DNA of pSLN (lane 2), or transcripts of pT7-ToMV (lane 3). The products of standard sequencing reactions were used as size markers. The positions of the primer used (arrows) and the nucleotide insertion in pSLN (black bar in diagram 2) are indicated schematically.

In this communication, we show that a plasmid containing a ToMV cDNA clone fused to the CaMV 35S RNA promoter and the NOS polyadenylation signal can be used to infect plants by rubbing the DNA onto the leaves. The resulting viruses are infectious on local-lesion hosts and on systemic host plants of ToMV. Furthermore, the virus progeny resulting from cDNA infection experiments cannot be distinguished from viruses obtained from an infection with in vitro transcripts of analogous constructs. However, various host plants of ToMV exhibited quite different responses to infection with the ToMV cDNA construction. Whereas *C. quinoa* was highly susceptible to DNA inoculation, tomato, which is the native host of ToMV, showed a high degree of resistance to DNA infection. Even when we apply more than 100 μg of pSLN to either very young or older tomato plants, we have never succeeded in achieving even a local infection on this host plant. Our results are in good agreement with observations published recently by Mori et al. (9). When these authors tried to infect barley plants, a systemic host, and *Chenopodium hybridum* plants, a local-lesion host, with cDNA of brome mosaic virus, only *C. hybridum* showed the expected host response. Taken together, these data indicate that *Chenopodium* species are highly susceptible to inoculation with cDNAs of plus-stranded RNA viruses. Further experiments with other viruses and a broad spectrum of host plants will be necessary, however, to investigate this phenomenon in more detail. Furthermore, by using a mutagenized ToMV cDNA, we show that the insertion in the Ω sequence of ToMV of G residues, which are normally absent from this region, can clearly be tolerated by the virus. Our

findings are in contrast to previous reports suggesting that a consecutive stretch of CAA repeats devoid of G residues at the 5′ end of the viral RNA is essential for the replication and uncoating of ToMV (10, 19).

In summary, although further experiments are needed to characterize our system in more detail, inoculation of *C. quinoa* plants with a viral cDNA under the control of the CaMV 35S RNA promoter seems to be a feasible alternative to the T7 in vitro transcription system for the generation of infectious plus-stranded RNA viruses from cloned cDNA.

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