The Ribonucleic Acid of Tomato Spotted Wilt Virus

By J. VAN DEN HURK, P. W. L. TAS AND D. PETERS

Laboratory of Virology, Agricultural University,
Binnenhaven 11, Wageningen, The Netherlands

(Accepted 11 February 1977)

SUMMARY

TSWV nucleic acid, extracted from purified virus with phenol-SDS, was non-infectious. Following electrophoresis of the extracted nucleic acid in 2% polyacrylamide gels containing 0.5% agarose, 3 major and 2 minor bands were observed. The amount of material contained in the different bands varied with the season. Bands 1, 2, 3 and 4 were sensitive to RNases and resistant to DNase. This was a strong indication of the presence of single-stranded RNA in these bands. Precipitation in 2 M-LiCl, effect of heating and mobility in gels containing an increasing percentage of acrylamide confirmed the single-stranded character. Band 1a was resistant to the action of RNases and stained with Coomassie brilliant blue. The mol. wt. of RNAs 1, 2, 3 and 4 (corresponding to bands 1 to 4) were estimated under both non-denaturing and denaturing conditions and appeared to be 2.5 to $2.7 \times 10^6$, $1.9 \times 10^6$, $1.7 \times 10^6$ and $1.3 \times 10^6$, respectively. Following dissociation of the virus with Nonidet P 40, an infectious zone could be isolated after sedimentation in an 8 to 43% glycerol gradient.

INTRODUCTION

Very little information exists concerning the nucleic acid of tomato spotted wilt virus (TSWV). Van Kammen, Henstra & Ie (1966) concluded that the nucleic acid was RNA on the basis of positive orcinol and negative diphenylamine reactions. Best (1968) arrived at the same conclusion from data obtained by paper chromatography and estimation of the base composition of the nucleic acid (guanine 38%, adenine 35%, cytosine 9% and uracil 18%). In the cryptogram of TSWV (R/*:*/*:S:S:Th; Ie, 1970) data are missing on the strandedness and molecular weight of the nucleic acid, and its percentage content in the virus particles.

This paper describes a study of the nucleic acid of TSWV, with respect to its type, segmentation, molecular weight and infectivity. In addition, the isolation of an infectious ribonucleoprotein complex obtained after dissociation of the virus with Nonidet P 40 is described.

METHODS

Virus purification. Plant material infected with TSWV-S was ground in 0.01 M-tris (pH 8.0) containing 0.01 M-sodium sulphite and 0.1% cysteine hydrochloride. The extract was centrifuged for 10 min at 10,000 g. The pellet was resuspended in buffer consisting of 0.01 M-tris, 0.01 M-glycine, 0.01 M-sodium sulphite and 0.1% cysteine hydrochloride (pH 7.9). After clarification for 10 min at 10,000 g, the suspension was spun for 30 min at 25,000 rev/min. The pellet was resuspended and incubated with antiserum against healthy plant.
material for 1 h. After removal of the precipitate the virus was subjected to a cycle of rate and equilibrium centrifugation in sucrose gradients (Tas, Boerjan & Peters, 1977a).

Purification of RNA. Sterile solutions and glassware were used throughout all operations. Pellets of purified virus were dissolved in 0.01 M-tris-HCl, 0.1 M-NaCl, 0.001 M-Na₂ EDTA, pH 7.6, containing 1 or 2% SDS. This mixture was stirred vigorously for 10 min at room temperature, cooled to 4°C, and an equal volume of phenol, saturated with buffer containing 8-hydroxyquinoline at a final concentration of 0.1%, was added. After stirring for 10 min at 4°C the aqueous phase was separated from the phenol phase by centrifugation at 3000 rev/min for 15 min at 4°C. The phenol phase was re-extracted with one third volume of buffer. The aqueous phases were pooled and re-extracted three times with one third volume of phenol saturated with buffer. The nucleic acid was precipitated from the final aqueous phase using 2.5 volumes of ethanol and a few drops of 3 m-sodium acetate, pH 5.5. Precipitation was completed by storage overnight at -20°C. The nucleic acid precipitate was pelleted by centrifugation in a table centrifuge at 3000 rev/min for 15 min and was washed three times with 70% ethanol containing 0.1% sodium acetate. The nucleic acid was stored at -20°C either in ethanol or in 0.15 M-NaCl and 0.015 M-Na-citrate, pH 7.2 (1 × SSC).

Electrophoresis of RNA. Polyacrylamide gel electrophoresis under non-denaturing conditions was performed by a modification of the method of Loening & Ingle (1967); electrophoresis under denaturing conditions was by the method of Reijnders et al. (1973). Acrylamide and bisacrylamide were re-crystallized according to Shepherd & Gurley (1966).

Gels containing 2% acrylamide and 0.5% agarose were polymerized in glass tubes (65 × 6 mm) at 37°C for 30 min. Gels were left at room temperature overnight and usually pre-swollen in electrophoresis buffer for 12 to 16 h before use. Upper and lower parts of the gel were removed with a sharp razor blade. The length of the gel was adjusted to 5.5 cm.

For the analysis of nucleic acid under non-denaturing conditions the gels were pre-run at 4 mA/gel in 0.036 M-tris-H₃PO₄, 0.03 M-NaH₂PO₄.2H₂O, 0.001 M-EDTA, pH 7.7, containing 0.2% SDS for 1 h at 4°C. Following this run the electrophoresis buffer was renewed. The nucleic acid, dissolved in electrophoresis buffer supplemented with 0.2% SDS and 5% glycerol, was layered on top of the gels through the electrophoresis buffer. Electrophoresis of RNA samples was performed at 4°C for 3 to 4 h at 4 mA/gel. After electrophoresis the gels were soaked in water for 2 to 3 h and scanned at 260 nm in a Beckman DU spectrophotometer equipped with a Gilford 2410 Linear Transport, an ERA attachment and a linear/log recorder. Gels were also stained with 0.01% toluidine blue O in 40% methanol, and destained in water or 1% acetic acid and stored in the dark.

Incubation with RNase and DNase. After completion of electrophoresis of the RNA, the gels were washed in 2× SSC for 16 h and incubated with 25 units/ml RNase T₁ grade III (Sigma), from Aspergillus oryzae, and 25 μg/ml RNase A type 1-A, 5× crystallized, from bovine pancreas, (Sigma), at room temperature in 2× SSC (15 ml/gel). The distribution of the extinction at 260 nm over the gel was measured before and after incubation.

To determine the sensitivity of the nucleic acid to DNase the gels were washed in 2× SSC containing 5 mM-MgCl₂ for 16 h and incubated with 5 μg/ml DNase I in the same medium (15 ml/gel).

Precipitation in 2 M-LiCl. Nucleic acid extracted from TSWV was mixed with an equal volume of 4 M-LiCl. This suspension was stored overnight at -20°C. The resulting precipitate was removed by centrifugation at low speed, and RNA in the supernatant was concentrated by precipitation with 2 vol. cold ethanol. The low speed pellet and ethanol precipitate were analysed by electrophoresis in polyacrylamide gels.
RNA of tomato spotted wilt virus

Denaturation of the nucleic acid by heating. Nucleic acid from TSWV, suspended in 0.01 M-EDTA, pH 7.0, was heated at 100°C for 3 to 10 min and then cooled rapidly in ice. The treated extracts were analysed by electrophoresis in polyacrylamide gels.

Estimation of molecular weights of the different RNA segments in polyacrylamide gels. The single-stranded RNA of cowpea mosaic virus middle component (CPMV RNAa) and bottom component (CPMV RNAb) were used as standards. The mol. wt. values were taken as 1.37 x 10^6 and 2.02 x 10^6, respectively (Reijnders et al. 1974). Double stranded RNA molecules consisting of the respective replicative forms of the RNAs of CPMV were kindly supplied by A. M. Assink (Assink, Swaans & Van Kammen, 1973).

Isolation of infectious ribonucleoprotein. Purified virus was treated with 0.05% Nonidet P-40 (Shell) for 2 min, layered on an 8 to 43% glycerol gradient in 1 x SSC, and centrifuged for 1 h at 40000 rev/min in an SW 60 Ti rotor of a MSE 65 centrifuge. The gradient was fractionated and the extinction of each fraction measured at 257 nm. The infectivity of the fractions was determined by inoculation on detached leaves of Petunia hybrida.

RESULTS

Infectivity

The nucleic acid isolated by the phenol-SDS method was non-infectious. Purified virus disrupted with 0.1% SDS and inoculated on to Petunia hybrida was also non-infectious. Several changes in the extraction conditions (buffer composition, presence or absence of bentonite and RNase inhibitors) failed to yield infective preparations.

Polyacrylamide gel electrophoresis

The nucleic acid was analysed by electrophoresis on 2% polyacrylamide gels containing 0.5% agarose (Fig. 1). In the electrophoretic diagrams obtained by scanning of the gels at 260 nm, 4 to 5 peaks were detected; they corresponded with the bands found by staining. The relative amount of material contained in the different bands varied with the season. The reason for this inconsistency is not understood. All further experiments to be described were performed with nucleic acid extracted from virus preparations purified in the period June–July. The virus could be purified from infected leaves in high yield and with a high degree of purity (Tas et al. 1977a).

The extinction profiles obtained at 260 nm always revealed bands 1, 3 and 4 clearly, while band 2 was seen only when a high resolution was obtained. In most cases component 2 appeared as a shoulder of component 3 in the electrophoretic patterns, although bands 3 and 2 were always clearly resolved upon visual observation of stained gels. Band 1a was not always visible; this may be attributable to the relatively small amount of this component.

The observed band patterns cannot be due to the effects of storage of purified virus at −20°C prior to extraction. The same species were found when nucleic acid was extracted from virus used directly after purification.

Incubation with RNase and DNase

The nature of the nucleic acids was investigated by incubating the gels after electrophoresis with RNase and DNase. Fig. 2 clearly shows that the bands which form peaks 1, 2, 3 and 4 in the profile and the single stranded CPMV RNAs are equally sensitive to RNases. Double-stranded CPMV RNA was resistant under the incubation conditions employed. Incubation without RNase did not change the observed band pattern (Fig. 2).
It is interesting to note that band 1α appeared to be rather resistant to the action of the enzymes. Following incubation of the gels with RNase and staining with toluidine blue O, band 1α was still visible, whereas bands 1, 2, 3 and 4 had disappeared.

Fig. 3 shows the band pattern observed after incubation of the gels with DNase. A test for the presence of RNase activity in the DNase preparation using single stranded CPMV RNA proved negative. From the almost complete disappearance of the calf thymus DNA peak following incubation with DNase for 12 h, it was concluded that the DNase preparation was active. Under these conditions no breakdown of TSWV nucleic acid was observed. From the results obtained by the incubation of the gels with RNases and DNase it was concluded that bands 1, 2, 3 and 4 consisted of RNA of a single-stranded nature. The material in band 1α appeared not to be sensitive to either RNase or DNase.

**Differential precipitation by salt**

Information about the nature of a nucleic acid can also be deduced from its behaviour in solutions of increasing salt concentration (Erikson & Franklin, 1966). Whereas single-stranded RNA is precipitated by 2 M-LiCl, double-stranded RNA, double-stranded DNA and transfer RNA are not precipitated under these conditions (Segal & Sreevalas, 1974). The procedure of Segal & Sreevalas was applied to the nucleic acid of TSWV (Fig. 4). The material forming bands 1, 3 and 4 on the gels was almost completely precipitated by 2 M-LiCl suggesting that they are single-stranded. Band 2 was also found but was not well
resolved in the electrophoretic pattern (Fig. 4) suggesting that the material present in this band exhibited the same behaviour as that forming bands 1, 3 and 4. The concentrated supernatant contained material forming band 1a and a relatively small amount of material forming band 3. The occurrence of the latter material in the supernatant suggested that the single stranded RNA was not completely precipitated by 2 M-LiCl. The material forming band 1a behaved differently from that forming bands 1, 2, 3 and 4.

Effects of heating at 100°C

Information about the nature of the nucleic acid was gained from an investigation of its sensitivity to denaturation at 100°C. The components of double-stranded nucleic acid will be separated from each other, resulting in a change in the mobility.

The positions of bands 1, 2, 3 and 4 did not change after heating (Fig. 5), suggesting that these bands consisted of single-stranded RNA. However, band 1a disappeared following this treatment. The material contained in the supernatant and pellet fraction obtained after precipitation with 2 M-LiCl was also heated at 100°C and analysed by electrophoresis in polyacrylamide gels. The material in the low speed pellet fraction appeared to be resistant to denaturation, since no change in the electrophoretic pattern occurred. Analysis of the supernatant fraction after heating revealed the presence of some peak 3 material while peak 1a could not be detected. This experiment furnished additional proof that band 1a consisted of material differing in properties to that contained in bands 1 to 4.
Fig. 3. Electrophoretic patterns of (a) calf thymus DNA and (b) TSWV RNA, scanned before and after incubation of the gels with DNase. The gels were incubated in 2 × SSC containing Mg-ions and 5 μg DNase 1/ml for 0 h (---), 5 h (-----) and 12 h (......) respectively.

Properties of band 1a

Determination of the extinction of the bands in the gels at 260 nm and 280 nm revealed that the 260/280 nm ratio of band 1a was lower (1.3 to 1.4) than that of the bands 1, 2, 3 and 4 (2.0 to 2.2). This pointed to possible contamination of band 1a with protein. Staining of a gel with Coomassie brilliant blue did not reveal protein in bands 1, 2, 3 and 4; however, band 1a reacted positively suggesting the presence of protein.

Electrophoretic behaviour of the RNA in gels with different percentages of acrylamide

Polyacrylamide gel electrophoresis in gels with differing amounts of acrylamide can be used to differentiate between single-stranded, double-stranded linear and double-stranded circular RNA (Harley, White & Rees, 1973).

The mobility of bands 1, 2, 3 and 4 was measured by electrophoresis in gels containing different concentrations of acrylamide (Fig. 6). Single- and double-stranded CPMV RNAs were used as standards. The curves obtained for bands 1, 2, 3 and 4 of TSWV nucleic acid were similar in shape to each other and to the curves of single-stranded CPMV RNA (RNA\(_{s}\) + RNA\(_{a}\)), stressing again their single-stranded character.
RNA of tomato spotted wilt virus

Fig. 4. Electrophoretic patterns of (a) TSWV RNA, (b) the pellet and (c) the supernatant fractions obtained after precipitation by 2 M-LiCl. The ethanol precipitate of the supernatant was resuspended in one tenth the original volume.

Molecular weight determination

The mol. wt. of the RNA segments corresponding to bands 1, 2, 3 and 4 were determined under both non-denaturing and denaturing conditions (Table 1). It was assumed that the RNA of TSWV was totally denatured when analysed in 8 M-urea and low salt concentrations at 60°C (Reijnders et al. 1973, 1974). The values obtained by the two methods were in good agreement (Table 1).

The ratio of the number of RNA segments present in bands 1, 3 and 4 (Fig. 1) was determined by dividing the peak area by the mol. wt. of the corresponding RNA. The ratio of the number of molecules of RNA 1, RNA 3 and RNA 4 was 1:10:1 for TSWV extracted in the months June-July, 2:8:3 for RNA extracted in February and 6:1:12 for RNA extracted in April.

Isolation of an infectious ribonucleoprotein complex from purified TSWV

Because the RNA extracted from TSWV appeared to be non-infectious, experiments were conducted to try to isolate an infectious ribonucleoprotein from purified virus.

The non-ionic detergent Nonidet P 40, at a final concentration of 0.05%, was used to dissociate TSWV. TSWV preparations treated in this manner exhibited a very low infectivity. No complete virus particles were seen when virus treated with Nonidet P 40 was observed by electron microscopy (Tas et al. 1977b).

To exclude the possibility that the residual infectivity was due to the presence of non-disrupted virus particles, two virus samples, one treated with Nonidet P 40 and the other
Fig. 5. Electrophoretic patterns of (a) TSWV RNA and (b) TSWV RNA heated for 5 min at 100 °C.

Table 1. Molecular weights of the RNA segments 1–4 under both non-denaturing and denaturing conditions

<table>
<thead>
<tr>
<th>Peak</th>
<th>Non-denaturing conditions (Loening &amp; Ingle, 1967)</th>
<th>Denaturing conditions (Reijnders et al. 1973)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(2.7 \times 10^6 \pm 10%)</td>
<td>(2.5 \times 10^6)</td>
</tr>
<tr>
<td>2</td>
<td>(1.9 \times 10^5 \pm 5%)</td>
<td>(1.9 \times 10^6)</td>
</tr>
<tr>
<td>3</td>
<td>(1.7 \times 10^6 \pm 5%)</td>
<td>(1.7 \times 10^6)</td>
</tr>
<tr>
<td>4</td>
<td>(1.3 \times 10^6 \pm 5%)</td>
<td>(1.3 \times 10^6)</td>
</tr>
</tbody>
</table>

untreated, were layered on separate gradients and centrifuged. The extinction profiles and the distribution of infectivity of the gradients are shown in Fig. 7. The extinction profile exhibited by detergent-treated virus consisted of 3 peaks (1 to 3). Peak 1 was due to Nonidet P 40 remaining at the top of the gradient. The nature of the material contained in peak 2 was unclear. The infectivity was contained in fractions in the region of peak 3. These peaks were not observed in the extinction profile of untreated virus and the infectivity was restricted to the pellet. These observations suggested that the infectivity of virus treated with Nonidet P 40 could not be ascribed to complete virus particles, but to an infectious component sedimenting more slowly than the complete virus particles.
RNA of tomato spotted wilt virus

DISCUSSION

The material in bands 1, 2, 3 and 4 was sensitive to RNases and resistant to DNase. This result pointed to the presence of single-stranded RNA segments in these bands. Precipitation in 2 M-LiCl, resistance to denaturation at 100°C and mobility in gels with increasing percentage of acrylamide confirmed the single-stranded character of these segments.

Band 1a, which was not always visible in the gel, appeared resistant to RNases. The material forming this peak could not be precipitated with 2 M-LiCl and appeared to be sensitive to heat. This indicated that the material in band 1a was not single-stranded RNA.

The presence of protein in band 1a was suggested by staining with Coomassie brilliant blue. There is no direct evidence for the presence of nucleic acid in band 1a because toluidine blue O also stains protein. It is, however, not plausible that band 1a consists solely of protein because such a protein would have a very high mol. wt. An association of protein with nucleic acid can account better for the occurrence of band 1a in the nucleic acid extract and can also explain the behaviour in 2 M-LiCl and the disappearance of band 1a from the profiles as a result of heating at 100°C.

The fact that the RNA extracted from TSWV was non-infectious could possibly be
ascribed to the production of specific fractures in the RNA during virus purification or extraction of the RNA. However, the observed RNA patterns in polyacrylamide gels did not suggest a simple fragmentation of a large RNA molecule.

If one assumes that infectious TSWV particles contain the equivalent of one of each of the 4 RNA segments the genome would have a mol. wt. of \(7.4 \times 10^6\). RNA segment 1 with a mol. wt. of \(2.5 \times 10^8\) can code, theoretically, for a maximum of about 260000 daltons of protein (Paucha, Seehafer & Colter, 1974) and since the sum of the mol. wt. of proteins 1 to 5 (Tas et al. 1977b) is more than 260000 it seems reasonable to assume that more than one RNA molecule is needed to code for the proteins of TSWV. The presence of a segmented genome predicts a high frequency of recombination, a prediction supported by the conclusions of Best & Gallus (1955). However, no recombinants have been characterized so far.

In the properties of its nucleic acid, TSWV shows some resemblance to influenza virus, namely single-stranded RNA that occurs in segments and is non-infectious. Heterogeneity in the RNA pattern has also been observed for influenza virus (Etchison et al. 1971; Palese & Schulman, 1976). It would be interesting to discover if other properties characteristic of influenza virus, such as genetic recombination, multiplicity reactivation and production of incomplete virus particles also occur with TSWV.

On the basis of the results obtained the cryptogram of TSWV may be written as: \(R/1:\Sigma 7.4/8:S/S:S/Th\). The classification of TSWV in a monotypic group (Harrison et al. 1971) is in agreement with the data presented here concerning the RNA and with data presented elsewhere (Tas et al. 1977b) concerning the protein composition of TSWV.

The authors thank Professor Dr Ir. J. P. H. van der Want for his stimulating support and discussions, and Dr A. Ziemiecki for his help in preparing the manuscript.
REFERENCES


(Received 19 October 1976)