Host Range and Purification of the Nucleic Acid of a Defective Mutant of Tobacco Mosaic Virus

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ABSTRACT

A defective mutant, existing in vivo as "free" nucleic acid, was isolated following treatment of tobacco mosaic virus strain U_1 (TMV-U_1) with nitrous acid. This mutant was transmitted to several solanaceous plants, in which it induced a variety of symptoms but its systemic movement was slow. An extraction medium was developed for isolating this mutant from leaves, and it was subsequently purified by polyacrylamide gel electrophoresis. Under conditions of virus reconstitution, incubation of mutant nucleic acid with TMV-U_1 protein, but not with bovine serum albumin, resulted in a 10- to 15-fold increase in its infectivity.

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Additional key words: TMV-RNA, TMV-protein, virus reconstitution.

Previous studies on defective strains of tobacco mosaic virus (TMV) have been concerned primarily with characterization of their nonfunctional proteins (5, 9, 11, 13, 15). Little attention has been given to their biological properties (6, 14) or characterization of their genomes. This report concerns host range studies, and standardization of a procedure for isolation and purification of the nucleic acid of a defective TMV mutant.

MATERIALS AND METHODS.—Plants were grown in steam sterilized soil in a greenhouse at 23 C. Strict precautions were observed for preventing contamination by TMV-U_1 of plants, glassware, and other equipment used for the mutant. TMV-U_1 was purified from Nicotiana tabacum L. var. 'Samsun' (12). Its protein was prepared by acetic acid treatment (2) and its ribonucleic acid (RNA) was isolated by phenol treatment in the presence of purified bentonite (4). All dilutions for infectivity tests were done in ice-cold 0.067 M phosphate buffer, pH 7.0, containing Celite (50 mg/ml) and assayed on N. tabacum 'Xanthi-nc' or on Phaseolus vulgaris L. 'Pinto'.

The mutant was isolated as follows: TMV (4 mg/ml) was treated with nitrous acid (12) at 25 C to < .001% survival after which the reaction mixture was cooled to 0 C and pH adjusted to 7.0. Samples of the treated virus were diluted and assayed on Xanthi-nc. After 3 days, five local lesions were selected at random and the tissues of each were individually homogenized. The resulting extracts were rubbed on young (3- to 4-leaf stage) Samsun plants. After about 3 weeks one plant showed symptoms typical of defective TMV strains (14). The defective nature of this isolate was confirmed by criteria established by Siegel et al. (14). Additionally, leaf-dip samples of this plant showed no viruslike particles by electron microscopy. Subsequently, this isolate was transferred and propagated in young Petunia hybrida L. plants maintained at 30 C.

Mortars and pestles and the extraction media were precooled prior to using them for extraction of the mutant from leaf tissue. Only leaves showing pronounced symptoms were used for virus extraction. These were excised, washed with deionized water, dried with absorbent paper, and cut into small strips. Weighed amounts of the tissue were cooled at 2 C for 4 to 5 hr. Inoculum for host range studies was prepared by homogenizing leaf tissue in 0.067 M phosphate buffer, pH 7.0, (1 g tissue/1 ml buffer) containing bentonite (100 mg/ml) and Celite, and the homogenate was immediately rubbed on plants. For nucleic acid extraction, 10 g of leaf
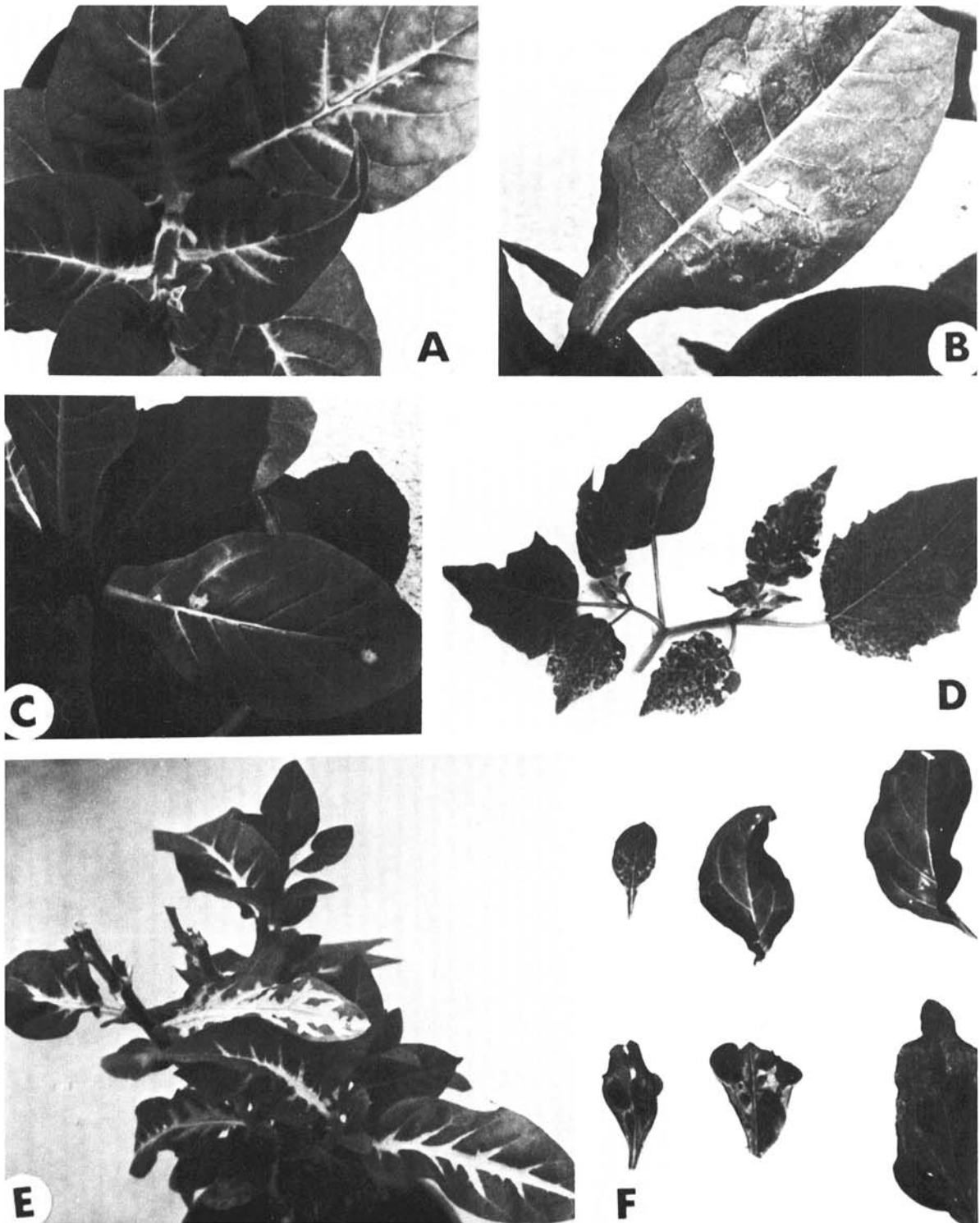
Fig. 1. Symptoms induced by the defective mutant following sap inoculation to: **A)** *Nicotiana tabacum* var. 'Samsun'; **B)** *N. tabacum* 'Java'; **C)** *N. sylvestris;* **D)** *Physalis peruviana;* **E)** *N. longiflora;* and **F)** *Petunia hybrida.*

tissue was homogenized in a medium prepared by combining the following solutions: 5 ml of a mixture of 0.25 M glycine, 0.125 M KH$_2$PO$_4$, and 0.75 M NaCl, pH 9.5; 3 ml phenol saturated with 0.067 M neutral phosphate buffer; 1.5 ml 2% sodium dodecyl sulfate, and 0.5 ml (100 mg) of purified bentonite. The emulsion was centrifuged 20 min at 7,000 g and the nucleic acids precipitated from the aqueous phase with 95% cold

ethanol. The nucleic acids were washed two to three times with ethanol, centrifuged, and dissolved in TP (0.05 M tris, 0.05 M Na_2HPO_4, pH 8.5) buffer (10) followed by a final, low-speed centrifugation. These preparations were used immediately or stored at −20 C.

Gradient polyacrylamide gels (2-12%) were prepared (1) in 7- × 76-mm tubes with Loening's buffer (7) which was also used for electrophoresis. Nucleic acid samples in 5% sucrose solution were layered on gels with the electrophoresis performed in Canalco vessels using an ISCO power supply (Model 490) at constant current (4mA/gel) for 2 hr at 25 C. Gels were stained with 0.01% methylene blue (dissolved in 0.4 M acetate buffer, pH 4.7) for 4 hr and subsequently destained with water. For eluting the mutant-RNA and TMV-RNA after electrophoresis, regions of unstained gels (in comparison to companion stained samples) containing the desired fractions were sliced and homogenized in a small volume of TP buffer. Sections from several gels were pooled to obtain enough sample for further studies. Neither rate zonal sucrose density-gradient centrifugation nor chromatography on methylated albumin Kieselguhr

Fig. 2. Electrophoresis of nucleic acid preparations in polyacrylamide gels. The electrophoresis was done for 2 hr at a constant current of 4mA/gel followed by staining with methylene blue. **A)** Healthy petunia; **B)** mutant-infected petunia leaves; and **C)** phenol-isolated TMV-RNA from virus.

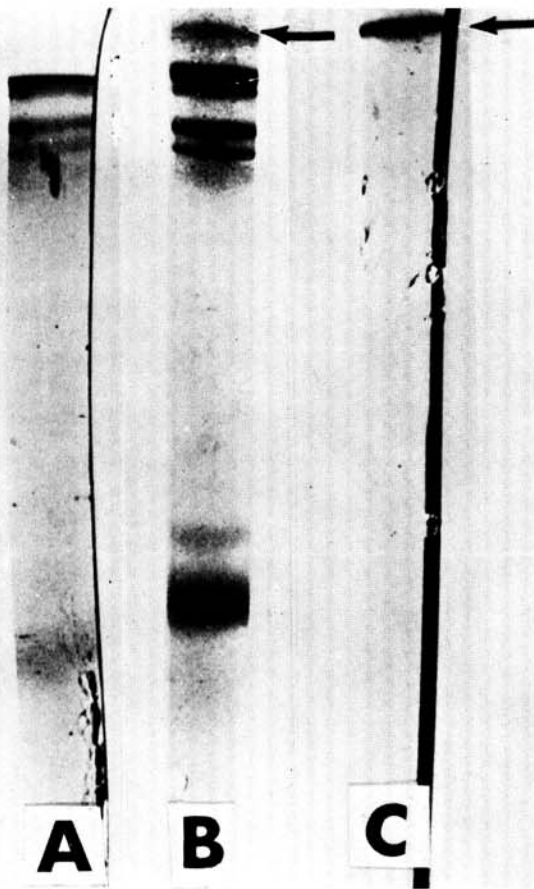columns (8) were satisfactory for separating the mutant nucleic acid from cellular RNA's.

Electrophoretically purified mutant-RNA and TMV-RNA were used for reconstitution with TMV protein (3). Reconstituted preparations were centrifuged at 5,000 g for 10 min and the supernatant, after dialysis (4 hr, 5 C) against 0.067 M neutral phosphate buffer, was assayed for infectivity or stored at 2 C in the presence of a crystal of thymol. For electron microscopy, uranyl formate-stained samples were examined with a RCA EMU 3G electron microscope. Serological tests were done in Ouchterlony gels prepared with 1% Ionagar in 0.067 M phosphate buffer, pH 7.0, containing 0.025% sodium azide.

RESULTS AND DISCUSSION.—Symptoms induced by the mutant on various hosts are shown in Fig. 1. The mutant-induced symptoms on Samsun tobacco were similar to those caused by the PM1 and PM2 strains of TMV (14). Bright yellow spots with irregular margins and occasional veinbanding developed on the inoculated leaves of *N. tabacum* 'Java' and *N. sylvestris* Speg. & Comes but only rarely was there any systemic movement of the mutant in these hosts. On *N. longiflora* L., the symptoms consisted of yellow veinbanding along the mid-vein and secondary veins, whereas on *Physalis peruviana* L., a variegated mosaic or yellow mottle developed. On *Petunia hybrida* a variegated or generalized yellow mottle and leaf deformation occurred, and only in this host was there any rapid movement of the mutant in inoculated and noninoculated plant parts. Furthermore, the systemic transport of the mutant in *P. hybrida* was accelerated by keeping the plants at 30 C without affecting the gross symptom expression or endogenous titer of the mutant. The mutant induced local lesions on Xanthi-nc *N. glutinosa* L., *Chenopodium amaranticolor* Costa & Reyn., and *P. vulgaris* Pinto were similar in size and appearance to those caused by TMV-U_1.

Upon assay, homogenates of individual mutant-induced lesions produced 1-5 (avg=2) lesions per half-leaf on Xanthi-nc vs. 40-62 (avg=48) by TMV-U.

Electrophoretic separation of nucleic acids from healthy and mutant-infected petunia leaves and purified TMV-RNA is shown in Fig. 2. The mutant-RNA and TMV-RNA (shown by arrows) moved little on polyacrylamide gels and had similar mobilities but were adequately separated from cellular RNA's. The specific infectivity of the mutant nucleic acid was increased 2- to 3-fold after electrophoretic separation. It was infectious when stored for 3 to 4 days at 2 C, but not after 7 days. The mutant nucleic acid was inactivated by incubation with 0.1 µg/ml of pancreatic ribonuclease for 1 hr at 25 C.

When purified mutant nucleic acid and TMV-RNA were incubated with TMV-protein under conditions favorable for virus reconstitution, approximately a 10- to 15-fold increase in their infectivities occurred (Table 1). Treatment with bovine serum albumin caused no increase in the infectivity of mutant-RNA or TMV-RNA. A direct comparison shows, however, that the specific infectivity of the reconstituted mutant was only one-half to one-third that of TMV. Electron microscopic examination of samples of the reconstituted mutant revealed many TMV-like particles. Comparable samples of TMV protein lacked these particles. Incubation of reconstituted mutant with 0.1 µg/ml of pancreatic ribonuclease (25 C, 1 hr)

TABLE 1. Infectivity of mutant nucleic acid and TMV-RNA before and after
incubation with TMV protein or bovine serum albumin in 0.1 M sodium pyrophosphate,
pH 7.2, for 8 hr at 25 C

	Infectivity[a]				
	Experiment No.				
	1	2	3	4	5
TMV protein[b] (660 μg/ml)	0[e]	0	0	0	0
Mutant nucleic acid[c] (33 μg/ml)	3	5	7	22	15
Mutant nucleic acid[c] (33 μg/ml) + TMV-U$_1$ protein (660 μg/ml)	72	65	83	210	149
Mutant nucleic acid[c] (33 μg/ml) + bovine serum albumin (660 μg/ml)			6		24
TMV-RNA[d] (33 μg/ml)	16	14	19	42	58
TMV-RNA[d] (33 μg/ml) + TMV-U$_1$ protein (660 μg/ml)	230	275	312	630	696
TMV-RNA[d] (33 μg/ml) + bovine serum albumin (660 μg/ml)			12	28	30

[a] Infectivity assays in experiments 1-3 were made on *Nicotiana tabacum* 'Xanthi-nc' and in experiments 4-5 on primary leaves of *Phaseolus vulgaris* 'Pinto'.

[b] TMV protein was prepared by acetic acid treatment (2).

[c] Electrophoretically purified mutant nucleic acid.

[d] TMV-RNA purified from virus by phenol and bentonite (4) and subjected to electrophoresis prior to use.

[e] No. of local lesions per half-leaf (out of four to seven) of Xanthi-nc or per primary leaf (out of 12 to 16) of 'Pinto'.

resulted in over 80% loss of its infectivity; apparently, only 15 to 20% of the mutant-RNA molecules were coated with TMV-U$_1$ protein and became resistant to ribonuclease attack. Homogenates of single lesions induced by ribonuclease-treated reconstituted mutant produced two to three lesions per Xanthi-nc half-leaf, indicating that these contained defective virus genome. When extracts of 10 such lesions were individually inoculated on young petunia plants, seven plants developed symptoms typical of the mutant. Preparations of reconstituted mutant were infectious after storage at 2 C for 8 to 10 days and occasionally up to 2 weeks, but not after 4 weeks.

The procedure of Hariharasubramanian & Siegel (5) was used to isolate and purify a protein from mutant-infected petunia plants; this protein was not recovered from healthy plants. This protein, however, gave no serological reaction with TMV-antiserum. The coat protein of the PM1 defective strain of TMV also gives no visible serological reaction with TMV antiserum (9). Upon electrophoresis in polyacrylamide gels in the presence of 8 M urea, this protein moved to the same position as TMV-protein; this is characteristic of another nonfunctional TMV coat-protein (11). Since further studies on this protein were not done, the relationship (if any) of this mutant with other defective TMV strains is uncertain.

The results of host range studies show that the defective mutant can be mechanically transmitted to several plant species, and based on symptom expression, it apparently remains in defective form in these hosts. During the past 4 years, when the mutant was maintained in petunia, there was no indication of its reversion to the parent wild-type strain, which suggests that it is genetically stable.

The similarities between the electrophoretic mobilities of mutant nucleic acid and TMV-RNA indicate that the former does not have a detectable deletion. The mutant nucleic acid did not band, however, as discretely as did TMV-RNA, possibly owing to some heterogeneity. The results of reconstitution experiments suggested that mutant nucleic acid can be coated with TMV-protein and ca. 15-20% of the reconstituted particles become resistant to ribonuclease. The fact that the infectivities of mutant-RNA and TMV-RNA were enhanced only upon reconstitution with TMV-protein, and not by treatment with bovine serum albumin, precludes the possibility of a nonspecific stabilization of the virus genome. Nonetheless, the use of this method in preserving genomes of defective TMV strains is limited since reconstituted preparations lose infectivity during in vitro storage. Appropriate conditions need to be examined and developed for extended storage of reconstituted mutant particles in stable and infectious form. Our studies suggest that the defectiveness of the TMV mutant described in this report is not related to an inherent instability of the virus nucleic acid, but probably results from a failure of the in vivo encapsidation process, as in other defective TMV strains, resulting from the formation of a nonfunctional coat-protein.

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