Evidence for Two Functional RNA Species and a 'Satellite' RNA in Tomato Black Ring Virus

(Accepted 19 January 1973)

Our recent studies have shown that raspberry ringspot virus (R/1:2-4/43 + 1-4/30 (or 2 × 1-4/46):S/S:S/Ne, nepovirus group) has two functional RNA species, of mol. wt. 2-4 × 10^6 (RNA-1) and 1-4 × 10^6 (RNA-2) (Harrison, Murant & Mayo, 1972a, b; Murant et al. 1972). There is also evidence that tomato top necrosis (Bancroft, 1968), tobacco ringspot (Harrison et al. 1972a) and cherry leaf roll (Jones & Mayo, 1972) viruses have divided genomes and we suggested (Harrison et al. 1972a) that this property is characteristic of nepoviruses. In this paper we present results with another nepovirus, tomato black ring virus (TBRV), in which we have found three RNA components. Two of these seem essential for infection and presumably comprise the genome of the virus. The third seems to be a 'satellite' RNA, analogous to that found in some cultures of tobacco ringspot virus (Schneider, 1969, 1971).

Two cultures of TBRV were used: (i) TBRV-S, the type culture of the 'beet ringspot' strain, originally obtained from <i>Arctium lappa</i> in Scotland (Harrison, 1958); (ii) TBRV-G, an isolate of the potato bouquet strain from Germany. Most of the work was done with TBRV-G.

The viruses were purified from systemically infected leaves of <i>Nicotiana clevelandii</i>, and preparations were centrifuged in sucrose density gradients and fractionated as described by Murant et al. (1972). Infectivity was assayed by counting the lesions produced in inoculated leaves of <i>Chenopodium amaranticolor</i>. RNA was extracted from the virus particles with pronase+SDS, heated in electrophoresis buffer containing 8 M-urea and fractionated by electrophoresis in polyacrylamide gels (Murant et al. 1972). Mol. wt. of RNAs were estimated as described by Murant et al. (1972) using ribosomal RNAs of known mol. wt. from pea tissue as standards. RNA fractions were prepared by extracting the u.v.-absorbing bands in the gels using tissue grinders (Harrison et al. 1972a). In each experiment a total of 200 to 400 μg RNA was electrophoresed in four to six gels. Each of the resulting RNA fractions was made up to 10 ml with 0-06 M-phosphate buffer, pH 8-0, and the amount of each RNA species in the inocula is expressed in terms of the dilution of these solutions (Table 1). To prepare inoculum, a fraction was mixed with other RNA fraction(s) or with buffer alone. Some RNA fractions were u.v.-irradiated for 4 min as described by Harrison et al. (1972a).

Three major RNA species were resolved in RNA preparations from TBRV-G (Fig. 1a). Assuming that all three are linear single-stranded molecules, mol. wt. estimates were 2.5 × 10^6 (RNA-1), 1.5 × 10^6 (RNA-2) and 0.5 × 10^6 (RNA-3). Similar results were obtained with RNA from TBRV-S. In co-electrophoresis experiments the mobilities of RNA-1 and RNA-2 of TBRV-G were respectively slightly less than those of RNA-1 and RNA-2 of raspberry ringspot virus.

RNA fractions from the TBRV-G gels were inoculated singly and in various combinations to <i>Chenopodium amaranticolor</i> test plants (Experiment 1, Table 1). Preparations of RNA-1 were moderately infective, but RNA-2 preparations had little infectivity. RNA-3 preparations did not induce lesions. Mixtures of the RNA-1 and RNA-2 preparations were more
Fig. 1. Polyacrylamide gel traces of RNA from (a) TBRV-G, (b) isolate B1, (c) isolate B1/2. Electrophoresis was in 2.2 % gels in 0.02 M-tris-phosphate + 0.002 M-EDTA + 0.2 % SDS, pH 7.8, at 7 V/cm for (a) 180 min, (b) 160 min, (c) 170 min. Electrophoretic migration is from left to right.

Table 1. Infectivity of RNA fractions from TBRV-G and TBRV isolate B1/2/2, inoculated singly and in different mixtures

| RNA species in inoculum† | Experiment 1 (all RNA species from TBRV-G) | | Experiment 2 (RNA-1 and RNA-2 from isolate B1/2/2; RNA-3 from TBRV-G) |
|--------------------------|---------------------------------------------|-----------------|
| RNA species in inoculum† | RNA-1 at 1/15 Without RNA-1 | RNA-1 at 1/3 | RNA-1 at 1/15 | RNA-1 at 1/75 Without RNA-1 |
| 1                        | 23 | — | 593 | 86 | 2 | — |
| 1 + 2                    | 61 | — | 631 | 207 | 38 | — |
| 1 + 3                    | 0 | — | 192 | 18 | 1 | — |
| 1 + 2 + 3                | 5 | — | 159 | 51 | 11 | — |
| 1 + 3 (u.v.)‡             | — | — | 521 | 103 | 3 | — |
| 1 + 2 + 3 (u.v.)‡         | — | — | 740 | 190 | 45 | — |
| 2                        | — | 2 | — | — | — | 3 |
| 3                        | — | 0 | — | — | — | 0 |
| 2 + 3                    | — | 0 | — | — | — | 0 |
| 3 (u.v.)‡                | — | — | — | — | — | 0 |
| 2 + 3 (u.v.)‡             | — | — | — | — | — | 0 |

* Figures are the total number of lesions produced in eight half-leaves of Chenopodium amaranticolor.
† RNA-1 was used at final dilutions of 1/3, 1/15 or 1/75; RNA-2 and RNA-3 were used at final dilutions of 1/3.
‡ The RNA-3 preparation used in these treatments was u.v.-irradiated for 4 min.
infective than RNA-1 preparations alone. In contrast, the infectivities of RNA-1 preparations and of mixtures of preparations of RNA-1 and RNA-2 were greatly decreased or abolished by adding RNA-3.

Two lesions produced by RNA-1 inocula in these tests were excised separately and virus propagated from them. RNA extracted from purified preparations of the two isolates (B1 and B2) contained RNA-1 and RNA-2, but much less RNA-3 than did the original isolate (Fig. 1b). Single lesions produced by RNA-1 inocula from isolate B1 yielded new isolates (B1/1 and B1/2) which produced no RNA-3 (Fig. 1c). The process was repeated a third time to yield isolate B1/2/2, which again produced RNA-1 and RNA-2 but not RNA-3. No RNA-3 was detected in virus purified from this isolate after five successive subcultures in *Nicotiana clevelandii*.

Experiment 2 in Table I shows the infectivity of various mixtures of RNA-1 and RNA-2 obtained from isolate B1/2/2, with RNA-3 obtained from the original culture of TBRV-G. The data, in addition to confirming those of Experiment 1, show that (i) the greater the dilution of RNA-1, the greater the proportional increase in lesion number obtained by adding RNA-2, whereas (ii) the proportional decrease in lesion number caused by adding RNA-3 to RNA-1, or to mixtures of RNA-1 + RNA-2, is similar at all concentrations of RNA-1; and (iii) u.v.-irradiated RNA-3 does not decrease lesion numbers.

Virus isolates were obtained from single lesions produced in Experiment 2 by inoculum containing RNA-1 + RNA-2 + RNA-3. RNA from these isolates produced gel traces resembling that in Fig. 1(a), showing that RNA-3 had been reintroduced into the culture. In contrast, lesions produced by inoculum containing RNA-1 + RNA-2, or by inoculum containing RNA-1 + RNA-2 + u.v.-irradiated RNA-3, yielded isolates that contained no detectable RNA-3.

To test the possibility that RNA-3 was from a contaminating virus, leaves inoculated with RNA-3 preparations were extracted using the normal virus purification procedure. No virus-like particles were found by electron microscopy, nor was RNA-3 detected in pronase/SDS extracts of the preparations. In contrast, virus particles were readily detected in preparations from leaves inoculated with RNA-1 + RNA-2.

We conclude from these results that TBRV resembles raspberry ringspot virus in having two essential RNA components: we failed to obtain virus cultures containing RNA-1 alone, and maximum infectivity occurred in preparations containing both RNA-1 and RNA-2. RNA-3, however, seems unnecessary for multiplication of TBRV because it can be eliminated from cultures containing it; moreover, it did not spontaneously recur, suggesting that it is not a normal by-product of TBRV replication. It does not induce lesions and we obtained no evidence that it multiplies on its own, yet when inoculated together with RNA-1 + RNA-2, it is found in the virus product; this does not happen, however, when RNA-3 is u.v.-irradiated. Taken together, these observations suggest that TBRV RNA-3 is analogous to the ‘satellite’ RNA that occurs in some cultures of tobacco ringspot virus (Schneider, 1969, 1971), although its mol. wt. is about six times greater. It is not clear how RNA-3 inhibits lesion formation, but its ability to inhibit is abolished by u.v.-irradiation.

As with raspberry ringspot virus (Harrison et al. 1972a, b), it is difficult with TBRV to obtain preparations of RNA-1 free from infectivity, suggesting that RNA-1 fractions from gels are contaminated with RNA-2. This in turn makes it difficult to detect enhancement of lesion number by adding RNA-2 to concentrated preparations of RNA-1. The enhancement is easier to detect with more dilute preparations of RNA-1, presumably because the chance of a site becoming infected both with RNA-1 and with the contaminating RNA-2
is much decreased by dilution. Therefore to determine whether one virus RNA influences
the number of lesions induced by another, RNA preparations likely to be contaminated
with other RNAs should always be inoculated at a range of concentrations.

We thank Agnes M. D. Lowson and Rhonda R. MacLagan for their valuable assistance.

Scottish Horticultural Research Institute
Invergowrie
Dundee
Scotland

A. F. Murant
M. A. Mayo
B. D. Harrison
R. A. Goold

REFERENCES

Harrison, B. D. (1958). Further studies on raspberry ringspot and tomato black ring, soil-borne viruses
Harrison, B. D., Murant, A. F. & Mayo, M. A. (1972a). Evidence for two functional RNA species in rasp-
Harrison, B. D., Murant, A. F. & Mayo, M. A. (1972b). Two properties of raspberry ringspot virus determined
by its smaller RNA. Journal of General Virology 17, 137–141.
General Virology 16, 349–358.
108–122.

(Received 15 December 1972)