

Characterization of tobacco mosaic virus isolated from tomato in India

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Tomato mosaic tobamovirus (ToMV) differs from the type strain of tobacco mosaic virus (TMV) in producing local lesions instead of systemic infection on *Nicotiana sylvestris*. An isolate collected from Kolar district of Karnataka which produced this differential host reaction was propagated in the greenhouse on *N. tabacum* cv. Samsun and purified. The virus is a rigid rod shaped particle with a coat protein of molecular weight 18 kDa and genomic RNA of size 6.3 kb. A cDNA library was constructed using a specific primer designed based on the conserved nucleotide sequence at the 3' non coding region of tobamoviruses. The cDNA library was screened for recombinant clones and the recombinant clone 82 with an insert of size 1.04 kb was sequenced in both directions. This sequence was compared with the genomic sequence of TMV and ToMV which showed 93.1 and 73.7 per cent identity, respectively. The sequence encompassed the 3' non coding region, the complete coat protein ORF and 467 nucleotides of the movement protein. The deduced amino acid sequence of the coat protein was compared with that of TMV and ToMV. This sequence was nearly identical to TMV with nine amino acid changes whereas thirty-two changes were observed with ToMV. This suggests that the virus under study is a strain of TMV and therefore we have named it as tobacco mosaic virus tomato strain from Karnataka, India (TMV(Tom-K)).

KARNATAKA is a major producer of tomatoes in India. Several tomato hybrids are grown all round the year.

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Tomato grown in Karnataka is distributed to other states in India and also exported to the Middle East. Tobacco mosaic virus (TMV) and tomato mosaic tobamovirus (ToMV) are two tobamoviruses known to naturally infect tomato and cause yield losses^{1,2}. Field surveys conducted during 1994–95 to determine the natural distribution and prevalence of the virus in tomato revealed that 688 of 1228 samples tested in enzyme linked immunosorbent assay (ELISA) and on local lesion host (*Nicotiana glutinosa*) were infected with TMV/ToMV (ref. 3). However, none of the isolates of tobamoviruses from India have been characterized with respect to the genomic sequence thus far.

In the current paper we report the purification and characterization of a tobamovirus infecting tomato from India, the second most prevalent virus after tomato leaf curl geminivirus on tomato.

A tobamovirus causing mosaic on tomato collected from a naturally infected tomato field in Kolar district of Karnataka was maintained on *N. tabacum* cv. Samsun by sap inoculation in an insect-proof glasshouse.

The virus under study was purified from infected *N. tabacum* cv. Samsun leaves using the method of Asselin and Zaitlin⁴. Purified viral samples applied on carbon-coated grids were stained with 1% uranyl acetate and examined under a JEOL 100CX transmission electron microscope.

The viral coat protein was analysed by SDS-PAGE as described by Laemmli⁵. The gel was stained with coomassie blue. Molecular weights were determined by plotting log molecular weight against migration distance using a set of marker proteins (Dalton Mark VII – L, SDS – 7, Sigma).

Viral RNA was isolated from purified virus essentially by the method of Murrant *et al.*⁶. Purified virus was incubated with an equal volume of RNA extraction buffer (0.1 M Tris, 0.01 M EDTA, 2% SDS, pH 7.5) at 55°C for 10 min. Following phenol–chloroform extraction, RNA was precipitated by adding 1/10 volume of sodium acetate and 0.8 volume of isopropyl alcohol. The isolated RNA was then run on a 1% agarose gel. Physalis mottle virus RNA of 6 kb and Sesbania mosaic virus RNA of 4.5 kb were used as markers.

Antisera were produced in rabbits against purified virions of the virus under study by giving three intramuscular injections in Freund's incomplete adjuvant at weekly intervals and one intravenous injection without the adjuvant in the fourth week. Serum was collected every week starting a week after the last injection. Antiserum titre was determined by ELISA. Double antibody sandwich ELISA (DAS-ELISA) was performed as follows.

Next, the procedure of Clark and Adams⁷ was employed and the concentration of the protein (γ -globulin) was adjusted to 1 mg/ml ($A_{280\text{ nm}} = 1.4$) and then conjugated with penicillinase (from Hindustan Antibiot-

ics Ltd.) at an enzyme: globulin ratio of 2:1 in the presence of 0.06% glutaraldehyde.

Flat-bottomed polystyrene micro titre plates (Corning, Sigma) were pre-coated with 100 μ l (100 ng) of γ -globulin in carbonate buffer pH 9.6. After 3h, the plates were blocked with 5% dried milk powder in PBS, coated with antigen from infected leaves (1 g/10 ml) extracted in phosphate buffered saline tween-20-poly vinyl pyrrolidone-ovalbumin (PBST-PVP-OA). Following overnight incubation and washing, the antigen was detected using penicillinase conjugated globulins of homologous antiserum at 1:1000 dilution. The substrate (2 mg sodium penicillin-G/ml bromothymol blue + 0.2 M NaOH, pH 7.2) was added and the disappearance of the blue colour at 620 nm was monitored.

The polyclonal antiserum to the virus was also used for penicillinase-based direct antigen coating ELISA (DAC-ELISA) which was done as described by Sudarshana and Reddy⁸. Micro titre plates were coated with antigen (infected leaf extract 1 g/10 ml) extracted in carbonate buffer pH 9.6 and diluted to 1:10. Following overnight incubation at 4°C, the plates were coated with crude antiserum diluted in PBST-PVP-OA cross-adsorbed with healthy leaf extract. F_c -specific anti-rabbit penicillinase conjugate was then added followed by the addition of the substrate (2 mg sodium penicillin-G/ml of bromothymol blue + 0.2 M NaOH, pH 7.2).

Using a specific primer synthesized corresponding to the 24 bases at the 3' end of TMV RNA (3' GGG CC/AT/A TGG GGG CCA A/TCC CCG GGT 5') the first strand cDNA was synthesized essentially following the protocol described by Sambrook *et al.*⁹. This first strand cDNA was then used as a template for the second strand synthesis.

Following gel filtration, the cDNA fractions were cloned in the *HincII* site of pUC 19 cloning vector. After ligation, the DNA was transformed in competent DH 5 α cells and spread on LB-Amp-X gal-IPTG plates. The colonies were initially screened by blue/white selection and later by plasmid isolation and restriction digestion with *XbaI* and *HindIII*.

Initially, the sequencing of the clone was performed manually by Sanger's dideoxy chain termination method¹⁰. The clone was later sequenced using a Perkin-Elmer ABI Prism DNA sequencer.

Nucleic acid and deduced amino acid sequences were analysed using the 'GCG' program package at the Bioinformatics Centre, IISc. The viral sequence determined was compared and aligned with the other tobamoviral sequences in the GenBank and EMBL data base using the 'FASTA' and 'GAP' programs.

The program 'CLUSTALW' was used to generate multiple alignment¹¹. The aligned sequences were analysed with the 'PHYLP' package of programs^{12,11} to infer phylogenies and to place a confidence limit on the phylogeny. The program 'SEQBOOT' was used to

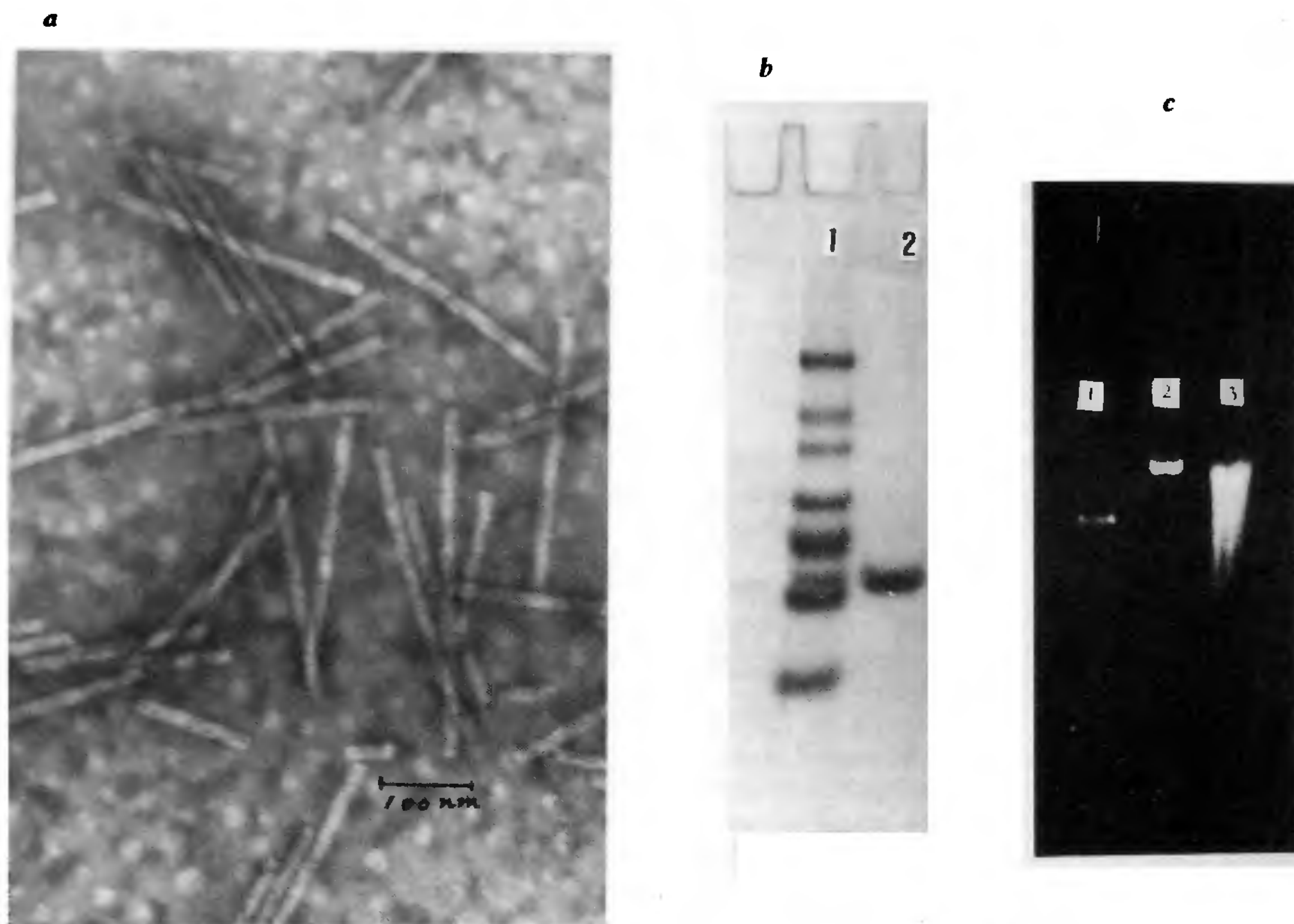


Figure 1. *a*, Electron micrograph of purified TMV (Tom-K) particles visualized by negative staining with 1% w/v uranyl acetate; *b*, Electrophoresis of the TMV (Tom-K) coat protein in 10% SDS-polyacrylamide gel stained with coomassie brilliant blue. Lane 1: Markers Type VII-L (Sigma) from top to bottom – bovine albumin (66,000); albumin egg (45,000), glyceraldehyde 3 – phosphate dehydrogenase (36,000); carbonic anhydrase (29,000); trypsinogen (24,000); trypsin inhibitor (20,100) and lactalbumin (14,200); Lane 2: Coat protein of TMV (Tom-K); *c*, Electrophoresis of TMV (Tom-K) RNA in 1% agarose gel stained with ethidium bromide. Lane 1: Sesbania mosaic virus RNA (4.5 kb). Lane 2: TMV (Tom-K) RNA. Lane 3: Physalis mottle virus RNA (6.2 kb).

produce 100 bootstrap¹⁴ replacement sequence alignments from the original 'CLUSTALW' aligned sequences. The bootstrapped samples were analysed by the maximum parsimony method using the program 'PROTPARS'. The trees produced by 'PROTPARS' were analysed by the program 'CONSENSE' to deduce a consensus tree. The unrooted phylogenetic tree was drawn using the program 'DRAWTREE'.

The virus under study was transmitted mechanically from tomato to tomato and tobacco with an efficiency of 100%. It induced mild to severe form of mosaic in tomato. Infected tobacco leaves showed light and dark green mottling, together with distortion and smaller size. The virus produced necrotic local lesions on *Datura metel*, *D. stramonium*, *Gomphrena globosa*, *Nicotiana tabacum* cvs. White Burley, Riwaka-1, L-1158, CTRI special, *N. glutinosa* and *N. sylvestris*. It produced chlorotic local lesions on *Chenopodium amaranticolor*. It may be noted that TMV gives systemic infection of *N. sylvestris* whereas ToMV produces necrotic lesions^{15,16}.

Table 1. Comparison of DAC-ELISA with local lesion assay for detection of the virus causing mosaic on tomato

Antigen dilution	Absorbance readings ^{a,b}	Lesions/cm ² on <i>N. glutinosa</i>
Infected 10-1	1.139	7.37
10-2	1.130	5.45
10-3	1.118	4.15
10-4	1.117	2.30
10-5	0.856	0.43
10-6	0.215	0.06
10-7	0.054	0.00
10-8	0.005	0.00
Healthy 10-1	0.011	0.00

^aMean absorbance values of five wells at 620 nm obtained after deducting the mean absorbance value of buffer control.

^bAbsorbance values were considered positive if they yielded OD values twice greater than the mean optical density of wells coated with healthy plant extracts.

When the same inoculum of the virus under study obtained from *N. tabacum* cv. Samsun was inoculated to


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1  CTGCGGGTTT CTGTCCGCTT TCTCTGGAGT TTGTGTCGGT ATGTATTGTT
51  TATAGAAATA ACATAAAATT AGGTTTGAGA GAGAAAATTA CGAGCGTGAG
101 AGACGGAGGG CCCATGGAAT CCACAGAAGA AGTTGTTGAT GAGTTCATGG
151 AAGATGTCCC TATGTCGATA AGGCTTGCAA AGTTTCGATC TCGAACCGGG
201 AAAAAGAGTG ATGTCTGAA AGCGAAAGTT AGTAGTAATG ATCGGTCGCA
251 GCCCAACAAG AACTATATAA ATGTTTAAGA TTATGGAGGA ATGAGTTTAA
301 AAAAGAATAA TTTAATCGAT GATGATTCGG AGACTTCTGT CGCCCAATCG
      Start codon
351 GATTCGTTTT AAATATGTCT TATAATATCA CTAATCCATC TCAGTTCGTG
401 TTCTTGTCGT CCGCGTGGGC CGACCCGATA GAGTTAATTA ATTTATGTAC
451 TAATGCCTTG GGTATCAGT TCCAAACACA ACAAGCTCGA ACTGTCGTTC
501 AAAGACAATT CAGCGAGGTG TGGAAACCTT CACCACAAGT TACTGTCTAGT
551 TCCCCTGACA GCGACTTTAA GGTATATAGG TACATTGCGG TATTAGAGCC
601 TCTAGTTACT CGACTATTAG GCGCTTTTGA TACTAGAAAT AGAATAATAG
651 AAGTTGAAAA TCAAGCAAAC CCCACGACTG CCGAAACGTT AGATGCTACT
701 CGTAGAGTAG AGGACGCAAC GGTGGCCATA AGGAGCGCGA TAAATAATTT
751 AATAGTAGAA TTGATCAGAG GAACTGGATC TTATAATCGG AGCTCTTTTCG
      Stop codon
801 AGAGCTCTTC TGGTTTGGTT TGGACCTCTA GTCCGGCAAC TTGAAGTAGT
851 CAAGATGCAT AATAAATAAC GGATTGTGTC CGTAATCACA CGTGGTGCGT
901 ACGATAACGC ATAGTGTGTT TCCCTCCACT TAAATCGAAG GGTGTGTCTT
951 TGGATCGCGC GGGTCAAATG TATATGGTTA ATATACATCC GTAGGCACGT
      Primer
1001 AATAAAGCGA GGGGTTTCGA TCCCCCGGTT ACCCCCGGTT GGGGCCGA

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Figure 2. Nucleotide sequence of the 3' terminal 1048 nucleotides of TMV (Tom-K). The start and stop codons of the coat protein ORF and the sequence of the primer used to generate the cDNA clones are underlined.

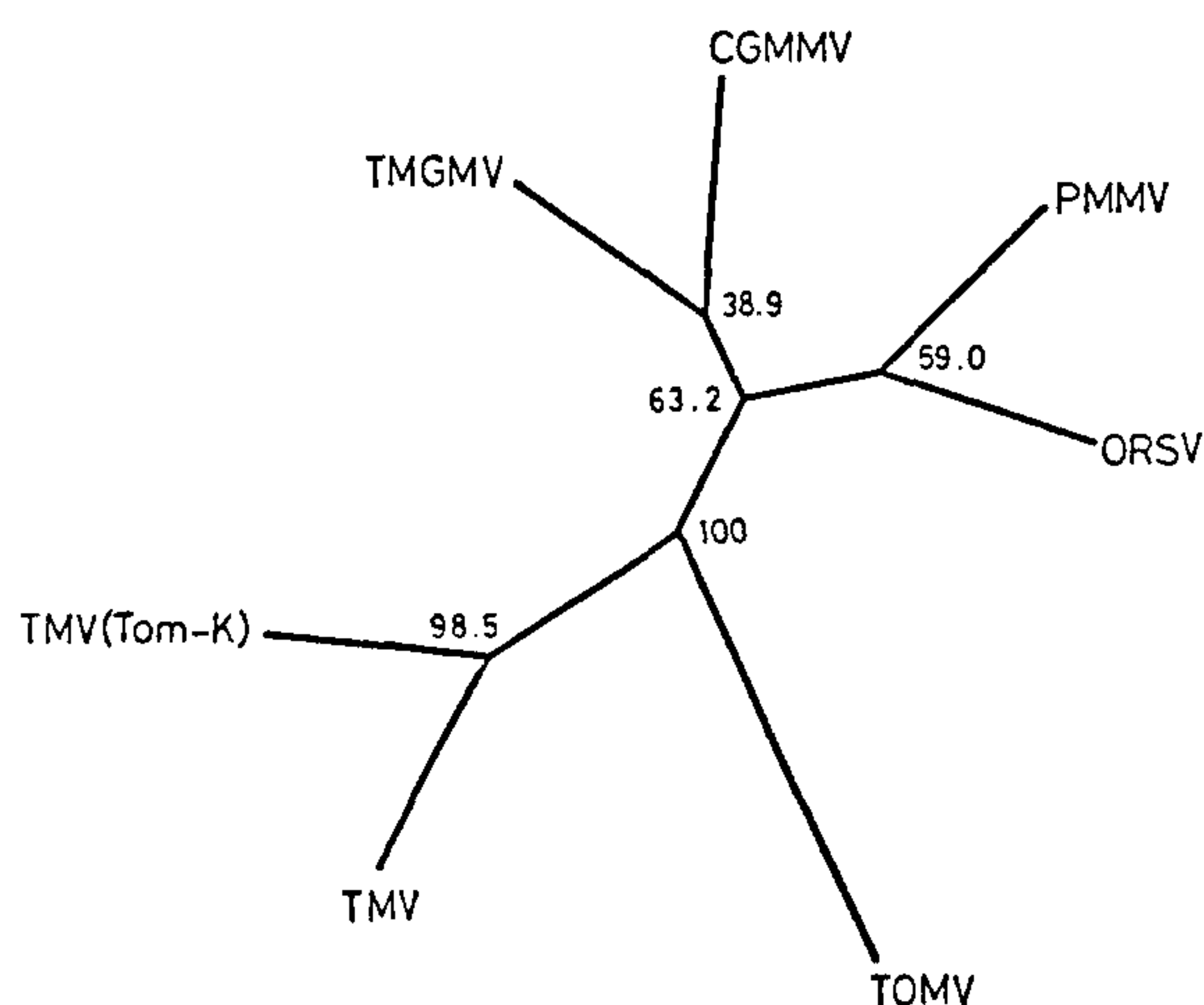


Figure 3. Phylogenetic analysis of tobamoviral coat protein. The dendrogram represents the consensus relationship obtained from 100 bootstrap samples for CP sequences. The number at the branch nodes indicate the number of times a particular branch appeared in all the trees that were used to determine the consensus tree.

N. glutinosa leaves of different sizes, they produced widely varying lesion numbers. But mean lesions/cm² were similar and varied from 6.09 to 6.88.

TMV (Tom-K)	MSYNITTPSQFVFLSSAWADPIELINLCTNALGYQFQTQARTVVQRQFSEVWKPSQVPT
TMV	MSYSITTPSQFVFLSSAWADPIELINLCTNALGNQFQTQARTVVQRQFSEVWKPSQVPT
ToMV	MSYSITSPSQFVFLSSVWADPIELNVCTNSLGNQFQTQARTTTVQQQFSEVWKPFPOST
	.;*****.*****;*.***;** *****.***;***** ** *
TMV (Tom-K)	VSSPDSDFKVYRYIAVLEPLVTRLLGAFDTRNRIIEVENQANPTTAETLDATRRVEDATV
TMV	VRFPDSDFKVYRYNAVLDPVLTALLGAFDTRNRIIEVENQANPTTAETLDATRRVDDATV
ToMV	VRFPDQVYKVYRYNAVLDPVLTALLGAFDTRNRIIEVENQSQPTTAETLDATRRVDDATV
	* .. ;*****.***;*.*** *****.*****;*****
TMV (Tom-K)	AIRSAINNLIIVELIRGTGSYNRSSFESSSGLVWTSPPAT
TMV	AIRSAINNLIIVELIRGTGSYNRSSFESSSGLVWTSPPAT
ToMV	AIRSAINNLIIVELIRGTGLYNQNTFESMSGLVWTSAPAS
	*****; **.*** **.;*** *****.***;

Figure 4. Comparison of the deduced amino acid sequence of the coat protein of TMV (Tom-K) with TMV strain vulgare and ToMV. Conservative substitutions are indicated by . or ; * indicates identities.

Purified virion preparations gave an yield of 80 mg/100 g of leaf tissue, assuming an extinction coefficient of 3.1 for a 0.1% solution at 260 nm. The ultra-violet absorption spectrum of the purified virus showed maximum absorption at 260 nm. The A₂₆₀/A₂₈₀ ratio of the virus was close to 1.2. Electron microscopy of purified virions of the virus under study showed that the particles were rigid rods measuring 300 × 18 nm in size (Figure 1a). The CP of the virus was shown to have a molecular weight of 18,000 daltons (Figure 1b). The virus was found to consist of a single species of RNA which moved the same distance as physalis mottle virus RNA of molecular weight 2 × 10⁶ daltons when subject to agarose gel electrophoresis (Figure 1c). The protein and nucleic acid molecular weights determined for the Kolar viral isolate agree with those reported in literature¹⁷⁻¹⁹.

The polyclonal antiserum produced against the virus under study reacted with the infected sap and the purified virus to give titres of 1/10,000 and 1/32,000, respectively, in penicillinase-based DAS-ELISA. The penicillinase globulin conjugate had a titre of 1/2000 when tested in DAS-ELISA. Table 1 (ELISA results) shows that the virus can be detected in infected leaf extracts in the dilution range of 10⁻¹ (1.14) to 10⁻⁷ (0.054) using an antiserum dilution of 1:1000. In comparison, the local lesion host *N. glutinosa* detected the virus in tomato leaf extracts diluted up to 10⁻⁶.

Among the recombinant clones generated, clone 82 released an insert of size 1.04 kb upon digestion with *Xba*I and *Hind*III. Automated DNA sequencing of this clone in both the directions resulted in the sequence of the 3' terminal 1048 nucleotides of the virus (Figure 2). Comparison of this sequence with other members of the tobamovirus group showed that this sequence aligned at nucleotide 5711 of TMV and ended at 6395, the 3' terminal nucleotide of TMV²⁰. It is interesting to note that the sequence obtained matched well with that of TMV (93.1% identity) whereas it had much less homology with ToMV (73.7% identity). This suggests that though this isolate produces local lesion on *N. sylvestris*, it is a

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Table 2. Per cent identity of the coat protein and 3' non coding region of TMV (Tom-K) with other tobamoviruses

Virus	Nucleotide sequence of CP	3' NC	Deduced amino acid sequence of CP	Ref.
Tobacco mosaic virus strain vulgare (TMV)	92.6	97.6	94.3	20
Tomato mosaic virus (ToMV)	72.7	77.3	78.6	21
Odontoglossum ring spot virus (ORSV)	65.8	69.5	70.5	22
Pepper mild mottle virus (PMMV)	64.5	63.3	68.2	23
Tobacco mild green mosaic virus (TMGMV)	62.9	35.7	66.7	24
Cucumber green mottle mosaic virus (CGMMV)	45.7	56.0	35.8	25

strain of TMV and not a strain of ToMV. This is further supported by the data presented in Table 2 which depicts the per cent identity of the coat protein and 3' non-coding region of the present isolate with other tobamoviruses. Figure 3 shows the dendrogram of the tobamoviral CP sequences. Even here TMV (Tom-K) clusters with TMV CP rather than with ToMV CP. Comparison of the CP sequence of TMV (Tom-K) with the TMV strain vulgare²⁰ showed that there are 9 amino acid changes between TMV CP and that of the Indian isolate (Figure 4). Out of these, 5 of the changes are conservative substitutions. On the other hand, 32 amino acid changes were observed between TMV (Tom-K) and ToMV²¹. Thus the analysis of the 3' terminal nucleotides of the Indian isolate clearly establishes that the isolate is a strain of tobacco mosaic and hence is called tobacco mosaic virus tomato strain (TMV (Tom-K)).

The nucleotide sequence reported in this paper has been submitted to GenBank and assigned the accession number AF 126505.

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