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Expression of tobacco mosaic virus RNA in transgenic plants

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Summary. Tobacco mosaic virus (TMV) is a message-sense, single-stranded RNA virus that infects many *Solanaceae* plants. A full-length cDNA copy of TMV genomic RNA was constructed and introduced into the genomic DNA of tobacco plants using a disarmed Ti plasmid vector. Transformed plants showed typical symptoms of TMV infection, and their leaves contained infectious TMV particles. This is the first example of the expression of RNA virus genomic RNAs in planta.

Key words: Tobacco mosaic virus – Transgenic plant – In planta expression – Ti plasmid

Introduction

Recent progress in gene manipulation techniques in plant molecular biology has made available several novel methods for plant virology. One of these is the in vitro transcription system allowing production of infectious viral RNAs from their cDNAs (Ahlquist et al. 1984; Dawson et al. 1986; Meshi et al. 1986). In this system, cDNA copies of viral genomic RNAs are put downstream of a modified lambda phage promoter and used as templates for in vitro transcription. Capped transcripts from these templates are infectious upon inoculation onto plant protoplasts or leaves. With this system, various mutations can be easily introduced into the viral RNAs at the cDNA level to examine their effect(s) on viral functions (Ishikawa et al. 1986; Takamatsu et al. 1987).

Another application of genetic engineering for plant virology makes use of the Ti plasmid of *Agrobacterium tumefaciens*. More than one tandemly repeated copy of the plant DNA virus genome is placed in the T-DNA of the Ti plasmid. When the *Agrobacterium* strains carrying the engineered Ti plasmid are inoculated onto a host plant of the virus, viral DNAs escape from the T-DNA and spread systematically in the plant (termed agroinfection; Grimsley et al. 1986, 1987). Successful agroinfection has also been reported for a viroid (Gardner et al. 1986).

Although agroinfection can introduce the viral DNAs into plants, the resultant plants are not transgenic; only the cells at the bacterial inoculation site have viral DNA in their genome. On the other hand, Rogers et al. (1986) have introduced genomic DNAs of a gemini virus into the genomic DNA of petunia plants by Ti plasmid mediated transformation and demonstrated the replication of the virus in the transgenic plants.

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For plant RNA viruses, Baulcombe et al. (1986) have reported the succesful expression of cucumber mosaic virus (CMV) satellite RNA from its cDNA in transgenic tobacco plants, but there has been no report of the expression of viable viral genomic RNAs in planta. Here we modified the previously reported in vitro transcription system of tobacco mosaic virus (TMV) (Meshi et al. 1986) and combined it with the Ti plasmid mediated transformation system, resulting in successful integration and expression of TMV cDNA in the transgenic tobacco plants.

Materials and methods

Promoter modification. The 1 kb fragment containing the 35 S promoter was isolated from cloned cauliflower mosaic virus (CaMV) Cabb-S strain DNA (Franck et al. 1980; Guilley et al. 1982). The ends of the fragment were made blunt and *Bam*HI linkers were added. The 0.86 kb *Bam*HI-*Cla*I fragment was subcloned from this 1 kb fragment between the *Bam*HI and *Acc*I sites of M13mp8 to create mCaP35. A synthetic 23mer oligonucleotide was designed to anneal to the transcription initiation site of the 35 S promoter with two mismatches at its 5′ end, thus creating a new *Stu*I site at the transcription initiation site (Fig. 1). 100 pmol of this modifying primer was annealed to 5 pmol of mCaP35 single-stranded DNA and extended by DNA polymerase I large fragment. The resultant heteroduplex was cleaved at the *Pvu*II site in the M13 sequence and the 511 nucleotide extended modifying primer was isolated after strand separation. This 511 nucleotide sequence was then annealed to a M13 reverse sequence primer (17mer; P-L Biochemicals) and the second strand synthesized as above. After cleavage at the *Bam*HI site, the 325 bp fragment containing the modified 35 S promoter was cloned between the *Bam*HI and *Hinc*II sites of pUC18, creating pCaP35J. The DNA sequence of the modified 35 S promoter was verified by direct sequencing (Hattori and Sakaki 1986). In the course of sequencing, a one base change from T to A at position −269 of the promoter was found compared with the published sequence (Franck et al. 1980). This point mutation is unlikely to affect the promoter function (Odell et al. 1985).

pOKL4 construction. pCaP35J was digested with *Stu*I and *Pst*I, and the 5′ half of the *Pst*I-digested full-length TMV-L cDNA (Meshi et al. 1986) was cloned between these sites to make pLCaP51. The nucleotide sequence between the promoter-cDNA junction and the *Stu*I site at residue 204 of TMV-L cDNA in pLCaP51 was verified by DNA se-

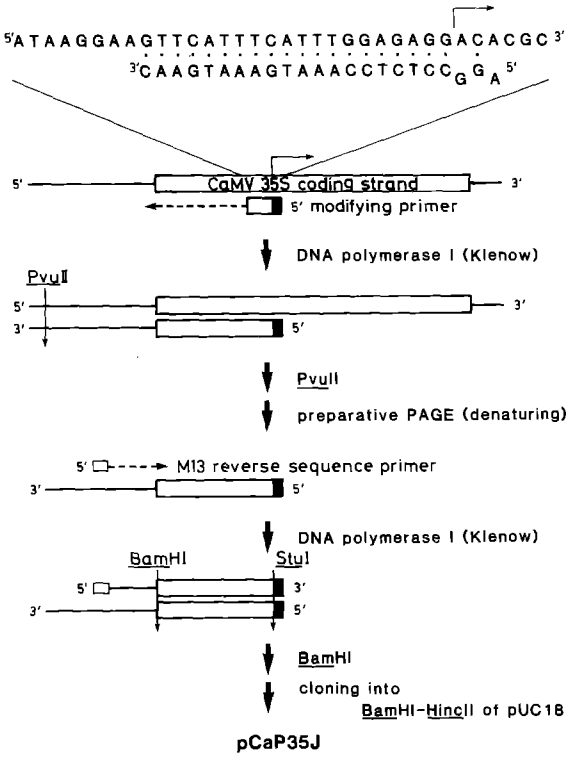

Fig. 1. Construction of the modified CaMV 35 S promoter. *Large boxes* represent the CaMV sequences, the *filled region* being the modified part. *Lines* indicate M13 sequences. *Small open boxes* represent the M13 reverse sequencing primer. The nucleotide sequences shown at the top are those of the coding strand of the CaMV 35 S promoter around the transcription initiation site (upper strand) and the 23mer modifying primer (lower strand). *L-shaped arrows* indicate the start site and the direction of transcription

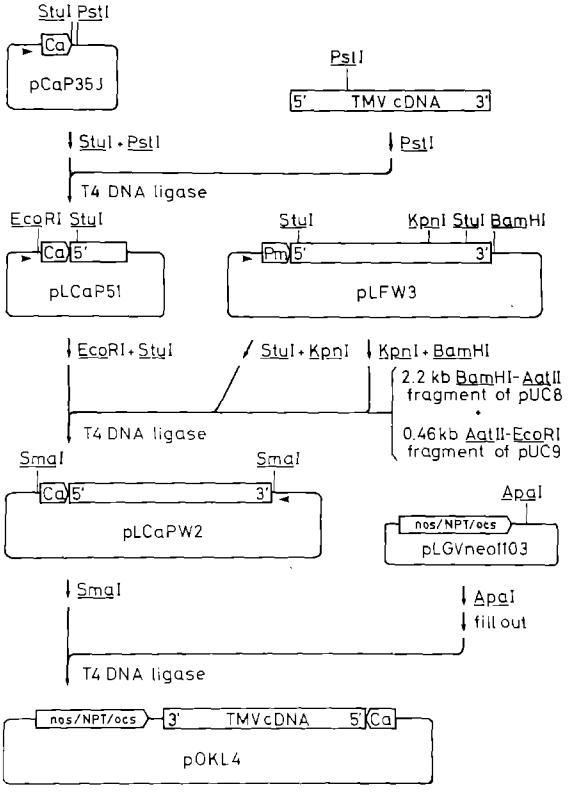

Fig. 2. Construction of the intermediate vector pOKL4. TMV-L cDNA sequences are *boxed*. Ca, modified CaMV 35 S promoter; Pm, modified lambda phage promoter, nos/NPT/ocs; chimaeric marker gene consisting of nopaline synthase promoter, neomycin phosphotransferase II structural gene and octopine synthase poly(A) signal. *Arrowheads* indicate the *lac* promoter of pUC

quencing (Guo and Wu 1982). The 4.2 kb *Stu*I-*Kpn*I fragment and the 2 kb *Kpn*I-*Bam*HI fragment were cleaved from pLFW3 (Meshi et al. 1986), and the 0.55 kb *Eco*RI-*Stu*I fragment containing the modified CaMV 35 S promoter and the 5′ portion of TMV cDNA was cleaved from pLCaP51. These fragments were ligated with the 2.2 kb *Bam*HI-*Aat*II fragment of pUC8 and the 0.46 kb *Aat*II-*Eco*RI fragment of pUC9 to make up pLCaPW2, which carried full-length TMV-L cDNA under the control of the modified CaMV 35 S promoter. This rather complicated construction was necessary because a construct carrying full-length TMV cDNA in the right orientation with respect to the *lac* promoter of pUC was toxic to its host. The 6.7 kb fragment containing the promoter-cDNA with only 20 bp of vector sequence was obtained from pLCaPW2 by *Sma*I digestion. This fragment was inserted into the filled-out *Apa*I site of the intermediate vector pLGVneo1103 (Hain et al. 1985), creating the intermediate vector pOKL4. The orientation of the promoter-cDNA in pOKL4 was verified to be as shown (Fig. 2).

Tobacco transformation. Intermediate vector pOKL4 or pLGVneo1103 (as control) were transferred to *A. tumefaciens* C58ClRif^r carrying the Ti plasmid vector pGV3850 (Zambryski et al. 1983) by conjugation (Van Haute et al. 1983). Transconjugants were selected for their antibiotic resistances, and the presence of the TMV cDNA sequence was verified by Southern analysis. Small leaf disks of tobacco plants (*Nicotiana tabacum* cv. Samsun) were infected with these *Agrobacteria* for 48 h, then transferred to a shoot-inducing medium containing 250 µg/ml Chlaforan and 100 µg/ml kanamycin. Resultant shoots were cut off and transferred to hormone-free medium containing kanamycin, and the transformants were selected for their ability to form roots on this medium.

Southern analysis. Total DNA was prepared from tobacco leaves by the small scale preparation method of Paszkowski et al. (1984). The DNA preparations were digested with *Bam*HI, and 5 µg samples of digested DNAs were electrophoresed in 1% agarose and blotted onto Z-probe membrane (Bio-Rad) by the alkaline blotting procedure (Reed and Mann 1985). The membrane was hybridized to ^{32}P-labelled CaMV 35 S promoter-TMV cDNA fragment. The copy number standards were reconstructions of *Bam*HI-digested pOKL4 plasmid and 5 µg *Bam*HI-digested total DNA from normal tobacco leaves. The amount of the standard pOKL4 DNA was calculated assuming the DNA content of tobacco cells to be 9.67 pg (Galbraith et al. 1983).

Results

Construction of modified plant promoter

To express TMV RNA from its cDNA copy in planta, it was assumed to be necessary to put the cDNA sequence

under the control of a promoter which is active in plant cells. For this purpose, we chose the promoter for the 35 S transcript of CaMV (Guilley et al. 1982), which has been shown to be capable of a relatively high level of expression of foreign genes in tobacco plants which is tissue non-specific (Odell et al. 1985). The successful production of infectious RNAs in vitro (Ahlquist et al. 1984; Dawson et al. 1986; Meshi et al. 1986) is assumed to be, at least in part, due to the coincidence of the 5′ end of the in vitro transcripts and the native viral RNAs, and Dawson et al. (1986) found that six extra nucleotides at the 5′ end of their in vitro TMV cDNA transcripts reduced the infectivity by 100-fold. Accordingly, we first modified the CaMV 35 S promoter so that transcription into RNA would start just at the 5′ end of the inserted sequence.

For this purpose, the sequence around the transcription initiation site of the CaMV 35 S promoter, which is $^{5'}$AGGACA$^{3'}$ with the second A being the initiation site, was converted to $^{5'}$AGGCCT$^{3'}$, the recognition sequence of *Stu*I. As *Stu*I cleaves $^{5'}$AGGCCT$^{3'}$ in the middle leaving blunt ends, any sequence inserted at this *Stu*I site would be transcribed from its 5′ end in plant cells. This construction was accomplished by oligonucleotide mutagenesis essentially as described (Goeddel et al. 1980; Ahlquist and Janda 1984) with some modifications (Fig. 1; see Materials and methods). As our 23mer modifying primer showed high specificity for the desired site, it was not necessary to use a large excess of the primer and/or reverse transcriptase as mentioned by Ahlquist and Janda (1984).

Construction of intermediate vector pOKL4

The TMV used here is a Japanese tomato isolate called TMV-L, whose entire genome of 6,384 nucleotides has been cloned and sequenced (Ohno et al. 1984). A full-length cDNA copy of TMV-L RNA was synthesized by primer extension as described previously (Meshi et al. 1986), and the 5′ half of *Pst*I-cut cDNA (1.8 kb) was cloned between the *Stu*I and *Pst*I sites of pCaP35J to create pLCaP51 (Fig. 2). The sequence of the promoter-cDNA junction and the 5′ portion of the cDNA was verified. Only this sequenced region was taken from pLCaP51 and recombined with the cDNA fragments from pLFW3 (Meshi et al. 1986) to make up a full-length cDNA copy. pLFW3 is a pUC9 derivative carrying full-length TMV-L cDNA under the control of the modified lambda phage promoter, and in vitro transcripts from this plasmid have been shown to be infectious, indicating that there are few mutations in the cDNA sequence in pLFW3. First we simply exchanged the P_M promoter region in pLFW3 with the modified CaMV 35 S promoter region from pLCaP51, but this construct was found to be toxic to its host *Escherichia coli*. This was presumably due to transcriptional expression of the TMV sequence driven by the upstream *lac* promoter, as we also observed that pLFW3 itself was highly unstable when the P_M promoter was derepressed. Thus the rather complicated construction of pLCaPW2 as described in Materials and methods was employed to put the TMV cDNA sequence in the opposite direction to the *lac* promoter. The 6.7 kb *Sma*I fragment of pLCaPW2 containing the entire promoter-cDNA sequence plus 20 bp of the vector sequence was isolated and inserted into the filled-out *Apa*I site of an intermediate vector pLGVneo1103 (Hain et al. 1985), carrying a kanamycin resistance gene as a marker for plant transformation, to create pOKL4, the intermediate vector for TMV cDNA integration into plant cells (Fig. 2).

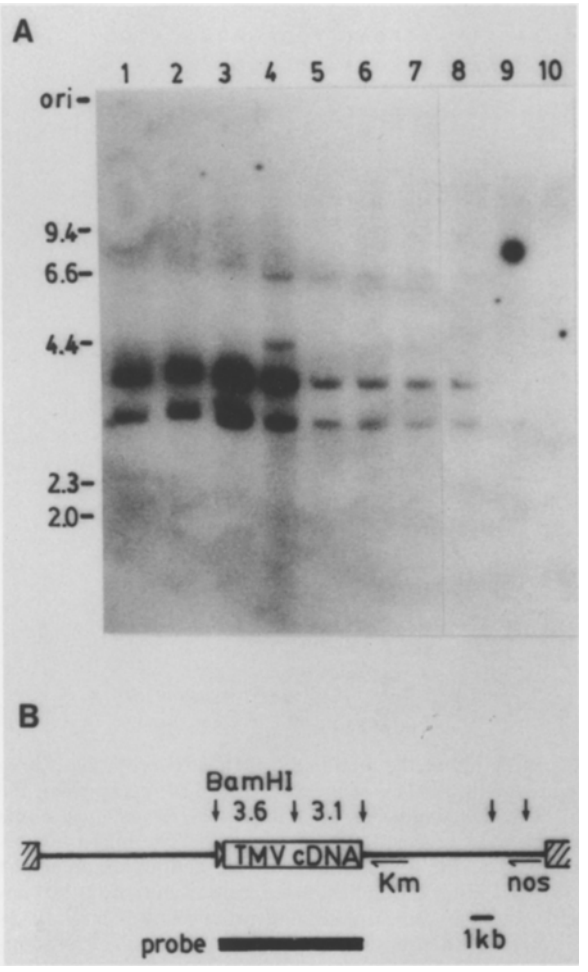

Fig. 3A, B. Southern blot analysis of total DNA from transformed plants (**A**) and the expected structure of the T-DNA in transgenic plants (**B**). **A** Lanes 1–3, 1, 2 and 5 copy number standards; lanes 4–8, five independent pOKL4 transformed tobacco lines; lane 9, pLGVneo1103-transformed tobacco; lane 10, normal tobacco. **B** Expected structure of the T-DNA region in the transgenic tobacco plants. The *open box* represents the TMV cDNA sequence. The *triangle* on the left of the TMV cDNA is the modified CaMV 35 S promoter. Km and nos represent the transformation marker genes for kanamycin resistance and nopaline synthesis, respectively. *Hatched boxes* show plant genomic DNA sequences. *Arrows* indicate the positions of *Bam*HI sites within the T-DNA region. Figures between the arrows are the expected size of the fragments (in kb). The *filled box* below indicates the probe fragment used in the Southern analysis

Integration of TMV cDNA sequence into tobacco genomic DNA

The intermediate vector pOKL4 was integrated into the T-DNA of the disarmed Ti plasmid vector pGV3850 (Zambryski et al. 1983) and the co-integrate was verified by Southern blotting. Small leaf disks of tobacco plants (*N. tabacum* cv. Samsun) were infected by *A. tumefaciens* carrying the co-integrate (Horsch et al. 1985). We obtained five kanamycin-resistant transformed plants which were tested for the presence of nopaline, another transformation marker, resulting from the expression of the nopaline syn-

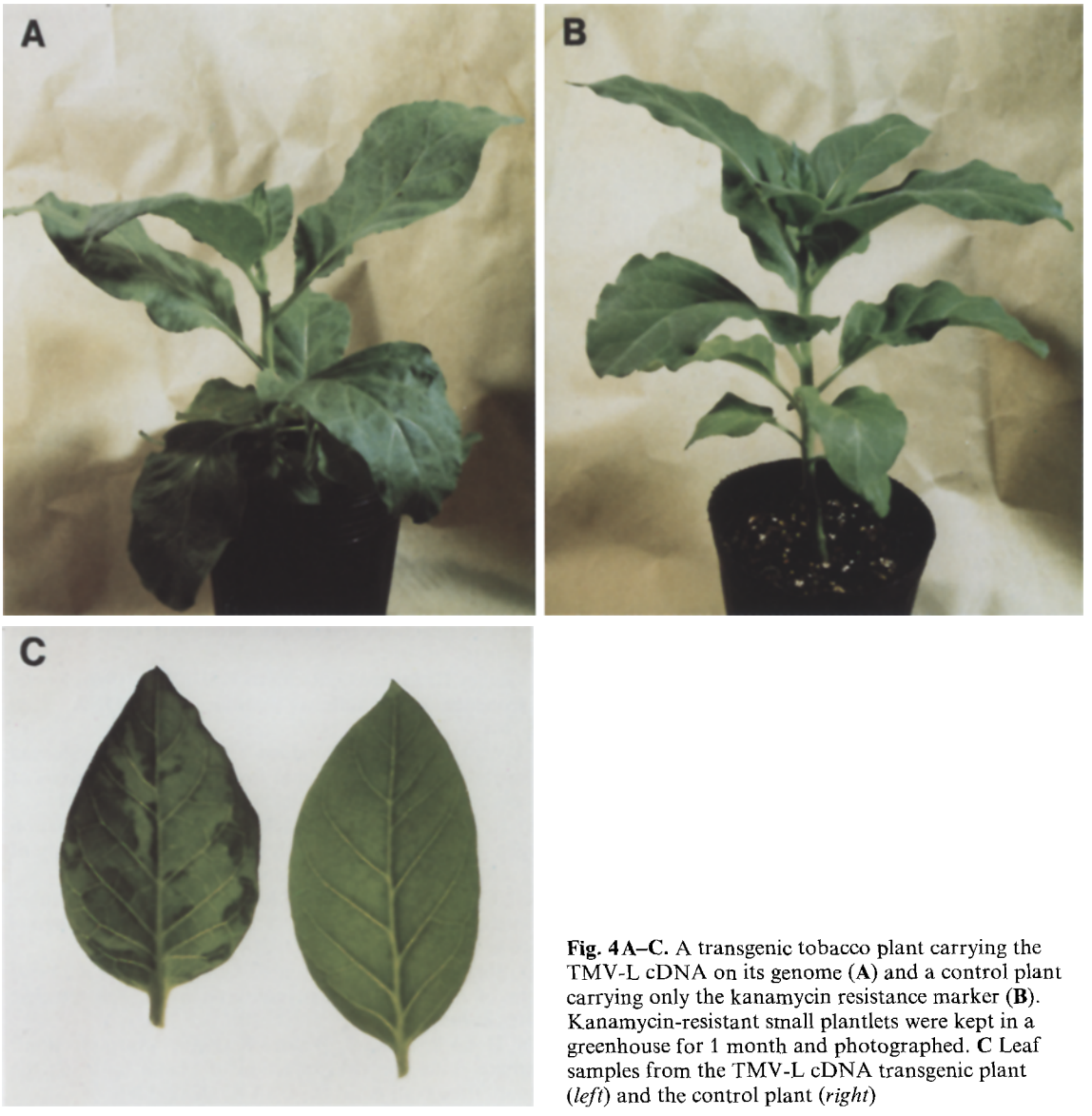

Fig. 4A–C. A transgenic tobacco plant carrying the TMV-L cDNA on its genome (**A**) and a control plant carrying only the kanamycin resistance marker (**B**). Kanamycin-resistant small plantlets were kept in a greenhouse for 1 month and photographed. **C** Leaf samples from the TMV-L cDNA transgenic plant (*left*) and the control plant (*right*)

thase gene on pGV3850 (cf. Fig. 3B). We found that three plants were nopaline positive while the other two were nopaline negative (data not shown). Loss of expression of the nopaline synthase gene in transformed plants has also been observed by others (Horsch et al. 1985).

Total DNA was isolated from the leaves of the transformed plants, digested with *Bam*HI, and subjected to Southern blot analysis using the 6.7 kb *Sma*I fragment of pLCaPW2 (Fig. 2) as a probe. Since there is one *Bam*HI site in the TMV cDNA sequence and two next to the *Sma*I sites at both borders of the promoter-cDNA sequence, the 3.6 kb and 3.1 kb fragments representing the 5′ and 3′ halves of the promoter-cDNA sequence, respectively, should be detected (Fig. 3B). As expected, two bands of 3.6 kb and 3.1 kb were detected from the *Bam*HI digests of the transformed plant DNAs (Fig. 3A). This indicates that the CaMV 35 S promoter-TMV cDNA sequence was integrated into the plant genome without large rearrangements or deletions. Faint upper bands of about 6.6 kb may be the result of incomplete digestion by *Bam*HI. In one plant, an additional band of about 4.4 kb was detected (Fig. 3A, lane 4); this may reflect some rearrangements during the transformation process in this plant. In addition, this plant had two to three copies of the CaMV 35 S promoter-TMV cDNA sequence per cell, while the other four plants had only a single copy (Fig. 3A; compare the band intensities with those of copy number standards in lanes 1–3). In a preliminary test, the kanamycin resistance of the R1 progeny from two of these four transgenic plants segregated in an approximately 3:1 ratio (data not shown).

Expression of TMV in transgenic plants

Every leaf of all five transgenic plants showed the typical symptoms of TMV infection: mosaic and wrinkling (Fig. 4). To confirm that these symptoms were due to viral multiplication, leaf samples of the transgenic plants were homogenized and inoculated onto local lesion host tobacco plants (cv. Xanthi nc). Lesions as large as those induced by control inoculation with purified TMV-L particles were observed (not shown). The number of lesions relative to control inoculation was of the same order as induced by inoculation with leaf homogenates of systemically infected plants that had been inoculated with TMV mechanically. We also de-

tected virus crystals by microscopic examination in the protoplasts derived from the transgenic plant leaves (not shown).

Discussion

Baulcombe et al. (1986) introduced tandemly repeated cDNA copies of CMV satellite RNA, with a plant promoter and termination signal, into the tobacco plant genome, and showed the production of biologically active satellite RNAs in the transgenic plants. Their strategy takes advantage of the fact that CMV satellite RNAs replicate through multimeric intermediates and that they are very small (335 nucleotides), and obviously cannot be used for the expression of viral genomic RNAs. Accordingly, we chose to introduce the cDNA copy of TMV genomic RNA with a modified CaMV 35 S promoter into the tobacco genome and succeeded in getting expression and replication of biologically active viral RNAs from this construct in planta. Recently, Garcia et al. (1987) introduced a cDNA copy of cowpea mosaic virus M-RNA with non-modified plant promoters into cowpea callus tissue and showed transcription and translation of the viral RNA in vivo, but replication of the cDNA transcripts was not demonstrated.

The promoter used here was modified so that the 5′ end of the in vivo transcripts would coincide with the 5′ end of the TMV RNA. On the other hand, we made no effort to define the 3′ end of the in vivo transcripts, since previous results suggested that this was not essential. In vitro transcripts from a circular template plasmid thought to have heterogeneous lengths of extra sequences at the 3′ end were still infectious, although their infectivity was decreased (Meshi et al. 1986). Several explanations are possible to account for this: termination of transcription near the TMV cDNA 3′ end by chance or some unknown factors; post-transcriptional processing of the primary transcripts; recognition of the internal TMV 3′ region in the transcripts by the TMV replication machinery and initiation of the minus strand synthesis from there. These possibilities cannot be distinguished from our results.

The system described here offers a novel method for investigations into the multiplication mechanism(s) and pathology of plant RNA viruses. Compared with the in vitro transcription system, our in planta system has the disadvantage of requiring a much longer time. However, in the in vitro system, in which infectious viral cDNA transcripts are inoculated onto plant leaves, the apparent replication of the virus is dependent on the infection (entering the cells) and transportation processes of the virus. Consequently, a mutation which blocks the cell to cell movement of the virus would result in the apparent loss of infectivity of the mutant. This may partly be overcome when protoplasts are used instead of whole plants, but it is hardly possible to observe the development of symptoms in protoplasts. With the in planta system, we can separate viral replication and development of symptoms from the viral infection and transportation processes, as every cell in the transgenic plants is expected to express the virus. Recently, the agroinfection system has been successfully used to infect whole plants with plant DNA viruses (Grimsley et al. 1986, 1987). Although the agroinfections reported so far have been restricted to DNA viruses and viroids, RNA viruses can be "agroinfected"; again, viral replication and transportation cannot be separated. Currently, we are constructing deletion mutants of TMV cDNA and introducing them into the tobacco genome to examine their effect(s) on viral replication and development of symptoms.

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