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Construction of Tobacco Mosaic Virus Subgenomic Replicons That Are Replicated and Spread Systemically in Tobacco Plants

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Two tobacco mosaic virus (TMV)-derived replicons, created by deletion of most of the 126/183-kDa open reading frame (ORF), replicated and systemically invaded tobacco plants when supported by wild type TMV. One RNA replicon contained an internal direct repeat of 476 nucleotides from the 3' end of the 30-kDa ORF. Although this RNA was replicated, most of the progeny were heterogeneous in size and smaller than the original transcript. A second TMV-derived RNA replicon, without any internally repeated sequences and containing a deletion of the 5' portion of the 30-kDa ORF as well as most of the 126/183-kDa ORF, was created and coinoculated with wild type TMV as helper. This RNA also was replicated efficiently and systemically invaded tobacco plants. An examination of the sequences of cDNA clones obtained after PCR amplification of the progeny population of this RNA replicon demonstrated that the observed size heterogeneity was due to deletions and insertions adjacent to the artificially created deletion junction. These data demonstrate that a TMV infection is capable of supporting an artificially created RNA replicon, similar to defective interfering RNAs or satellites. However, these dependent RNAs were replicated without noticeably interfering with wild type TMV symptoms or replication. © 1991 Academic Press, Inc.

INTRODUCTION

Many RNA viruses generate defective derivatives, consisting of genomic RNAs with internal deletions, which replicate along with the viral genome (Schlesinger, 1988). Replication of the helper virus is usually reduced by the replication of the defective RNAs (defective interfering RNAs, DIs). However, this is a characteristic that primarily is associated with RNA viruses of animals. Although most plant viruses have RNA genomes, a striking feature of these viruses is a general lack of DIs. Only the tombusvirus and carmovirus groups have been found to generate DIs (Hillman *et al.*, 1987; Burgyan *et al.*, 1989; Li *et al.*, 1989). However, many RNA plant virus groups do support a different type of dependent replicon: satellite viruses and satellite RNAs. In contrast to DIs, these dependent RNAs have little or no sequence similarity with the helper virus and do not appear to originate from the helper genome (Francki, 1985).

There must be requirements for generation or support of DIs that are not sufficiently provided by most plant RNA virus systems. One requirement for amplification of a dependent RNA appears to be that the RNA have specific *cis*-acting elements. Within the Sindbis virus "supergroup" (Goldbach *et al.*, 1990), the *in vitro* replicase complexes of several members have been found to amplify only related RNAs and this specificity

is thought to be associated with specific sequences (Lehtovaara *et al.*, 1982; Levis *et al.*, 1986; Tsiang *et al.*, 1988). *Cis*-acting elements required for RNA amplification of several plant viruses have been identified, which, in general, occur at the termini, within the terminal 100 nucleotides (nts) of the 5' end for positive-sense strand promotion, and within the 200 3' terminal nts for negative-sense strand promotion (Pacha *et al.*, 1990; Pogue *et al.*, 1990; Rao and Hall, 1990). Exceptions are the internal *cis*-acting element of brome mosaic virus RNA 3 (French and Ahlquist, 1987) and alfalfa mosaic virus (Van Der Kuyl *et al.*, 1990).

Dependent RNAs that replicate with a helper virus in protoplasts have been produced *in vitro* by internal deletions in genomic RNAs (Pogue *et al.*, 1990; Pacha *et al.*, 1990). However, for a dependent RNA to replicate and systemically invade an intact plant with the helper virus, it must also move from cell to cell and from leaf to leaf. With plant RNA viruses, this is thought to require encapsidation. Most importantly, the dependent RNA must be replicated and must move efficiently enough to effectively compete with the helper virus and not be left behind as the helper invades the plant.

Tobacco mosaic virus (TMV) is a member of the Sindbis supergroup, with a positive-sense, single-stranded RNA genome (6395 nts) that encodes at least four proteins (Goelet *et al.*, 1982). The 126-kDa protein and the 183-kDa readthrough protein are produced from the 5' open reading frame (ORF) of the virion RNA (Pelham, 1978). The 30-kDa movement protein and the

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17.5-kDa capsid protein are translated from 3' coterminal subgenomic mRNAs that are produced during replication (Siegel *et al.*, 1976; Hunter *et al.*, 1976; Beachy and Zaitlin, 1977). A satellite virus has been found naturally associated with one tobamovirus and can replicate with the help of any of several tobamoviruses (Valverde *et al.*, 1991). The only sequence similarity between the satellite virus RNA and the helper genome are two 50-nt sequences near the 3' termini (Mirkov *et al.*, 1989).

In this paper we report the construction *in vitro* of TMV-derived, dependent RNA replicons that replicate to high levels and systemically invade tobacco plants with TMV as helper. The replicons were produced by making approximately 5-kb deletions within the replicase ORFs of TMV. However, these dependent RNAs were not replicated homogeneously. Instead, the progeny consisted of heterogeneous populations with deletions and insertions that were located primarily at the original deletion/ligation junctions. These dependent RNAs did not affect the ability of TMV to replicate nor did they alter the effect of the virus upon the host.

MATERIALS AND METHODS

Construction of TMV-defective replicons

Transcripts of the wild type virus (TMV 204), strain U1 of TMV, and RNA replicons TMV-KKL and TMV-KL (Fig. 1) were derived from pTMV 204, a complete genomic clone of TMV (Dawson *et al.*, 1986). Nucleotide numbering is that of Goelet *et al.* (1982). pTMV-KKL was derived from pTMVS3-28 and pTMV-KK1. pTMVS3-28 has the coat protein ORF replaced with a *Xho*I restriction endonuclease site (Dawson *et al.*, 1988). pTMV-KK1 has a *Xho*I restriction endonuclease site (3 non-TMV nts 3' of TMV nt 4901) inserted between the 183- and the 30-kDa ORFs (Lehto and Dawson, 1990). pTMV-KKL was created by removal of most of the 126/183-kDa ORF and the 5' half of the 30-kDa ORF of pTMVS3-28 using the restriction endonucleases *Sma*I (258) and *Pvu*II (5236), to create the intermediate plasmid pTMV-DS3-28. An 11-bp fragment, *Xho*I (5 nonTMV nts 3' of nt 5712)–*Nsi*I (6202), of pTMV-DS3-28 was then replaced with the 1302-bp *Xho*I (4901 + 3)–*Nsi*I (6202) fragment of pTMV-KK1, creating pTMV-KKL, which has a direct repeat of 476 bp of the 3' region of the 30-kDa ORF (5237–5712) (Fig. 1B).

Plasmid pTMV-KL (Fig. 1C) was created from pTMV 204 by *Sma*I (258) and *Pvu*II (5236) digestion followed by religation resulting in the deletion of most of the 126/183-kDa ORF and the 5' portion of the 30-kDa ORF (TMV nt 259–5236). Both mutant constructs were con-

firmed by restriction endonuclease and DNA sequence analysis.

RNA transcription and plant inoculation

RNA transcripts of the *Pst*I linearized TMV plasmids were produced with *E. coli* RNA polymerase (Ahlquist and Janda, 1984; Dawson *et al.*, 1986). Helper virus was amplified by inoculating the leaves of *Nicotiana tabacum* (L.) cv Xanthi-nc plants with pTMV 204 transcripts and local lesions were harvested after 3–4 days. To establish systemic infections containing dependent RNAs, the lower leaves of *N. tabacum* cv Xanthi plants were inoculated with extracts from lesions containing TMV 204 virions and 18 hr later the same leaves were inoculated with RNA transcripts of pTMV-KKL or pTMV-KL. Noninoculated, upper leaf tissue from plants inoculated 10 days earlier with TMV 204 virions and pTMV-KKL RNA transcripts (KKL/TMV) was used as inoculum for subsequent passage of the virus.

Virion isolation and preliminary analysis

Upper, noninoculated leaf tissue was harvested 3 weeks after inoculation and virions were isolated and fractionated on linear sucrose gradients essentially as described by Beachy and Zaitlin (1977). RNA was prepared from virions as described by Chirgwin *et al.* (1979), except the CsCl fractionation was omitted. The virion RNAs were analyzed by electrophoresis in formaldehyde–formamide denaturing agarose gels and transferred to nitrocellulose followed by hybridization with nick-translated probes (Ausubel *et al.*, 1987).

In vitro amplification and cloning

First strand cDNA was synthesized from 1 μ g of virion RNA (Maniatis *et al.*, 1982) utilizing Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories). Methylmercuric hydroxide was used to denature the RNA and synthesis was primed with 100 pmol of mixed oligonucleotide hexamers (Pharmacia LKB Biochemicals).

Polymerase chain reaction (PCR) amplification of cDNA was optimized for the production of TMV subgenomic replicon cDNA while eliminating the polymerization of full-length TMV 204 cDNA. Twenty cycles were completed with a 1-min incubation period at each temperature (94, 60, and 72°C). The polymerase reaction mixture was 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 0.2 mM of each dNTP, and 20 units/ml of *Taq* polymerase (Cetus Corp.). Three sets of primers, each at a final concentration of 50 pM, were used to amplify regions of the dependent progeny RNAs.

In order to produce the nearly full-length dependent progeny DNA a one-fifth aliquot of the first strand cDNA synthesis product was used as the DNA template with the first primer set. The 5' primer, 21(-), which hybridized to the 3' terminus of the negative-sense strand, consisted of nine 5' nts that contained a *Sa*I restriction endonuclease site (underlined) and TMV nts 1-20, 5'-GACGTCGACGTATTTTACAAC-AATTACC-3'. An internal 3' primer, 6291(+), was used that hybridized to TMV nts 6292-6312 and by the substitution of 1 nt (6304) incorporated a *Bam*HI restriction endonuclease site (underlined), 5'-GACCCGCGGGAT-CCAAGACAC-3'.

A 100th aliquot of the initial amplification product was used to reamplify segments of this PCR-produced DNA, using two other primer sets. The 3' portion of the progeny PCR DNA was amplified using primers 6291(+) and 5681(-) (nts 5658-5680, 5'-TTTAATCG-ATGATGATTCGGAGG-3'). The 5' portion of the progeny PCR DNA was amplified using primer 21(-) and 5672(+) (nts 5673-5692, 5'-CGGCGACAGTAGCC-TCCGAA-3').

The DNA produced from the initial PCR amplification of KL progeny cDNA was first phosphorylated and the single-stranded ends were filled using the Klenow fragment of *Escherichia coli* DNA polymerase I (Ausubel *et al.*, 1987). cDNA clones were produced by ligating the *Sa*I and *Bam*HI restriction endonuclease cut PCR DNA into pBluescript II SK (Stratagene). A few clones were obtained using *Sa*I and *Cla*I (5667) restriction endonucleases. The plasmid DNA was transfected into *E. coli* JM109 cells (Hanahan, 1983) and clones containing potential KL progeny were identified by their ability to hybridize to a probe complementary to TMV nt 5541-5603. The sequence of each of the clones was determined using 2',3'-dideoxynucleotide 5'-triphosphates, a variety of primers, and Sequenase Version 2.0 (U.S. Biochemical Corp.), essentially as described in Sanger *et al.* (1977).

RESULTS

RNA replicon KKL

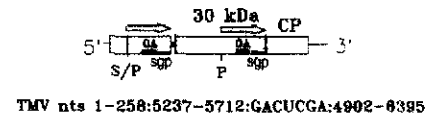
The TMV-derived replicon, KKL, was the first RNA tested to determine whether TMV can support a *trans*-replicating defective RNA. KKL (2235 nts) has most of the 126/183-kDa ORF (4644 nts) deleted and the 3' half of the 30-kDa ORF (containing the coat protein subgenomic mRNA promoter) is duplicated and inserted between the 5' 258 nts of TMV 204 and the 30-kDa ORF (Fig. 1B).

RNA transcripts of pTMV-KKL along with TMV 204 virus were used to inoculate the lower leaves of Xanthi tobacco plants. After the viral symptoms were visible in

A) Wild Type TMV-U1 (TMV 204) -- TMV nts 1-6395



B) TMV Derived Replicon KKL - 2235 nucleotides



C) TMV Derived Replicon KL - 1417 nucleotides

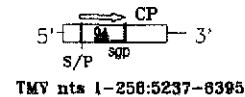


Fig. 1. Schematic representation of tobacco mosaic virus (TMV)-derived RNAs. TMV nucleotide (nt) numbering is of the positive-sense strand and is from Goelet *et al.* (1982). Transcripts of wild type TMV (depicted in A) were created from a full-length genomic copy of TMV (pTMV 204) and were used as the helper virus for the defective replicons. Replicon KKL (B) was created by a deletion of TMV nts 259-4900 and by the insertion of nts 5237-5712 (denoted by the arrow), resulting in a direct repeat of the 3' portion of the 30-kDa ORF. The designation below the schematic is the numbered TMV nts and the nonTMV nts (conferring the *Xho*I restriction endonuclease site) that are contained in this replicon. Replicon KL (C) was created by a deletion of nts 259-5236 from TMV 204. The subgenomic mRNA promoter region (sgp) for the coat protein gene (CP) is displayed as well as the origin of assembly (OA) region. The restriction endonuclease sites used in the construction of the TMV-dependent mutants are S, *Sma*I (258); P, *Pvu*II (5236); and N, *Nsi*I (6202). S/P refers to the deletion junction, utilizing the *Sma*I and *Pvu*II restriction endonucleases.

the upper noninoculated leaves, an extract of these leaves was used as inoculum to infect a second set of plants. The virus was subsequently passaged two more times from systemically infected tobacco leaf tissue. There was no noticeable effect on the symptoms of the plants inoculated with TMV 204 plus RNA transcripts of pTMV-KKL (KKL/TMV) compared to control plants inoculated with TMV 204 alone. When transcripts from pTMV-KKL were used as the sole inoculum, plants developed no symptoms, nor were any viral-like particles recovered from them.

Systemically infected leaf tissue was harvested from the third passage plants infected with TMV 204 alone and those infected with KKL/TMV, and virions were isolated and fractionated on a linear sucrose gradient. Virions prepared from TMV 204-infected leaves fractionated primarily into one uv-absorbing peak, whereas profiles of virions from KKL/TMV-infected tissue consisted of two prominent peaks (Fig. 2). When virions from these peaks were viewed by transmission electron microscopy, virions from the TMV 204 peak and peak B of the KKL/TMV sucrose gradient were predominantly the length (300 nm) of wild type TMV (Fig. 3A).

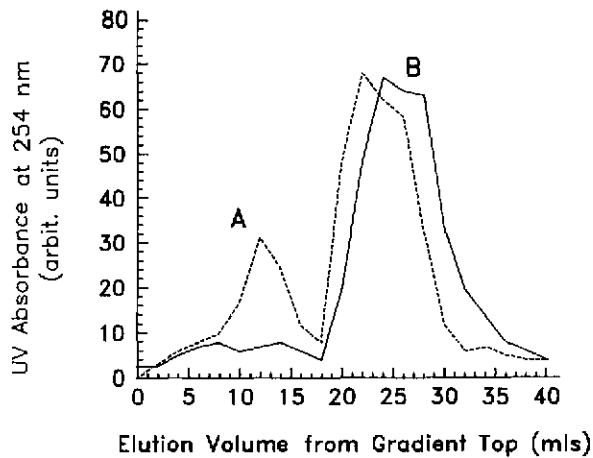


Fig. 2. Ultraviolet absorbance profile of virion preparations fractionated on a linear 10–40% sucrose gradient. The solid line represents the profile of virions prepared from Xanthi tobacco plants infected with TMV 204 virions alone. The profile of virions prepared from leaves infected with TMV 204 virions plus pKKL transcripts (KKL/TMV) is displayed as the dashed line.

Peak A of the sucrose gradient of KKL/TMV did not contain a homogeneous population of small virions of the expected size of KKL (105 nm). Instead it consisted of a heterogeneous population of virions, between 40 and 150 nm, with most approximately 60–75 nm in length (Fig. 3B).

Virion RNAs were extracted and fractionated in a denaturing agarose gel, transferred to nitrocellulose paper, and hybridized to ^{32}P -labeled DNA that contained nts 5080–6395 of TMV. Total TMV 204 RNA migrated primarily as a single major band consisting of the full-length genome of TMV (Fig. 4, lane 1). Total RNA from KKL/TMV virions resolved into multiple bands (Fig. 4, lane 3), one that comigrated with the full-length genomic RNA band and a population of bands that predominantly were smaller than the *in vitro* transcript of pTMV-KKL (the 2247-nt standard). Analysis of KKL/TMV RNA from fractionated virions from the sucrose gradient peak A (Fig. 2) showed a population of virion RNA bands (Fig. 4, lane 4) that was not found in the peak A fraction of the TMV 204 gradient (Fig. 4, lane 2). Although there was substantial size heterogeneity, the predominant RNAs of the population resulting from the introduction of pTMV-KKL transcripts were smaller than the original pTMV-KKL transcripts. When virions from peak A of Fig. 2 were sedimented through a second sucrose gradient, there was further fractionation with the RNAs prepared from virions in the upper shoulder of the peak migrating as a diffuse band smaller than the 1527-nt standard (Fig. 4, lane 5).

These data demonstrated that a TMV-derived, defective replicon could be propagated with wild type TMV in tobacco plants without greatly affecting the

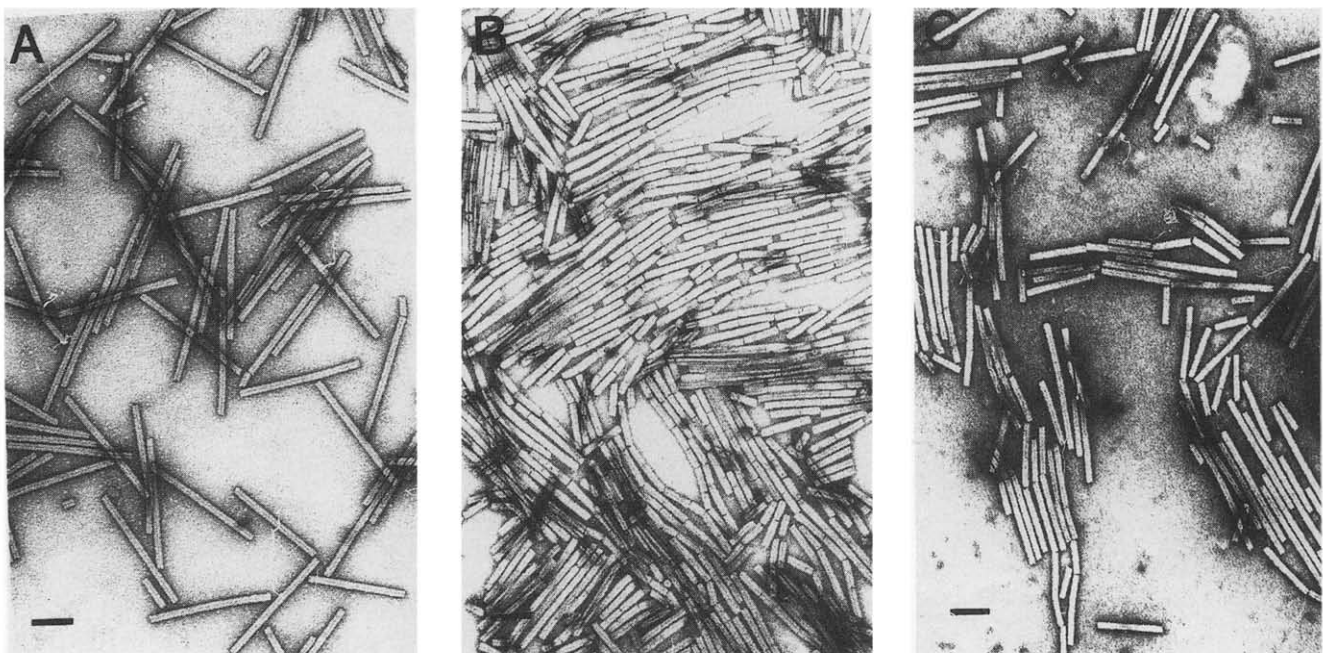


Fig. 3. Transmission electron microscopy of virions stained with 2% uranyl acetate: (A) wild type sized TMV virions (300 nm in length) obtained from peak B of the sucrose gradient presented in Fig. 2, (B) virions from peak A of the sucrose gradient of KKL/TMV virions (Fig. 2), and (C) unfractionated virions, derived from KKL/TMV-infected plants. The length of the bar located in the lower left corner of each micrograph represents approximately 100 nm.

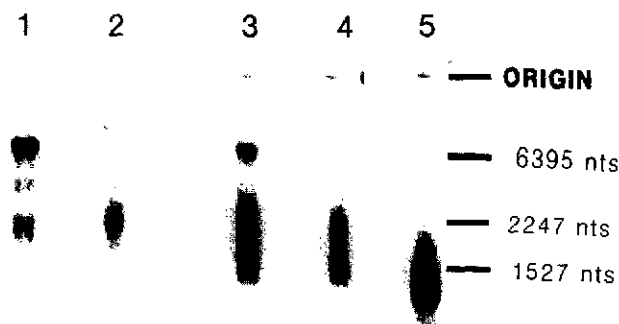


FIG. 4. Northern blot hybridization analysis of RNA populations prepared from sucrose gradient fractionation of virions derived from TMV 204 and KKL/TMV-infected tobacco tissue. Three micrograms of RNA were electrophoresed in a 1.2% agarose formaldehyde-formamide denaturing gel. Lane 1, total TMV 204 virion RNA; lane 2, RNA from sucrose gradient peak A (Fig. 2) of TMV 204 virions; lane 3, total KKL/TMV virion RNA; lane 4, RNA from sucrose gradient peak A (Fig. 2) of KKL/TMV virions; and lane 5, virion RNA from the upper shoulder of a second gradient of the virions found in the KKL/TMV sucrose gradient peak A. RNA was hybridized to a nick-translated probe containing the 3' portion of pTMV 204 (5080–6395). The migration of RNA transcripts used as the size standards is marked at the extreme right of the figure: 6395 nts, pTMV 204 transcript; 2247 nts, full pKKL transcript; and 1527 nts, partial pKKL transcript.

accumulation of helper virions or the symptoms induced by the helper virus. However, the original pTMV-KKL transcript appeared not to be faithfully reproduced. Most of the short virions (Fig. 3B) and the RNAs from them (Fig. 4, lane 4) were smaller than expected, suggesting that some sequences of the original transcripts were lost during replication. In an attempt to identify which sequences were missing, this RNA preparation was analyzed by Northern blot hybridizations and direct RNA sequencing. When probes specific to the origin of assembly, the coat protein ORF, or either the 5' or 3' untranslated regions were used in blot hybridizations, the results were essentially identical to those shown in Fig. 4, lane 4, suggesting that these sequences were retained in most of the RNAs (data not presented). When the population of RNAs was directly sequenced using a primer to initiate in the coat protein ORF or 150 nts from the 5' terminus, a unique sequence was obtained, again suggesting that these sequences were retained in most of the RNAs. However, when sequencing was initiated from the origin of assembly (at nt 5502), the sequence was unique for about 100 bases and then stops occurred in all lanes, suggesting the lack of a unique sequence in this area within the RNA population (data not presented).

To further characterize the heterogeneous progeny RNAs of KKL virions, the PCR, optimized to amplify the small, deleted TMV RNAs, was used to amplify cDNAs of these RNAs. Since attempts to amplify cDNAs utiliz-

ing a primer to the 3' terminus of TMV resulted in multiple bands, probably due to the high C+G content of this area, an internal 3' primer was utilized. The nearly full-length sequence of the defective replicons could be amplified using primer 6291(+) which hybridizes to TMV nts 6292–6312 and the 5' primer, 21(–), which hybridizes to the complement of TMV nts 1–20 (see Fig. 5A). Amplification of other small TMV-related RNAs, such as subgenomic mRNAs or fragmented TMV RNAs, which would lack either or both termini, was prevented using these primers. All primer sets, along with the PCR reagents, were always run without any DNA template and none of these control reactions produced any DNA that was detected by electrophoretic analysis in nondenaturing agarose gels (Fig. 5C, lane 1). Although these primers would also bind to the wild type TMV genomic cDNA, short polymerization times were designed to favor the amplification of smaller DNAs. When cDNA of TMV 204 RNA was used as the template for the PCR reaction, essentially no DNA was detected (Fig. 5C, lane 2). When cDNA of KKL/TMV RNA was amplified by this assay, a heterogeneous pattern, ranging in size from 300 to 2200 bp, with a concentration of bands between 900 and 2200 bp, was observed (Fig. 5C, lane 3). The 2161-bp standard was the PCR product obtained when cDNA of DNase treated *in vitro* transcripts of pTMV-KKL were used (shown as the marker to the left of lane 3). Some of the progeny RNAs of the KKL/TMV infection were the size of the original transcript, but many of the progeny RNAs were smaller.

The location of the size heterogeneity within the KKL progeny molecules was determined by reamplification of a small aliquot of the initial PCR-produced DNA (shown in Fig. 5, lane 3) and by using primer sets (Fig. 5A) designed to amplify 5' and 3' segments of the PCR DNAs. Control PCR DNA produced using the 3' primer set [5681(–) and 6291(+)] with cDNA of DNase treated *in vitro* transcripts of pTMV-KKL as the template migrated as two bands of 1473 and 655 bp (markers to the left of lane 4), reflecting the two binding sites for one of these primers [5681(–)]. When the 3' portion of the KKL progeny PCR DNA was reamplified using this primer set, a single band of approximately 650 bp (Fig. 5C, lane 4), which comigrated with the smaller of the two control DNA bands, resulted. Reamplification of PCR DNA from full-length TMV 204 cDNA (shown in Fig. 5, lane 2), using this primer set, resulted in a very faint band of similar size (data not shown). These data demonstrated that the 3' approximately 700 nts of the progeny RNAs were homogeneous and that the additional binding site in the repeated coat protein subgenomic RNA promoter region of KKL was not present in most of the progeny RNAs.

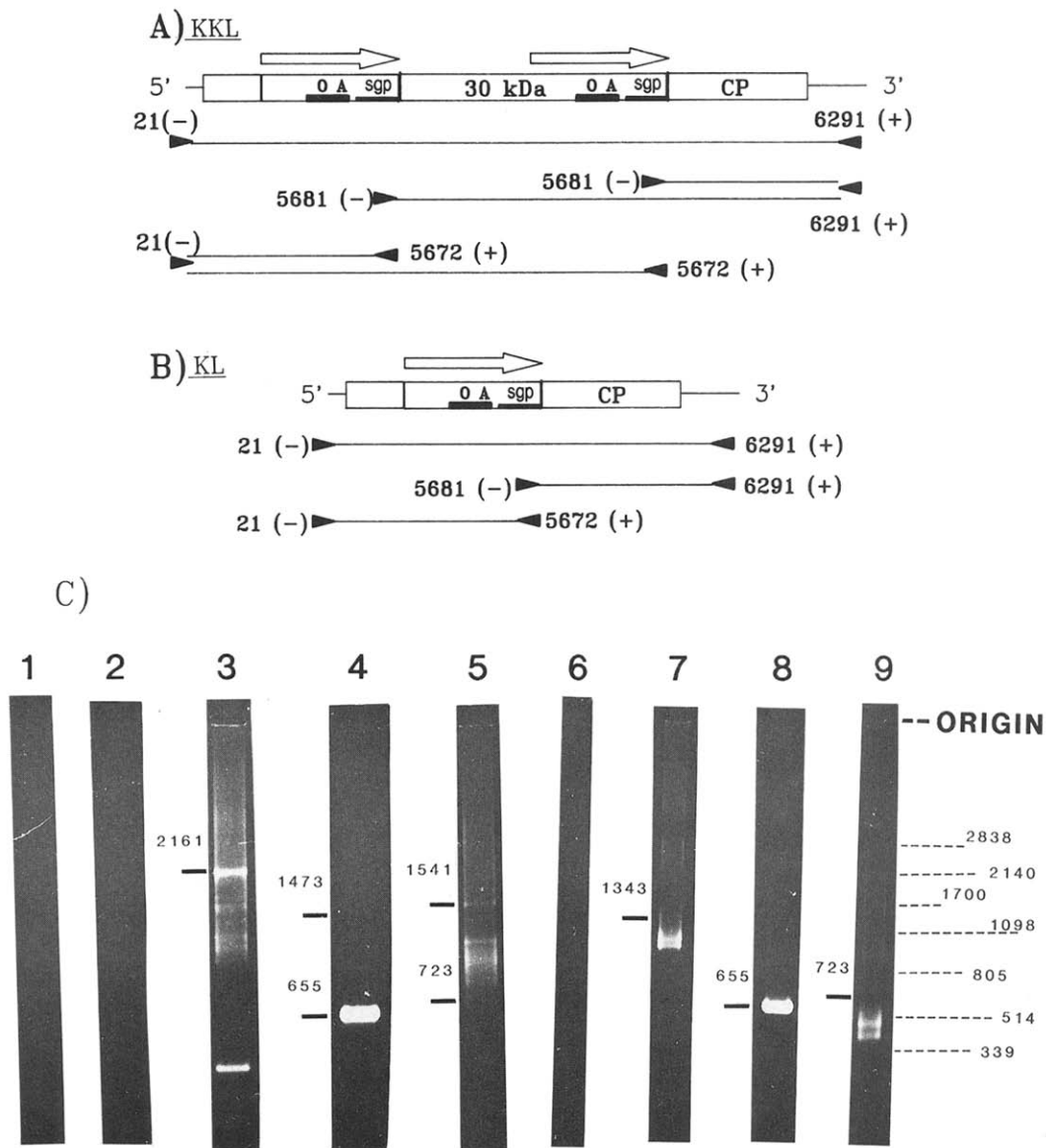


Fig. 5. PCR amplification analysis of cDNA derived from TMV 204, KKL/TMV, or KL/TMV total virion RNA under reaction conditions designed to amplify only the defective replicon progeny cDNA. Schematic representation of the inoculated transcripts KKL (A) and KL (B) are presented as in Fig. 1 along with the relative position of the primers and the expected products (arrowheads and connecting lines) of the two amplification steps. The initial PCR procedure used the first primer set [primers 21(-) and 6291(+)] and amplified the nearly full-length defective replicon cDNA. A small aliquot of the product of this PCR amplification was reamplified using two other primer sets designed to amplify either the 3' [primers 5681(-) and 6291(+)] or the 5' [primers 21(-) and 5672(+)] terminal segments of the defective replicon progeny PCR-produced DNA. The open arrow above the schematics denotes the repeated region found in the KKL defective replicon and amplification of either terminal segment of this replicon would be expected to result in two DNA products. (C) Nondenaturing 1.5% agarose gel electrophoretic patterns of ethidium bromide-stained PCR-amplified DNA. All primer sets were subjected to a PCR amplification reaction without the addition of an exogenous DNA template and lane 1 shows the typical products from such a PCR analysis, here using the first primer set. Analyses of cDNAs associated with the replicon KKL are presented in lanes 2 to 5. Lane 2 is the PCR product using template cDNA prepared from TMV 204 total virion RNA, with the first primer set, 21(-) and 6291(+). This same primer set produced the PCR products seen in lane 3 when KKL/TMV virion RNA-derived cDNA was amplified. The secondary PCR amplifications of this KKL progeny PCR-produced DNA are displayed in lane 4 when the 3' primers, 5681(-) and 6291(+), were used and in lane 5 when the 5' primer set, 21(-) and 5672(+), was used. Analyses of cDNAs associated with the replicon KL are presented in lanes 6 to 9. Lane 6 shows the results of the initial amplification of cDNA derived from TMV 204 virion RNA using the first primer set, and lane 7 is the profile of the products of KL/TMV virion RNA-derived cDNA amplification with this same primer set. Reamplification of the KL progeny PCR DNA seen in lane 7 using the 3' primer set and the 5' primer set is displayed in lanes 8 and 9, respectively. The markers to the left of each lane denote the migration of the standard PCR products using cDNA derived from DNase-treated KKL transcript (lanes 3-5) and DNase-treated KL transcript (lanes 7-9). The markers to the extreme right depict the migration of selected bands from a *Pst*I-digested λ DNA electrophoretic pattern used as size standards. All the standard markers are labeled according to the number of base pairs contained in the DNA associated with that band.

The DNA produced by the 5' primer set [21(-) and 5672(+)], using cDNA of pTMV-KKL transcripts, resulted in two bands of 1541 and 723 bp (markers to the left of lane 5), because of the two binding sites for one of these primers [5672(+)]. However, a heterogeneous smear with discrete bands resulted when the PCR DNA representing the 5' portion of the KKL progeny was analyzed. These data suggested that the 5' portion of the KKL progeny was heterogeneous, ranging from approximately 700 to 1300 bp (Fig. 5C, lane 5) due to various sized deletions within the 5' half of KKL. When PCR DNA of full-length TMV 204 cDNA (Fig. 5, lane 2) was reamplified using this set of primers, no detectable bands were observed (data not shown).

The 3' half of the pTMV-KKL transcripts, which included the 3' portion of the 30-kDa ORF, the coat protein ORF, and most of the 3' untranslated region, was retained in most of the progeny RNA. However, within this population of RNAs, there appeared to be extensive deletions within their 5' sequences.

RNA replicon KL

In an attempt to determine whether a deleted, dependent RNA could be replicated and transported throughout the plant with more stability than that observed for KKL, two modifications were made. One, a procedural modification was to reduce the time of propagation of the dependent RNA. Progeny virions were isolated from the upper leaves of the first plants inoculated. The second modification was to alter the sequences contained within the replicon. One factor now known to greatly affect the stability of sequences within the TMV genome became evident as this work was being done. Directly repeated sequences facilitate the loss of sequences between the repeats if they are not required for the replication of the virus. For example, TMV mutants with additional copies of the coat protein (Beck and Dawson, 1990), 30-kDa (Lehto and Dawson, 1990) ORFs, or the coat protein subgenomic mRNA promoter region (Dawson *et al.*, 1989) quickly lost the repeats and the intervening sequences upon replication. Thus, the second RNA replicon examined, KL, did not have directly repeated sequences, while the majority of the 126/183-kDa ORF and the 5' half of the 30-kDa ORF (4979 nts) were removed (Fig. 1C). The 3' half of the 30-kDa ORF, containing the origin of assembly and the subgenomic promoter for the coat protein, was ligated to the 5' 258 nts of TMV resulting in a 1417-nt RNA. The coat protein ORF and the full 3' untranslated region were not changed.

Transcripts of pTMV-KL, along with wild type TMV 204 virus as helper (KL/TMV), were used to inoculate Xanthi tobacco plants and systemically infected leaves

were harvested 3 weeks after inoculation of the lower leaves. As in the KKL/TMV infection, no significant change in symptomology was observed, compared to plants infected with only TMV 204. An examination of several electron micrographs suggested that virions from KL/TMV-infected tissue consisted of full-length virions and a heterogeneous population of small virions (Fig. 3C), which seemed to be less heterogeneous than that observed for the KKL/TMV virions (Fig. 3B). Sucrose gradient profiles of TMV 204 virions resulted in, predominantly, a single peak, while a KL/TMV virion profile contained two peaks, essentially as shown for KKL/TMV virions (Fig. 2). A comparison of the relative yields of full-length virions of KL/TMV compared to that of TMV 204 alone demonstrated that the support of the defective KL population by the helper, TMV 204, resulted in little or no significant reduction of helper virus yields. In two experiments, the total yield of virions from KKL/TMV-infected leaves was 3.4 and 4.3 mg/g fresh weight compared to the yield of virus from TMV 204-infected leaves of 2.7 and 3.7 mg/g fresh weight, respectively.

To characterize the KL progeny virion RNA, cDNA synthesis followed by PCR was used to amplify total viral RNAs, using PCR reaction times designed to prevent amplification of the helper cDNA, as described for the characterization of KKL progeny. Primers were used that bound to the complement of TMV nts 1-20 [21(-)] and to TMV nts 6292-6312 [6291(+)]. Amplification of the cDNA prepared from virion RNA from TMV 204-infected plants resulted in no detectable DNA (Fig. 5C, lane 6). The KL standard, derived from a PCR analysis which used these primers and cDNA from DNase treated *in vitro* transcripts of pTMV-KL, migrated as a single band at 1343 bp (shown as the marker to the left of lane 7 of Fig. 5). DNA representative of total KL/TMV virion RNA consisted primarily of two diffuse bands of approximately 1000 and 1200 bp (Fig. 5C, lane 7), both considerably smaller than the KL standard.

To locate the region responsible for the smaller size and heterogeneity of the KL progeny population, two primer sets were used to reamplify either the 3' or the 5' half of the initially produced PCR DNA (Fig. 5B). Reamplification of the 3' half of the progeny PCR DNA, using primers 5681(-) and 6291(+), resulted in a single, homogeneous band that comigrated with the PCR DNA (655 bp) produced using the same primers and cDNA to DNase-treated pTMV-KL transcripts (Fig. 5C, lane 8, and the marker to the left). When the 5' portion of the progeny PCR DNA was reamplified using primers 21(-) and 5672(+), a heterogeneous profile resulted (Fig. 5C, lane 9), which migrated primarily as two bands about 200-300 bp smaller than the respective standard (723 bp) which used PCR DNA to cDNA of DNase-treated

pTMV-KL transcripts (shown as markers to the left). Amplification analyses of TMV 204 PCR DNA (Fig. 5, lane 6) resulted in a faint band using the 3' primer set and none using the 5' primer set (data not shown).

These data demonstrated that the KL progeny RNA population consisted predominantly of two size classes, about 200 and 300 nts smaller than the original RNA transcript, and that the size heterogeneity was due to internal deletions within the 5' region. This was reinforced by sequence analyses of the PCR DNA shown in Fig. 5C, lane 7. Four different primers within the 3' 850 nts of the progeny PCR DNA resulted in unique sequences that matched those sequences found in the 3' region of pTMV-KL, whereas sequences using primers within the 5' region gave mixed sequences (data not shown).

To further characterize the 5' heterogeneous regions of the KL progeny virion RNAs, PCR DNA derived from two separate viral preparations was cloned into the pBluescript II SK plasmid. The first four cDNA clones shown in Table 1 and Figure 6 were cloned using PCR DNA that amplified the 5' region of the KL progeny, nts 1–5692 (Fig. 5C, lane 9). The remaining 26 cDNA clones were derived from a second viral preparation and the almost full-length PCR DNA of the KL progeny was cloned, nts 1–6304 (Fig. 5, lane 7). This sampling of the KL progeny indicated that the RNAs ranged in size from less than 1000 to almost 1500 nts, with most about 1000–1200 nts, and one (C6) was only 236 nts. The sequences of these clones are summarized in Table 1 and the 5' portion of the representative RNAs are schematically drawn in Fig. 6.

DNA sequence analyses of the cDNA clones that were derived from KL progeny RNA showed that the heterogeneity was due to deletions and in a few cases additions at the original deletion and ligation junction of pTMV-KL (nt 258:5237; see Figs. 1 and 6). All except two clones had the 3' approximately 920 bp (TMV nt 5380–6304) intact. Few nucleotide substitutions were found. Only four clones (3C12, C28, C76, and C79) contained nucleotides not consistent with the TMV 204 sequence (Goelet *et al.*, 1982). This usually was an adenosine being replaced by a guanosine residue. Four clones (3C12, C28, C62, and C64) contained nucleotide insertions (usually one) between the 5' and 3' border sequences at the deletion junction (Table 1).

One modification observed in all the cDNA clones was the deletion of sequences adjacent to the original hybrid junction of pTMV-KL, nts 258:5237 (Fig. 6). The deletion of the border sequences 5' of the original pTMV-KL junction were heterogeneous ranging between 70 and 235 nts and averaging about 100 nts. Seven of the clones had the start codon for the 126/183-kDa ORF deleted. Of these, clone C64 retained

TABLE 1

SUMMARY OF THE SEQUENCE ANALYSES OF 30 CLONES OBTAINED FROM PCR AMPLIFICATION OF KL PROGENY cDNA

Clone	5' TMV terminus	3' Region of TMV
KL	1–258	5237–6395
3C12	1–500 TTTT	5449–5667 (T to C:161; A to C:246; A to G:281 and 469)
3C4	1–165	5377–5667
3C5	1–86	5183–5667
9C5	1–134	5381–5667
C1	1–27: 45–53	5326–6304
C2	1–116	5385–6304
C6	1–80	6291–6376: 6237–6304
C9	1–425	5455–6304
C21	1–102	5374–6304
C26	1–102	5374–6304
C27	1–166	5346–6304
C28	1–451: A	5263–6304 (A to G:292)
C31	1–176	5405–6304
C34	1–44: 368–372	5378–6304
C35	1–187	5382–6304
C38	1–468	5377–6304
C45	1–87	5387–6304
C47	1–109	5376–6304
C48	1–306	5386–6304
C54	1–485	5252–6304
C55	1–115	5375–6304
C56	1–59	5301–6304
C57	1–306	5386–6304
C61	1–64	5338–6304
C62	1–74: A	5375–6304
C64	1–23: T	5303–6304
C76	1–45	5412–6304 (A to G:5432)
C79	1–158	5350–6304: 6199–6304 (A to G:5439)
C85	1–66	5419–6304
C86	1–186	5381–6304

Note. Nucleotide numbering is taken from Goelet *et al.* (1982). The clone KL indicates the construction of the original TMV mutant transcript. The next four are KL progeny that were cloned to include the *Cla*I site (5667). The remaining KL progeny clones included an engineered *Bam*HI, site (6304). Nucleotides shown between the 5' TMV terminus and the 3' region of TMV denote inserted nucleotides found in four of these clones (3C12, C28, C62, C64). Denoted in parentheses are the base substitutions found in four clones (3C12, C28, C76, C79), with the TMV nucleotide location for this exchange indicated. Duplication of TMV nucleotides within the 3' untranslated region found in two clones, C6 and C79, are presented.

only 23 nts of the 5' terminus. Four additional clones retained less than 100 nts of the TMV 5' end, 12 retained 100–200 nts, while none retained nts 200–258 of the original 5' terminus.

Deletions of sequences 3' of the original KL junction also occurred, with 15–218 nts removed, averaging about 150 nts. However, these deletions were less heterogeneous. Half of the deletions ended within the

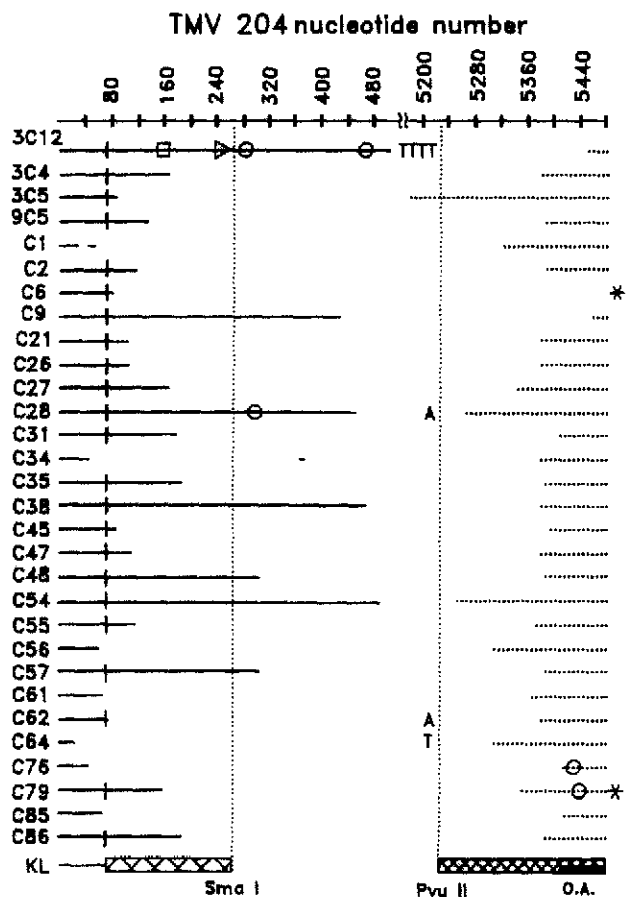


Fig. 6. Schematic representation of the 5' portion of the sequence determined for each of the clones obtained after the PCR amplification of the KL progeny cDNA. A detailed presentation of this data is found in Table 1. The line at the top of the figure represents the positive-sense TMV RNA nucleotide numbering of Golet *et al.* (1982), showing TMV nts 1–520 and 5160–5480. The schematic at the bottom of the figure represents KL, the original transcript, with the vertical dashed lines delineating the deletion junction. The remaining horizontal lines represent the KL progeny clones obtained after PCR amplification. The solid horizontal lines on the left represent the locations of nucleotides sequenced for each clone that correspond to those that are in the 5' terminus of TMV, and the vertical bar indicates the presence of the start codon for the 126/183-kDa ORF. The dotted horizontal lines on the right represent the location of the nucleotides sequenced for each clone that correspond to those within the 3' portion of the TMV 30-kDa ORF. The nucleotides to the left of some of these dotted lines are the nucleotides found inserted between these regions. The seven symbols denote the approximate location of base substitutions not consistent with the TMV sequence. A circle represents the location where a guanosine replaced an adenosine, the square shows where a cytosine replaced a thymidine, and the triangle denotes where a cytosine replaced an adenosine. The asterisks on the far right denote two clones that contained a duplication of portions of the 3' untranslated region.

same 14-nt area (TMV nts 5374–5387) and eight ended within a 5-nt region (5374–5378). This area is slightly 5' of the origin of assembly. Two clones, 3C12 and C9, had a portion of the origin of assembly region

removed. However, both retained the 3' portion of this area, which is thought to play an essential role in encapsidation (Turner *et al.*, 1988).

Another type of modification, found in nine clones, was the addition of sequences not found in the original pTMV-KL transcript, but found in the wild type helper virus. These additions extended beyond the original hybrid junction. One clone, 3C5, had an addition of 54 nts to the 3' border of this junction (nts 5183–5236), while 172 nts were removed from the 5' border. Eight clones (3C12, C9, C28, C34, C38, C48, C54, and C57) had 48–242 nts added to the 5' border of the original KL junction. Each of these insertions coincided with a deletion of 15–218 nts from the other border. One clone, C34, appeared to have deletions within the added sequences. The 5' end consisted of nts 1–44, followed by nts 368–372, and then the 3' portion consisting of nt 5378 and progressing to the 3' terminus. An additional clone, C1, also appears to have resulted from multiple deletions, but without the addition of nonKL sequences.

There were two cDNA clones with a substantial duplication of similar sequences within the 3' untranslated region. One (C79) maintained the 3' portion of the 30-kDa ORF and the coat protein ORF, while the other (C6) had these sequences deleted, so that only the 3' untranslated region remained (Table 1).

DISCUSSION

TMV-derived RNAs with more than two-thirds of the genome deleted, when coinoculated with a competent TMV genome as helper, replicated as dependent replicons, assembled into virions, and spread throughout the plant with the helper virus. The TMV replication complex was therefore capable of replicating the sub-genomic replicons created from TMV. This ability of the viral replicase complex to replicate an RNA *in trans* is similar to that reported for other viruses in the Sindbis virus "supergroup" (Lehtovaara *et al.*, 1982; Levis *et al.*, 1986; French and Ahlquist, 1987; Van der Kuyl *et al.*, 1990; Pacha *et al.*, 1990; Pogue *et al.*, 1990), but apparently dissimilar to that of picornaviruses (Kuge *et al.*, 1986; Hagino-Yamagishi and Nomoto, 1989). Although no defective interfering RNAs (DIs) have been found naturally associated with tobamoviruses, these artificially created RNAs not only replicated efficiently in initially inoculated cells, but were competitive enough to move with the helper virus during propagation throughout the entire plant, similar to the naturally occurring dependent replicons of plants: DIs, satellite viruses, and satellite RNAs.

A major difference between the TMV-dependent RNAs and typical DIs or satellites was that these TMV

subgenomic replicons did not interfere with the replication of the helper virus. Normal concentrations of the helper virions were produced in addition to the high levels of subgenomic virions. In most virus-plant associations in which a dependent RNA replicates with the helper virus, the symptoms produced by the virus are also altered. However, neither of the TMV-dependent RNAs that we examined had a noticeable effect on symptoms caused by TMV in tobacco. In a parallel system, the satellite virus that replicates with TMV also does not alter symptom production, although its replication does appreciably decrease helper virus yields (Valverde *et al.*, 1991).

The progeny of both deletion mutants were heterogeneous, with most of the population being smaller than the input transcripts. This heterogeneity was similar to that described for naturally produced DIs originating from genomic RNAs in numerous virus systems (Kaariainen *et al.*, 1981; Jennings *et al.*, 1983; Barrett *et al.*, 1984; Makino *et al.*, 1985; Kuge *et al.*, 1986; Hillman *et al.*, 1987; Li *et al.*, 1989). Sequence analyses of PCR DNA and cDNA clones of the progeny of KL demonstrated that the 5' terminus and a large 3' region, containing the origin of assembly, coat protein subgenomic mRNA promoter, coat protein ORF, and the untranslated 3' end generally were retained, whereas various deletions and occasionally insertions occurred at the original deletion junction. It is possible that replication of mutant transcripts KKL and KL through a single cycle in protoplasts would have resulted in a more homogeneous progeny, as has been demonstrated with bromoviruses (French and Ahlquist, 1987; Pacha *et al.*, 1990; Pogue *et al.*, 1990). However, forcing the TMV subgenomic replicon to propagate in intact plants not only required efficient replication, but efficient encapsidation, cell-to-cell movement, and long-distance movement. Because of the probable requirement of encapsidation for efficient long-distance movement (Sanger, 1968; Siegel *et al.*, 1962; Dawson *et al.*, 1988), our sampling of progeny RNAs was probably prejudiced by our isolation from systemically infected tissue. The heterogeneity observed in the progeny population probably reflects multiple selection pressures resulting from the *in planta* propagation. Some of the sequences retained in these progeny RNAs are *cis*-acting elements required for replication and/or encapsidation. While other sequences might not be absolutely required, they may enhance *efficient* replication or movement.

Previous work using mutations in the TMV genome defined regions that can be deleted without blocking replication and viability of the helper virus. Mutants with the 30-kDa and/or the coat protein ORFs deleted retain the ability to replicate (Meshi *et al.*, 1987; Taka-

matsu *et al.*, 1987; Dawson *et al.*, 1988). Also, the virus will replicate with deletion of one of the three (Dawson *et al.*, 1988) or even two of the three (Takamatsu *et al.*, 1990) pseudoknots found in the TMV 3' untranslated region between the coat protein ORF and the tRNA-like region (Van Belkum *et al.*, 1985). At the 5' end, the terminal 8 nts and nts 48–61 have been found to be required for replication of genomic TMV (Okada *et al.*, 1990). However, the experiments reported here with the dependent RNAs measure different requirements. The dependent RNAs were replicated *in trans* without a requirement for gene expression, but with the requirement to compete with the wild type population during systemic infection of the plant. These experiments suggest that little 5' sequence is required because some of the progeny RNAs retained less than 100 nt of the 5' end (11 of 30 cDNA clones) with one having only the 5' 23 nts. Even though TMV mutants with deletions in the coat protein ORF and the untranslated 3' end retain the ability to replicate, virtually all of the modified progeny of KL retained a large area of the 3' region unmodified. This might suggest that for a TMV-based replicon to replicate and spread efficiently in tobacco little modification of this region can be tolerated. Other mutants of TMV that we have examined with insertions that separated the coat protein ORF from the 3' untranslated region have replicated much more poorly than similar mutants with the same insertions between the 30-kDa and coat protein ORFs (Dawson *et al.*, 1989; Beck and Dawson, 1990; Lehto and Dawson, 1990). For competitive replication and invasion of intact plants, the requirements of a TMV-based RNA appear to be *cis*-acting sequences which apparently consist of less than 100 5' nt, but perhaps a large 3' region containing considerably more than the negative-sense strand promoter and the origin of assembly.

A possible mechanism to explain how the diverse KL progeny arose results from the proposed ability of viral replicase complexes to dissociate and switch templates. Two types of copy-choice strand switching have been proposed to explain recombination and production of DIs in viral systems (for reviews, see Lazzarini *et al.*, 1981; King, 1988). In homologous recombination, restart is favored by the nascent RNA finding complementary or near-complementary sequences, which would preferentially favor restart at the same sequence within the genome from which the replicase complex dissociated, although not necessarily on the same template molecule. Alternatively, heterologous recombination does not require complementary binding between nascent chain and template chain upon replication restart which can therefore occur at various positions. The deletion of sequences from the TMV dependent RNAs could be the result of the replicase

3C12	... GAAAG ACAGT AAACG TGAGA TTTT	ATTGA ACTAT GACGGAGGGC...	C35	... AAGAGTTAA TGGAG TTTGT	CGCTC GTGAC GTCGGTCTGT...
3C4	... CGTCTTACG TTCTC TGGAG	ACACA GCGGT TTTGTCTCGG...	C38	... GACGTTFCGAG TTCTC TGGAG	ACATC ATGCG TTTGTCTCGG...
3C5	... CACAC AGACA TGCTC GGTGG	GCTAC CACAT ACAAAAGGAT...	C45	... ACACAGACAG TTTGT GTCGG	CTACC ACATC TGTGTATTGT...
9C5	... CAACTCCTTG CTGGA GTTGG	GTCAA TGATC TGTCGGTGTG...	C47	... CTTTGCTGGA TTTCT CTGGA	CACCT TCCGA TTTGTCTCGG...
C1A	... ACCAA CAACA ACAAA CAACA	ACAAA CAACA TTACAATTAC...	C54	... GCGGCACGAA AAGAA AAGAT	GGCCA GAAAG TTCAGTTCAA...
C1B	... ATTACAATTA AGTTT TAGTT	CTATT TACAA AATATTAGAA...	C55	... TGGACACTGT CTTTC TCTGG	CCGAG GAAAC AGTTT GTGTC...
C2	... GGACACTGTC AGTTT GTGTC	CGAGG AAACA GGTGTGTATT...	C56	... ATTACTATT AGGAC GCGAT	ACAAT TACAA GAAAAACGTC...
C6A	... GGCAT ACACA ATCGA AGGGT	CAGAC AGCTA TGTGTCTTGG...	C48	... CAAAATGCCG GTTTG TGTGG	TGCAT TCGT GTGTGTATTG...
C6B	... ATCCC CCCGT CCGTA ATCAC	TACCC CCGGT ACGTGCTGCG...	C57	... TATTTACAAT TATTA GAAAT	TACAA TGCCA GTGAAGATGT...
C9	... GTTCAAGGGA GAGAG ACGGA	CGAGC ATATG GGGCCATGG...	C61	... TACAATGGCA CTTTC TCTGG	TGCAT TCGT AGTTT GTGTC...
C21	... ACATCAGCTT GC TTT CTCTG	TGCTG GACAC GAGTTTGTGT...	C62	... AATTCCAAC GAGGC GATGA	AAACA CAAC AAAACGTCTG...
C26	... GTCTTTACGA ATG TG AAGAT	CACAG CGGTT GTCAGCGGGT...	C64	... CAAAACACAT GAAAT AATAT	TACAA TTAAT AAAATTAGGT...
C27	... GCTGCATGCC TCAGT TCAAG	CAACC TGGAC GTCGGTCCCA...	C76	... AAAGCGTCGT GAAGA TGTCA	CTTTA CGACA GCCGGTTTCT...
C28	... CACAG CGGT GTTTA TAGAA	GAAGA GTTTA ATAATATAAA...	C79A	... TCTTG GATCC TGAGG TAGTC	CGCGG GTCAA AAGATGCATA...
C31	... ACAAACAACA TGCAA ATTCC	TTACA ATTAC CTACGTTGTG...	C79B	... TTTCAATTA TATAA AATTA	CAATG GCATA GGTTT GAGAG...
C34A	... ATTCCCTACG TCTCT GAGAT	GATCA TTGAC TTGTGTCGGT...	C85	... GAAGATTTA CTGGA GTTGG	ACGCT CGTGA TGTCTG GTGTC...
C34B			C86		

Fig. 7. TMV positive-sense nucleotide sequences found for each of the KL progeny clones (bold type), bordering the deletion junction between the 5' TMV terminus (upper left) and the 3' portion of the 30-kDa ORF (lower right) and a comparison of these sequences to those found at the 5' (upper right) and the 3' (lower left) ends of the deleted fragment (italic type). Indicated between these progeny junctions are the inserted nucleotides found in four of the clones (3C12, C28, C62, C64). When two clones contained the same junction (C21/C26 and C48/C57) the sequences from one is represented. When a clone contained two junctions (C1, C6, C34, and C79) both are shown, with the 5' junction denoted as A and the second junction denoted as B. Similarities found between progeny junction border sequences and the deleted fragment terminus sequences are indicated by a solid underline, when they occurred adjacent to the junction border (and are arbitrarily assigned to the 5' progeny junction border), and by a dashed line, when the similarities are not adjacent to the progeny junction border.

complexes restarting at alternative areas on a dependent RNA or switching to a different dependent RNA, as is thought to occur in the generation of natural DI populations. The addition of sequences that were not part of the original KL transcript, but were contained in the helper genome, could have resulted from the TMV replicase first producing a nascent chain using the helper as a template and then changing templates to a dependent RNA template.

The progeny junctions were examined for possible mechanism(s) that might explain how they could have been generated by comparing nucleotides that would have occurred on each side of the border regions prior to the formation of the present junctions. These sequences, expressed in Fig. 7 as TMV positive-sense, show that approximately one-half of the clones have borders containing some sequence similarity. The best examples of complementarity occurred in clones C1A (9 of 10 identical nts), C85 (8 of 10), C47, C61, C76 (7 of 10), C27, C35, and C86 (6 of 6). Half of the clones had at least 3 or 4 of 5 identical nts near the recombinant junction. In contrast to another report (Jennings et

al., 1983), the identical nts often occurred 1 or 2 nts from the observed junction (C27, C35, C55, and C86). When it was possible to assign these similarities to either of the progeny junctions most were found to occur on the 5' border junction, which might suggest the direction of RNA synthesis during which strand switching most often occurred. In contrast, the other half of the clones sequenced had no obvious sequence similarity associated with the junctions. These data suggest that the progeny were produced by both types of recombination mechanisms.

The high degree of heterogeneity, even after propagation in only one set of plants, suggests that the frequency of recombination was relatively high. In part, this may have been the result of our constructing an unstable replicating RNA, since we had no examples of naturally occurring DIs of TMV to use as a guide. Work with other viruses that employed *in vitro* transcripts to express defective replicons was based on naturally isolated DI populations, for example, DIs of Sindbis (Levis *et al.*, 1986), polio (Kaplan and Racaniello, 1988; Hagino-Yamagishi and Nomoto, 1989), and murine

coronavirus (Makino and Lai, 1989). Some of the KL progeny cDNA clones that we obtained are now being prepared as transcripts to examine their ability to be replicated.

Since the *in vitro*-constructed TMV subgenomic replicons replicated so well in inoculated plants, why are DIs of TMV not found in nature? During propagation in plants, preferentially selected RNAs from the heterogeneous population did not appear to become dominant. It is possible, after multiple passages, that the heterogeneous population would continue to be modified, until *cis*-acting elements are lost and the RNAs no longer are able to effectively invade plants along with the helper virus and thus will eventually be lost. It also is possible that subgenomic replicons do naturally occur in TMV infections. Although the *in vitro*-created replicons replicated to high levels when "seeded" at a high inoculum level, low levels of similar RNAs may occur in natural TMV infections. TMV virion RNA, when analyzed by gel electrophoresis, normally exhibits a heterogeneous smear of smaller RNAs. It would be interesting to characterize those RNAs with PCR technologies.

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