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# Effects of Two TMV Strains on the Synthesis and Stability of Chloroplast Ribosomal RNA in Tobacco Leaves*

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*Summary.* Multiplication of TMV-strains *vulgare* (light-green/dark-green mosaic symptoms) and *flavum* (severe yellow/green mosaic) had different effects on the ribosomal RNA of tobacco leaf chloroplasts. *Vulgare* inhibited chloroplast ribosomal RNA synthesis while having no effect on cytoplasmic ribosomal RNA synthesis (Fig. 2). *Flavum* inhibited chloroplast ribosomal RNA synthesis more severely than *vulgare*, and caused an earlier degradation of chloroplast ribosomal RNA than in control or *vulgare*-infected leaves (Fig. 1). *Flavum* also inhibited cytoplasmic ribosomal RNA synthesis. A connection between these differing effects on chloroplast ribosomal RNA metabolism and severity of visible symptoms is suggested, and discussed in relation to a possible influence on symptoms of denatured virus coat protein.

## Introduction

Hirai and Wildman (1969) reported recently that TMV strain U1 (*vulgare*) inhibited chloroplast ribosomal RNA synthesis while not affecting cytoplasmic ribosomal RNA synthesis. Protein synthesis by chloroplasts isolated from virus-infected leaves was found to be inhibited. They suggested that these effects on chloroplast metabolism give rise to the light-green mosaic symptoms of infection.

In view of the very different visible symptoms produced by different strains of TMV, it is of interest to compare the consequences of their multiplication for chloroplast metabolism. I have examined the effects of infection by TMV strains *vulgare* (light-green symptoms) and *flavum* (severe yellowing and early leaf collapse) on chloroplast ribosomal RNA synthesis and stability. The results suggest a correlation between the effects of virus multiplication on chloroplast ribosomal RNA synthesis and degradation, and the severity of visible symptoms.

## Materials and Methods

Tobacco (*Nicotiana tabacum*) var. "Samsun" plants were grown in a greenhouse at $23 \pm 2°C$ with continuous illumination of 3,500 lux night (Osram L-Fluora tubes) and *ca.* 7,500 lux day. 9 cm long leaves on 20 cm-tall plants were dusted with carborundum and inoculated by rubbing with either sterile phosphate buffer, 0.066 M, pH 7.0 (Control); 0.1 % TMV-strain *vulgare* in phosphate buffer, or TMV-strain *flavum*-infected leaves ground in phosphate buffer. The leaves were washed with running tap-water immediately after inoculation.

*Radioactive Incubation.* A detached leaf was placed with its petiole in 0.5 ml water containing 0.5 mc $^{32}$P-phosphate. This was completely taken up in 60—90 minutes. The leaf was then floated on water until a total $^{32}$P incubation time of 5 hours was reached. This method ensured equal uptake of $^{32}$P by all treatments.

* Abbreviations: TMV = Tobacco Mosaic Virus; RNA = Ribonucleic acid; DNA = Deoxyribonucleic acid; m = millions (in molecular weight values).

Bacterial ribosomal RNAs and chloroplast ribosomal RNAs have similar electrophoretic mobilities (Loening and Ingle, 1967). Although the $^{32}P$ incubations were done under non-sterile conditions, I do not think that labelling of bacterial ribosomal RNA influenced the labelling pattern of chloroplast ribosomal RNA: when old tobacco leaves (which do not synthesise chloroplast ribosomal RNA) were labelled in this way, no radioactivity was found in the bacterial/chloroplast ribosomal RNA position.

*RNA Extraction.* 0.4 g leaf material was homogenised for 10 seconds in a Bühler Homogeniser (E. Bühler, Tübingen, Germany) at *ca.* 40,000 r.p.m. in 8 ml 30 mM tris-chloride, pH 7.6, containing 0.5 % 1,5-naphthalenedisulphonic acid, disodium salt (Eastman Kodak) (Hastings and Kirby, 1966) and 5 mM Cleland's Reagent, at 0°C. Immediately after homogenisation, sodium tri-iso-propylnaphthalenesulphonate (Eastman Kodak) and sodium 4-aminosalicylate were added to final concentrations of 1 and 5 % respectively (Parish and Kirby, 1966). The homogenate was shaken with an equal volume of phenol-cresol mixture (Kirby, 1965: redistilled phenol 1,000 g; redistilled m-cresol 140 ml; 8-hydroxyquinoline 1 g; water to saturate). Thereafter extraction was at 10°C. The aqueous phase was recovered by centrifuging for 20 minutes at 10,000 × g. Sodium chloride was added to a final concentration of 0.3 M and the phenol-cresol extraction repeated twice. Nucleic acids were precipitated from the final aqueous phase by addition of two volumes of ethanol and 12 hours at −20°C. The nucleic acids were collected by low-speed centrifugation and dissolved in 2 ml 0.15 M sodium acetate pH 6 containing 0.5 % sodium dodecyl sulphate, and were dialysed against this solution for 24 hours at room temperature. Nucleic acids were precipitated by ethanol at −20°C and the precipitate was washed with ethanol, dried and dissolved in 0.2—1.0 ml electrophoresis buffer containing 5 % sucrose.

*Electrophoresis.* Fractionation of nucleic acids was on polyacrylamide gels (Loening, 1967), 7 cm long, 6 mm diameter, containing 2.4 % acrylamide and 0.12 % bisacrylamide. The buffer had a pH of 7.8 and contained tris 36 mM; $NaH_2PO_4$ 30 mM; EDTA 1 mM and 0.2 % sodium dodecyl sulphate. About 15 μg of RNA in 10 μl electrophoresis buffer were applied to each gel. Electrophoresis was for 2.5 hours at room temperature at 50 V and 5 mA per gel. The gels were scanned optically at 265 nm in a Joyce-Loebl Chromoscan. Radioactive gels were then frozen in solid $CO_2$ and sliced transversely at 0.5 mm intervals with a Mickel Gel Slicer (Mickel Laboratory Engineering Co., Gomshall, Surrey, England). The slices were dried on a film base and counted in a Packard 460 chromatogram scanner.

*Chlorophyll.* Chlorophyll content was determined by the method of Comar and Zscheile (1942). 1 g of leaf was homogenised in the Bühler homogeniser in 8 ml 80 % acetone. The pigment was extracted into diethyl ether and chlorophyll concentration calculated from the absorbances at 600, 644 and 662 nm.

## Results

### *Changes in Chloroplast Ribosomal RNA in Control Leaves*

Electrophoresis of nucleic acids from young (5 cm long) control leaves (Fig. 1 a) shows DNA at 0.8 cm; 1.28 m[1] and 0.71 m cytoplasmic ribosomal RNA at 2.5 and 3.8 cm and 1.1 m and 0.56 m chloroplast ribosomal RNA at 2.9 and 4.4 cm. The chloroplast RNA species were present in approximately equimolar amounts: this contrasts with previous reports on other tissues, such as French bean and raddish (Loening and Ingle, 1967; Ingle, 1968) and spinach, pea, tomato and tobacco (Spencer and Whitfeld, 1966) where the 1.1 m component was always reduced or absent.

[1] Bishop, Claybrook and Spiegelman (1967) and Loening (1969) have shown a linear relationship between log molecular weight and electrophoretic mobility. I will refer to all RNA species by molecular weight values determined by reference to the plant cytoplasmic ribosomal RNAs, and using Loening's (1969) molecular weight values for these molecules of 1.28 m and 0.71 m.

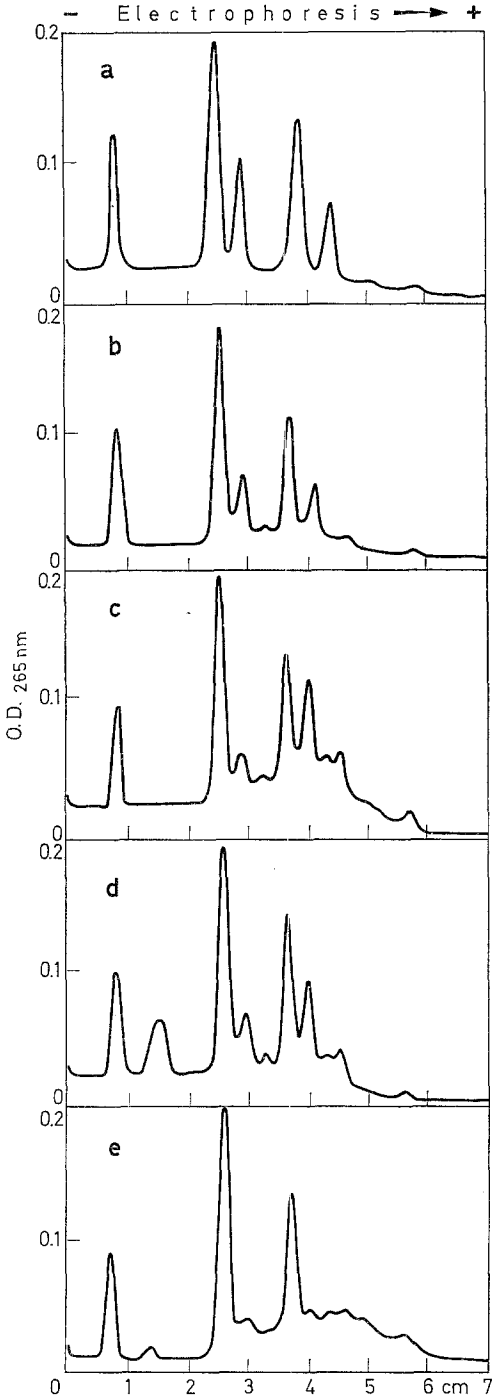


Fig. 1

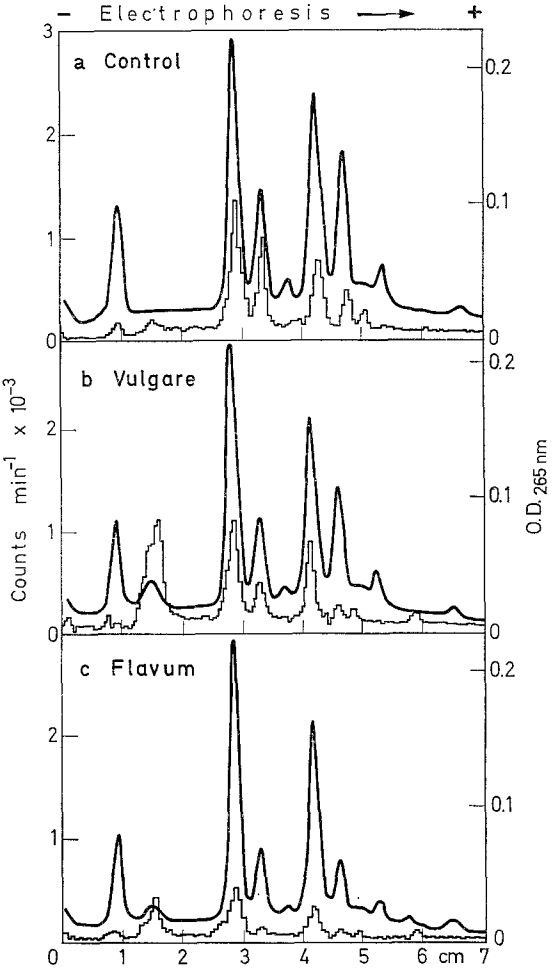


Fig. 2

Fig. 1 a—e. Electrophoresis of nucleic acids from tobacco leaves. a 5 cm long healthy leaf. b 9 cm long healthy leaf, at time of inoculation. c Control leaf, 17 cm long, 4 days after sham inoculation of 9 cm leaf. d Leaf 4 days after inoculation with TMV-strain *vulgare*. e Leaf 4 days after inoculation with TMV-strain *flavum*

Fig. 2 a—c. Electrophoresis of tobacco leaf nucleic acids labelled with $^{32}P$ and extracted 2 days after inoculation with a Control; b TMV-*vulgare*; c TMV-*flavum*

During growth of the leaf (Fig. 1 b, c) the 1.1 m chloroplast ribosomal RNA was degraded, but the 0.56 m peak persisted. Small peaks of molecular weights 0.9 m (3.3 cm); 0.45 m (4.2 cm); 0.40 m (4.6 cm) and 0.20 m (5.8 cm) appeared. Ingle (1968) has presented evidence suggesting the origin of these peaks by the degradation of the chloroplast ribosomal RNA, principally involving the larger component.

*Ribosomal RNA Synthesis*

Electrophoresis of control treatment nucleic acids labelled 2 days post-inoculation shows labelling of cytoplasmic and chloroplast ribosomal RNAs (Fig. 2a) The origin and significance of the small radioactive peak at 5.0 cm are unknown Apart from this, none of the minor optical density peaks was labelled, supporting their origin by *in vivo* degradation rather than by degradation during RNA extraction. The small labelled peak at 1.5 cm is identified as the cytoplasmic ribosomal RNA precursor (Loening, 1967; Fraser, 1968).

Both virus infected treatments showed optical and radioactivity peaks of TMV-RNA at 1.6 cm The accumulation and synthesis of strain *vulgare* RNA was greater than of *flavum* RNA (Fig 2b, c). Both viral treatments showed a small peak of radioactivity at 6 cm which was not present in the control. Its origin and significance are unknown.

Labelling of cytoplasmic ribosomal RNA in *vulgare*-infected leaves was slightly lower than in the control, but synthesis of chloroplast ribosomal RNA was reduced to 40 % of the control value. This confirms the results of Hirai and Wildman (1969). The inhibition of chloroplast ribosomal RNA synthesis in *flavum*-infected leaves was stronger than with *vulgare*; in addition the labelling of cytoplasmic ribosomal RNA was reduced to about 30 % of the control value.

*Ribosomal RNA Stability*

Leaves infected with TMV-*vulgare* showed a pattern of chloroplast ribosomal RNA degradation qualitatively and temporally similar to that in the control (Fig. 1). But in *flavum*-infected leaves, the degradation of chloroplast ribosomal RNA was accelerated. 2 days after inoculation with *flavum*, the amounts of 1.1 m and 0.56 m were lower than in the control (Fig. 2a, c). Unlike control or *vulgare*-infected leaves, where the 0.56 m molecule persisted while the 1.1 m was degraded, the 0.56 m RNA in *flavum*-infected leaves was also degraded. By 4 days after inoculation, little 1.1 m and 0.56 m chloroplast ribosomal RNA remained in *flavum*-infected leaves, and a large amount of material smaller than 0.56 m, probably degradation products, was present (Fig. 1e). The stabilities of the 1.28 m and 0.71 m cytoplasmic ribosomal RNAs in both types of virally infected leaves were similar to those in the control (Fig. 1c—e).

## Discussion

There are therefore considerable differences between the two strains of TMV in effect on chloroplast ribosomal RNA metabolism. The severer visible symptoms produced in *flavum* infection may therefore be a consequence of the severer inhibition of chloroplast ribosomal RNA synthesis (and possibly other types of

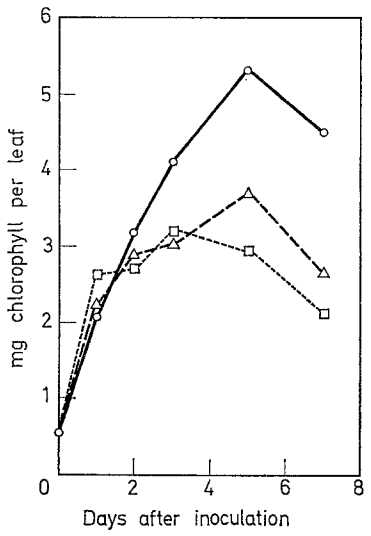


Fig. 3. Chlorophyll content per leaf after inoculation with TMV-*vulgare* (△— —△); TMV-*flavum* (□- - - -□); control (○——○)

chloroplast RNA synthesis), and the degradation of chloroplast ribosomal RNA by this strain, as the two effects must lead to a virtual cessation of protein synthesis inside the chloroplasts. In addition, the severe inhibition of cytoplasmic ribosomal RNA synthesis by *flavum* might also play a part in controlling the stability of the chloroplasts. The role of nuclear RNA synthesis and cytoplasmic protein synthesis in chloroplast metabolism is not yet fully defined, but there is some evidence for specification of chloroplast enzymes by nuclear genes (review by Kirk and Tilney-Basset, 1967).

An alternative explanation for the origin of yellow symptoms has been advanced by Jockusch and Jockusch (1968). They demonstrated that where yellow symptoms were produced, either by yellow mutants such as *flavum* or by multiplication of TMV mutants with temperature-sensitive coat proteins at high temperatures, greater amounts of insoluble virus coat protein were present. They argued that the insoluble proteins may lead to disruption of cell organelles, in the case of chloroplasts liberating chlorophyllase from some bound state and leading to chlorophyll breakdown and yellowing. This hypothesis might be extended to explain the accelerated breakdown of chloroplast ribosomal RNA in *flavum*-infected leaves by liberation of ribonuclease in disrupted chloroplasts.

However, there are certain arguments against the liberation of spatially separated enzymes through disruption of structure by denatured proteins being the sole cause of yellow symptoms.

1. Measurements of chlorophyllase activity (Peterson and McKinney, 1938; G. Paulsen-Oehlen, unpublished data) show that *flavum*-infected leaves attain a much higher chlorophyllase activity than control or *vulgare*-infected leaves. Clearly it is important to distinguish between whether this increased activity is a result of liberation and activation of pre-existing enzyme, or stimulation of enzyme synthesis. As the assays were done on acetone-dry powder extracts, which

would tend to eliminate any *in vivo* spatial segregation of enzyme and substrate, stimulation of new enzyme synthesis seems more probable. This would argue in favour of a directive influence of *flavum* multiplication on chloroplast metabolism rather than a merely passive influence through disruption of structure.

2. My results suggest that the breakdown of chloroplast ribosomal RNA begins much earlier than that of chlorophyll in *flavum*-infected leaves. From Fig. 2 c, where about 50 % breakdown of chloroplast ribosomal RNA is recorded by 48 hours after inoculation with *flavum*, it is clear that the effect on chloroplast ribosomal RNA stability is effected very early in virus multiplication, indeed must be effected before the majority of virus protein has been synthesised. On the other hand, Fig. 3 shows that the degradation of chlorophyll in virus-infected leaves did not become significant until three days after inoculation, and that degradation of chlorophyll in *flavum*-infected leaves first became more pronounced than in *vulgare*-infected leaves even later. Some degree of simultaneity of inception of degradation of chloroplast ribosomal RNA and chlorophyll, and an earlier distinction in chlorophyll contents in *flavum* and *vulgare*-infected leaves might be expected if disruption through denatured proteins were the sole cause.

3. The TMV mutant Ni 118 has temperature-sensitive coat protein (Jockusch, 1968), but produces green symptoms at high temperatures.

In view of these doubts, experiments are being carried out in an attempt to discover the relative importance of the effects on RNA synthesis and stability and the effects of denatured proteins in the determination of visible symptoms.

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