A Variant of Tobacco Rattle Virus: Evidence for a Second Gene in RNA-2

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SUMMARY

The isolation of YS, a spontaneous variant of the CAM strain of tobacco rattle virus, is described. Unlike CAM, YS produced yellow symptoms in several species of host plant. In experiments with mixtures of virus particles from the two strains, this character was shown to be controlled by RNA-2. However, the coat proteins of YS and CAM were indistinguishable by serology, by electrophoresis in polyacrylamide gels or by tryptic peptide mapping. It is concluded that the mutation responsible for the yellow symptoms is not in the coat protein gene, but elsewhere in RNA-2.

INTRODUCTION

Tobacco rattle virus (TRV) preparations contain a mixture of long and short rod-shaped particles, each particle containing a single RNA molecule of a size proportional to the particle length. Only the long particles are independently infective (Harrison & Nixon, 1959) and therefore presumably carry the information for an RNA replicase system. Such infections produce only uncoated long RNA (RNA-1). Short particles are not infective alone but, when mixed with long particles, give rise to infections producing coated particles of both lengths. Moreover, when a mixture of short and long particles from different strains is used as inoculum, both lengths of particle in the progeny virus have the serological specificity and amino acid composition of the short particle parent (Sänger, 1968; Ghabrial & Lister, 1973). Thus the short RNA (RNA-2) carries the coat protein gene.

In the CAM isolate of TRV, the mol. wt. of RNA-2 is $0.7 \times 10^6$ (Cooper & Mayo, 1972). The mol. wt. of the coat protein is about 22000 (Mayo & Robinson, 1975), which accounts for only about one-third of the coding potential of RNA-2. There is therefore space in RNA-2 for at least one more gene. Most other strains of TRV have RNA-2 molecules larger than that of CAM, with mol. wt. up to $1.3 \times 10^6$ (Harrison, 1970), and could include additional genes.

Lister & Bracker (1969), using a group of TRV isolates from Oregon, showed that a determinant for yellow symptoms was present on RNA-2 of one of their strains. However, they did not determine whether this was a function of the coat protein gene, or of another gene in RNA-2, although the very close serological relationship between the strains suggested that the coat protein gene was similar in all of them.

This paper describes a spontaneous variant of the CAM strain of TRV which, unlike its progenitor, produces yellow symptoms in host plants. Evidence is presented that the determinant for these symptoms is in RNA-2, but is not the coat protein gene.
METHODS

Virus purification. Both CAM and YS isolates of TRV were purified from systemically infected leaves of *Nicotiana clevelandii* as described by Cooper & Mayo (1972). Long and short particles in such preparations were separated by two cycles of sedimentation in sucrose density gradients containing phosphate buffer (0.017 M, pH 7.3) and 0.002 M-EDTA.

Electron microscopy. Lengths of TRV particles were measured from electron micrographs of phosphotungstate-treated preparations (magnification ×20000) using a binocular microscope fitted with a micrometer eyepiece.

Serology. Precipitin tests were made in tubes (0.7 × 12.5 cm), by mixing 0.5 ml of a fourfold dilution of heat-clarified sap from systemically-infected *Nicotiana clevelandii* leaves with an equal volume of appropriately diluted antiserum against CAM nucleoprotein, prepared as described by Cooper & Mayo (1972), and incubating the mixture at 37 °C. Immunodiffusion tests in 0.5% agarose gel were done as described by Robinson (1973b).

Polyacrylamide gel electrophoresis. Six mm-diam. 5% (w/v) polyacrylamide gels containing 8 M-urea were prepared in the pH 4.5 buffer system as described by Duesberg & Rueckert (1965). Ten to thirty-five μg virus protein in 7.6 M-urea + 0.05 N-acetic acid was loaded on to each gel, and electrophoresed for 45 min at 2 mA/gel. Gels were stained with Coomassie blue in methanol:water:acetic acid (5:5:1; v/v/v) and destained in the same mixture.

Tryptic peptide maps. Virus protein was prepared, oxidized and digested with diphenyl carbamyl chloride-treated trypsin (Sigma) as described by Ghabrial & Lister (1973), except that the digestion was allowed to continue for 24 h. Samples of the lyophilized digests suspended in pyridine:acetic acid:water (10:0.4:90; v/v/v; pH 6.5) were spotted on Polygram CEL 400 thin layer plates (Machery-Nagel & Co., Düren) and subjected to electrophoresis at 500 V for 70 min in the same buffer. The dried plates were then developed by ascending chromatography at right angles to the direction of electrophoresis, in pyridine:acetic acid:n-butanol:water (50:15:75:60) for 6 to 7 h. After a final drying, the plates were stained with 0.3% ninhydrin in collidine:acetic acid:ethanol (3:10:87).

RESULTS

Isolation of the YS variant

The variant was isolated from a plant showing a single yellow spot in a systemically-infected leaf among otherwise symptomless *Nicotiana clevelandii* plants being used for routine propagation of the CAM strain of TRV. When sap from the spot was inoculated to more *N. clevelandii* plants, a yellowish mosaic appeared in the youngest leaves. Several more subcultures in *N. clevelandii*, each time using material from the yellow areas of the leaves, produced steadily increasing proportions of yellow leaf in the mosaic. Finally the variant was purified by two consecutive single lesion isolations in *Chenopodium amaranticolor*, and propagated in *N. clevelandii* (Robinson, 1973a). The variant was designated YS.

Electron microscopy of sap from *Nicotiana clevelandii* leaves systemically infected with YS, using several negative stains, revealed no virus-like particles other than the typical rods of TRV. The length distribution of particles in purified preparations of YS (Fig. 1) showed the presence of two types of particle with modal lengths of about 50 and 200 nm, characteristic of the CAM strain of TRV (Harrison & Woods, 1966).
Second gene in RNA-2 of TRV

Symptoms in host plants

*Nicotiana clevelandii* plants infected with YS were severely stunted, and developed a mosaic pattern of yellowish-green and green areas in the systemically-infected leaves (Fig. 2a). Later the leaves became necrotic, and frequently the plants died. In contrast, CAM-infected plants were almost indistinguishable from healthy *N. clevelandii* (Fig. 2a). YS, unlike CAM, also produced yellow symptoms in *N. debneyi*, *N. rustica*, *Spinacia oleracea*, *Lycopersicon esculentum* and *Gomphrena globosa*. Symptoms appeared more rapidly in the summer than in the winter.

Following infection with YS, *Nicotiana tabacum* ‘White Burley’ plants of the line in regular use in this laboratory were severely stunted and developed a pattern of bright yellow areas in the systemically-infected leaves. In contrast, ‘White Burley’ plants grown from seed obtained from the John Innes Institute continued to grow almost as rapidly after infection with YS as did uninfected plants; occasionally yellow spots were observed in systemically-infected leaves, but usually there were no visible symptoms of infection (Fig. 2b). However, inoculation to *Chenopodium amaranticolor* showed that systemically-infected leaves of both lines contained a high concentration of infective virus, and extracts from infected, but symptomless, plants of the John Innes line produced a yellow mosaic in plants of the SHRI line. Inoculation with CAM produced a few necrotic marks in inoculated and non-inoculated leaves of both lines of tobacco, but no yellow symptoms.

Particle mixing experiments

Table 1 shows the occurrence of yellow symptoms in *Nicotiana clevelandii* inoculated with mixtures of long and short particles, in which one component was derived from YS and the other from CAM. The ratio of numbers of short to long particles varied between about 400:1 and 16:1. Of 11 plants inoculated with mixtures containing long particles from CAM and short particles from YS, 10 developed yellow symptoms; in contrast, no plants which received the reciprocal mixture became yellow. Therefore, the genetic determinant for the yellow symptoms is carried by the short particles (i.e. by RNA-2) of YS. Plants inoculated with long particles of either strain alone did not become yellow.
Comparison of the coat proteins of YS and CAM

In precipitin tests in tubes, antiserum against the CAM strain of TRV reacted to 1/128 with heat-clarified sap from *Nicotiana clevelandii* infected with either YS or CAM. In immunodiffusion tests this antiserum gave single precipitin lines against purified preparations of each of the isolates (Fig. 3), with a reaction of identity between them.

Electrophoresis in 5% (w/v) polyacrylamide gels in the presence of 8 M-urea separates proteins primarily on the basis of charge. In this system both CAM and YS coat proteins migrated as single zones, and a mixture of the two was not resolved.

Tryptic peptide maps of either CAM or YS protein each contained about 26 ninhydrin-positive spots (Fig. 4), which is about the number expected from published amino acid analyses of TRV protein. The positions of corresponding spots on the two maps were closely similar.
Second gene in RNA-2 of TRV

Table 1. Symptoms in Nicotiana clevelandii inoculated with mixtures of particles from YS and CAM

<table>
<thead>
<tr>
<th>Source</th>
<th>Long particles</th>
<th>Concentration* (μg/ml)</th>
<th>Short particles</th>
<th>Concentration* (μg/ml)</th>
<th>Plants with yellow symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>YS</td>
<td>0.5</td>
<td>CAM 10</td>
<td></td>
<td></td>
<td>0/3†</td>
</tr>
<tr>
<td>YS</td>
<td>0.1</td>
<td>CAM 10</td>
<td></td>
<td></td>
<td>0/3</td>
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<tr>
<td>YS</td>
<td>0.5</td>
<td>CAM 2</td>
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<tr>
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<td>0.1</td>
<td>CAM 2</td>
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<td>0/3</td>
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<tr>
<td>CAM</td>
<td>0.5</td>
<td>YS 10</td>
<td></td>
<td></td>
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<td>CAM</td>
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<tr>
<td>YS</td>
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<td></td>
<td></td>
<td>0/3</td>
</tr>
<tr>
<td>CAM</td>
<td>0.5</td>
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<td>0/3</td>
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</tbody>
</table>

* Final concentration in the mixed inoculum.
† Number of N. clevelandii which developed yellow symptoms/number infected.

Fig. 3. Immunodiffusion test of purified preparations of CAM and YS isolates with antiserum (AS) against CAM strain of TRV.

DISCUSSION

The YS variant was selected from a population consisting predominantly of the CAM strain of TRV on the basis of its ability to produce yellow symptoms in Nicotiana clevelandii. It is identical to its progenitor in gross morphology, including particle length distribution, and by serological tests, properties which distinguish it from all reported European isolates of TRV. It therefore represents a spontaneous mutation during culture under glasshouse conditions rather than a chance contamination with an independent isolate from outside.

Particle mixing experiments showed that the mutation responsible for the yellow symptoms is in RNA-2, which carries the gene specifying the amino acid sequence of the coat
Fig. 4. Maps of tryptic peptides from coat proteins of (a) CAM strain and (b) YS strain of TRV. Separation was from the origin (*) by electrophoresis in the horizontal dimension (cathode on the left), followed by chromatography in the vertical dimension.
Second gene in RNA-2 of TRV

protein (Sänger, 1968; Ghabrial & Lister, 1973). However, the coat proteins of YS and CAM could not be distinguished by serology, by polyacrylamide gel electrophoresis using a system which separates proteins mainly by charge, or by the positions of their tryptic peptides in a two-dimensional electrophoretic-chromatographic separation. The coat proteins are therefore certainly very similar and most likely identical in amino acid sequence. It must be concluded that the mutation responsible for the yellow symptoms lies elsewhere on RNA-2, and indeed this is the first clear evidence for the existence of another gene in RNA-2.

The nature of the product of the gene defined by the YS mutation is at present unknown, but it is expressed in several host species. The mutation may lead to synthesis of an altered gene product that, unlike its counterpart in CAM infection, itself gives rise to the yellow symptoms, or conversely, it may result in failure to express a function which in CAM infection prevents the yellowing reaction. In either instance, the symptom expression probably represents the visible end-product of a complex series of events in the infected plant, and is influenced not only by the genotype of the virus but also by the genotype of the host plant, as shown by the different reactions of two lines of ‘White Burley’ tobacco. Nevertheless comparison of the physiology and biochemistry of leaves infected with YS and with CAM should prove a fruitful approach to the general question of the mechanism of virus-induced yellowing.

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REFERENCES


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