Unequal Variation in the Two Genome Parts of Tobraviruses and Evidence for the Existence of Three Separate Viruses

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SUMMARY

Hybridization experiments using complementary DNA copies showed that 14 tobravirus isolates could be divided into three groups, comprising ten of tobacco rattle virus (TRV) serotype I-II, one of TRV serotype III, and three of pea early-browning virus (PEBV), respectively. RNA from members of each group has much nucleotide sequence homology with RNA of other members of the same group but little or none with RNA from members of the two other groups. The ten TRV serotype I-II isolates share extensive sequences in their RNA-1 species, but show much diversity in their RNA-2 species and in the antigenic relatedness of their particles. However, homology was found between the RNA-2 sequences of isolates that are serologically closely related. Because strain CAM (TRV serotype III) is as different from isolates of TRV serotype I-II in nucleotide sequence, antigenic specificity and biological properties as is PEBV, it is now considered to be a separate virus and a return to its original name, pepper ringspot virus, is recommended.

INTRODUCTION

The tobravirus group includes only two definitive members, tobacco rattle virus (TRV) and pea early-browning virus (PEBV). The nucleoprotein particles of each of these viruses are of two predominant lengths, referred to as long (L) and short (S). L particles of different isolates have rather similar modal lengths: 185 to 197 nm for TRV (Harrison & Woods, 1966) and 200 to 220 nm for PEBV (Bos & van der Want, 1962; Harrison, 1966), whereas the S particles in different isolates range from 50 nm to 115 nm in modal length. The genome consists of two single-stranded RNA species (RNA-1 and RNA-2) contained in L and S particles respectively (Harrison & Robinson, 1978). The particle protein gene is in RNA-2 (Sänger, 1968).

A remarkably wide range of serological variation is found among TRV isolates. Isolates were formerly separated into three serotypes (Harrison & Woods, 1966) but intergrading isolates are known to occur in serotypes I and II, and it seemed better to combine these in an antigenically somewhat heterogeneous group known as serotype I-II. However, the distinctness of serotype III, represented by an isolate from Brazil, has been confirmed by subsequent work (Harrison & Robinson, 1978). Two serotypes of PEBV have been reported, comprising British and Dutch isolates respectively (Gibbs & Harrison, 1964; van Hoof, 1969). A Dutch isolate of PEBV is serologically distantly related to a TRV serotype I-II isolate (Maat, 1963), but no such relationship was detected using a British isolate of PEBV (Gibbs & Harrison, 1964).

Tests for nucleotide sequence homology, by hybridization with complementary DNA (cDNA) copies, showed that the RNA-1 species of two TRV serotype I-II strains are almost identical, whereas that of a serotype III strain is quite different (Robinson, 1983). The RNA-2 species of the three strains had no detectable sequence homology with one another. It was suggested that, within TRV serotype I-II, the nucleotide sequence of RNA-1, like its size, may be strongly conserved, whereas the RNA-2 sequence may be very variable. Support for this hypothesis came from tests on TRV isolates from narcissus, all of which were detected by hybridization with cDNA to RNA-1 of TRV strain PRN, but fewer than half of which reacted with antiserum to the same TRV strain (Harrison et al., 1983).

To get a more general picture of nucleotide sequence relationships within the tobravirus group, nucleic acid hybridization experiments have now been done with 14 isolates of TRV and...
PEBV, chosen for the diversity of their other properties. The results confirm and extend previous findings, and are considered to justify the separation of TRV serotype III as a distinct virus.

METHODS

Virus isolates. The following isolates of TRV were used. ORY, the yellow strain of Lister & Bracker (1969), isolated from potato in Oregon, U.S.A. The culture used (OR3) had been freed of a mutant RNA-2 species (Robinson, 1983). ORM and ORS, the mild and severe strains, respectively, of Lister & Bracker (1969), isolated from the same source as ORY. SYM, a strain from spinach in Japan (Tomaru & Nakata, 1967). NZP, an isolate from pea in New Zealand (Jones & Young, 1978). RQ, an isolate obtained from a cucumder bait plant grown in soil from a field at SCRI (Cooper, 1972). RH, an isolate from Helianthus rigidus growing at Broughty Ferry, Scotland (R. I. Hamilton, unpublished results). ASH, an isolate from Fraxinus mariesii growing in England (Cooper et al., 1983), kindly supplied by Dr J. I. Cooper. CAM, the original serotype III strain, obtained from Bidens sp. in Brazil (Harrison & Woods, 1966).

The following isolates of PEBV were used. SP5, the type strain of the British serotype, originally obtained from pea growing at Sporle, Norfolk, England (Gibbs & Harrison, 1964). SHE, an isolate obtained in 1970 from pea grown in soil from Shernbourne, Norfolk. E116, a typical isolate of the Dutch serotype, recovered from pea seeds kindly supplied by Dr L. Bos.

Isolates ORY, ORM, ORS, HSN, NZP, CAM and E116 were held under licence from the Department of Agriculture and Fisheries for Scotland.

Purification of virus particles and extraction of RNA. All isolates were propagated in Nicotiana clevelandii. Virus particles were purified from leaf extracts in either 0.067 M-phosphate, pH 7.3 or 0.02 M-borate, pH 8.3, as described for strain ORY by Robinson (1983). Some preparations were heated at 50 °C for 10 min and centrifuged at low speed to remove residual impurities (Harrison & Woods, 1966). L particles of strain SYM were obtained as described by Kurppa et al. (1981). RNA was extracted from virus particles as described by Robinson et al. (1983).

Complementary DNA-RNA hybridization. Preparation of 3H-labelled cDNA copies of unfractionated virus RNA and of RNA-1, conditions for hybrid formation and conditions for assay using S1 nuclease were as described by Robinson et al. (1980).

Serological tests. Immunosorbent electron microscopy (ISEM) was performed as described by Roberts & Harrison (1979). Tube precipitin tests were done as described by Harrison & Woods (1966).

Electron microscopy. Lengths of virus particles were measured from electron micrographs of specimens stained at pH 6.5, either with 2% ammonium molybdate after fixation in osmium tetroxide, or with 2% sodium phosphotungstate, and were recorded and classified using an ID-TT-20 Tektronix digitizer linked to a 4051 graphic system.

RESULTS

Properties of the lesser known virus isolates

Most of the isolates included in this study are already characterized but there is little or no published information on others.

TRV isolate RQ has particles with modal lengths of 76 and 189 nm. It induced symptoms similar to those of strain PRN in Chenopodium amaranticolor, N. clevelandii and Phaseolus vulgaris, and neither isolate infected Pisum sativum or Vicia faba systemically. It caused symptoms similar to, but somewhat less severe than, those of strain PRN in N. tabacum cv. Samsun NN. Despite these similarities, isolate RQ is not closely serologically related to strain PRN. In tube precipitin tests, antiserum to isolate RQ (homologous titre 1/1024) did not react at dilutions of 1/2 or greater with particles of strain PRN or strain CAM. In similar tests, antiserum to PEBV strain SP5 (homologous titre 1/256) did not react (least dilution tested, 1/4) with RQ particles. However, the results of ISEM tests indicated that TRV isolates RH and ASH are antigenically strongly related to isolate RQ (Table 1).

TRV isolate HSN has particles with modal lengths of 65 and 188 nm and produced symptoms similar to those of strain PRN in C. amaranticolor, N. tabacum cv. Samsun NN and P. vulgaris. In tube precipitin tests with PRN antiserum, a serological differentiation index (Van Regenmortel & Von Wechmar, 1970) of 2 was found.

PEBV isolate SHE has particles with modal lengths of 103 and 216 nm and caused symptoms
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Table 1. Immunosorbent electron microscopy of strains RH and ASH using antisera to various tobaviruses

<table>
<thead>
<tr>
<th>Antiserum* to strain</th>
<th>Virus strain</th>
<th>RH</th>
<th>ASH</th>
</tr>
</thead>
<tbody>
<tr>
<td>RQ</td>
<td>47 000†</td>
<td>1184</td>
<td></td>
</tr>
<tr>
<td>PRN</td>
<td>700</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>SYM</td>
<td>700</td>
<td>NT§</td>
<td></td>
</tr>
<tr>
<td>ORY</td>
<td>700</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>CAM</td>
<td>700</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>Control†</td>
<td>800</td>
<td>31</td>
<td></td>
</tr>
</tbody>
</table>

* Grids were coated with antiserum diluted 1000-fold.
† Untreated grids.
§ NT, Not tested.

Figures are number of particles per 1000 μm² of grid.

similar to those of strain SP5 in \textit{N. clevelandii} and \textit{P. vulgaris}; both isolates infected \textit{P. sativum} and \textit{V. faba} systemically. In tube precipitin tests, isolate SHE reacted with antiserum to strain SP5 but was antigenically distinguishable (serological differentiation index about 3) from it. Isolate SHE is therefore considered to belong to the British serotype of PEBV.

Sequence homology in RNA-1 of TRV isolates

Robinson (1983) found that the maximum extents of hybridization of cDNA copies of RNA-1 (cDNA-1) of strain SYM with RNA-1 of strain SYM and of strain ORY were the same. Similar results were obtained when cDNA-1 of strain SYM was hybridized with unfractionated RNA of strains SYM or ORY, to a $R_0$ value of 0.5 mol./l; after correction for the S1 nuclease resistance of cDNA in the absence of RNA (Gonda & Symons, 1978), 53% of the cDNA hybridized with RNA of strain SYM, and 52% with RNA of strain ORY. In tests summarized in Table 2, no hybridization was observed between cDNA-1 of strain SYM and unfractionated RNA of strain CAM, but this cDNA did hybridize to RNA of all the other eight TRV isolates, to extents indicating sequence homology (calculated according to Gonda & Symons, 1978) of from 100% (RNA of strain ORS) to 66% (RNA of strain HSN). Thus, at least two-thirds, and in some instances essentially all, of the sequences represented in cDNA-1 of strain SYM are shared by all ten representatives of TRV serotype I-II.

Sequence homologies in unfractionated RNA

To obtain further information on the extent of genome homologies, cDNA copies were made from unfractionated RNA preparations of TRV isolates SYM, ORY, PRN, RQ, ASH and CAM, and of PEBV isolates SHE and E116, and each of these was hybridized with unfractionated RNA of the whole range of representative tobravirus isolates. The apparent percentage sequence homology for each combination is listed in Table 2. For each cDNA preparation the apparent percentage sequence homology observed depends on the relative proportions of copies of RNA-1 and RNA-2 in the preparation. Thus, although homology values obtained with each cDNA preparation and different RNA preparations are directly comparable, those obtained with different cDNA preparations are not. Furthermore, the two ways of comparing each pair of isolates need not necessarily give the same value of apparent percentage sequence homology. The 14 isolates clearly fall into three groups, containing respectively the ten isolates of TRV serotype I-II, the sole representative of TRV serotype III, and the three isolates of PEBV. Isolates within each group showed substantial sequence homology with one another. Conversely, cDNA of isolates in each group hybridized only slightly or not at all with RNA of isolates in the other two groups; the small extents of hybridization recorded for some of these combinations were not detected consistently and it is doubtful that they represent sequence homologies.
Table 2. Apparent percentage sequence homology between RNA preparations from 14 tobraviruses

<table>
<thead>
<tr>
<th>RNA of strain</th>
<th>cDNA to RNA-1 of TRV-SYM</th>
<th>cDNA to unfractionated RNA of strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SYM</td>
<td>ORY</td>
</tr>
<tr>
<td>SYM</td>
<td>100*</td>
<td></td>
</tr>
<tr>
<td>ORY</td>
<td>99</td>
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<td>PRN</td>
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<td>67</td>
</tr>
<tr>
<td>HSN</td>
<td>66</td>
<td>63</td>
</tr>
<tr>
<td>NZP</td>
<td>84</td>
<td>54</td>
</tr>
<tr>
<td>RQ</td>
<td>61</td>
<td>67</td>
</tr>
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</tr>
<tr>
<td>ASH</td>
<td>77</td>
<td>54</td>
</tr>
<tr>
<td>CAM</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>SP5</td>
<td>ND†</td>
<td>0</td>
</tr>
<tr>
<td>SHE</td>
<td>ND†</td>
<td>0</td>
</tr>
<tr>
<td>E116</td>
<td>ND†</td>
<td>0</td>
</tr>
</tbody>
</table>

* Apparent percentage sequence homology, calculated according to Gonda & Symons (1978), in tests using unfractionated RNA preparations hybridized to an Rₚₜ value of 0.5 mol s/l. Each value is the mean of at least two determinations, using the same preparation of cDNA and one or more preparations of RNA.
† ND, Not done.

Within TRV serotype I-II, isolates showed varying degrees of sequence homology. Using cDNA of strain SYM, 65% homology was observed with strain ORY, which shares essentially all of its RNA-1 sequences and none of its RNA-2 sequences with strain SYM (Robinson, 1983). None of the other TRV serotype I-II isolates showed significantly more homology with strain SYM than did strain ORY, suggesting that they all have RNA-2 sequences substantially different from those of strain SYM. RNA of strains ORY, ORM and ORS each hybridized with cDNA of strain ORY to the same extent, showing that these three strains have very similar sequences in both their RNA components. This suggests that they are derived by small-scale mutational events from a common ancestor, which is consistent with their derivation from a single field isolate and with their very close serological relationship (Lister & Bracker, 1969). With RNA of the remaining serotype I-II isolates, cDNA to strain ORY showed between 50 and 56% homology, suggesting that, like strain SYM, they have substantial homology in RNA-1 and little or none in RNA-2. Similarly, with cDNA to isolates PRN, RQ and ASH, RNA of many of the serotype I-II isolates showed similar degrees of homology. The homology revealed in reactions of cDNA of strain PRN with RNA of isolate NZP was greater than that with RNA of other isolates, suggesting the existence of some common sequences between the RNA-2 species of isolates PRN and NZP, and consistent with their apparently close serological relationship (Jones & Young, 1978). Another cluster of isolates that showed greater homology than most in this serotype comprised isolates RQ, RH and ASH. The homology between these three isolates seemed particularly great when cDNA to isolate ASH was used, but this preparation apparently contained an unusually high proportion of copies of RNA-1, making it less discriminating of differences among RNA-2 species. However, the grouping was also evident using cDNA to isolate RQ, but seemed less close. This grouping fits well with the results of serological tests (Table 1).

Among the PEBV isolates, a range of homologies similar to those among TRV serotype I-II isolates was evident. The two examples of the British serotype, isolates SP5 and SHE, showed 90% sequence homology, whereas isolate E116 of the Dutch serotype showed less, but still substantial, homology with these two.

**Discussion**

The results reported here confirm the hypothesis (Robinson, 1983) that the nucleotide sequence of RNA-1 is strongly conserved among isolates of TRV serotype I-II. There are evidently much less strong constraints on variation in the sequence of RNA-2, which can differ
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to such an extent that no common sequences are detectable by hybridization tests. However,
among the ten isolates studied, there are three groups that share some or all of their RNA-2
sequences. These groups comprise: (i) strains ORY, ORM and ORS; (ii) isolates PRN and
NZP; (iii) isolates RQ, RH and ASH. For each of these groups there is evidence of relatively
close serological relationships between their members (Lister & Bracker, 1969; Jones & Young,
1978; and Table 1). Isolates SYM and HSN each stand somewhat apart from these three groups,
although isolate HSN is moderately closely related serologically to strain PRN and seems to
show some homology with its RNA-2.

The two PEBV isolates of the British serotype clearly share most of the sequences in both their
RNA species. Isolate E116 of the Dutch serotype has only partial homology with these two, and
it seems likely that this will prove to be in RNA-1. Indeed, a more extensive study of PEBV
strains (Robinson et al., 1984) shows a pattern of RNA sequence homologies similar to those of
TRV serotype I-II isolates.

On the basis of hybridization tests, strain CAM (TRV serotype III) is as distinct from TRV
serotype I-II as is PEBV, and there seems now to be little justification for classifying all
tobraviruses as isolates of two viruses. The existence of distant serological relationships between
isolates of TRV serotype I-II and of TRV serotype III (Harrison & Woods, 1966; Kurppa et al.,
1981) and between isolates of TRV serotype I-II and PEBV (Maat, 1963) could be taken as a
reason for considering all tobraviruses to be isolates of a single virus, but this would obscure the
practical distinction between PEBV isolates, which cause diseases in legumes, and other
tobravirus isolates, which cause diseases in non-legumes (Harrison & Robinson, 1978).

The idea that TRV serotype I-II and PEBV are distinct viruses is supported by the failure to
make pseudo-recombinants between them (Lister, 1968). Similarly, Frost et al. (1967) failed to
make pseudo-recombinants between isolates of TRV serotype I-II and serotype III, although
Lister (1969) apparently succeeded in making two pseudo-recombinants containing RNA-1 of a
serotype III strain and RNA-2 of strain ORY. However the tests used to identify these pseudo-
recombinants did not conclusively exclude the possibility that they were in fact variants of strain
ORY.

The pattern of nucleotide sequence homologies among tobraviruses, together with their
serological and biological properties, therefore suggests that they should be regarded as isolates
of three distinct viruses. For TRV serotype III isolates a return to the original name, pepper
ringspot virus (Kitajima et al., 1969; Kitajima & Costa, 1969), seems appropriate. Although
most Brazilian tobraviruses probably are isolates of this virus, strain CAM is the only one that
has been widely used in laboratory studies. Indeed, many studies on "TRV" have used strain
CAM, and it now seems unsafe to assume that properties of this strain are necessarily also
typical of those of TRV.

The pattern of sequence homologies among TRV serotype I-II isolates raises interesting
questions about their evolution. It seems certain that their RNA-1 species have derived from
a common ancestor, but much less certain that their RNA-2 species have done so. A model in
which the two genome parts have separate origins, and in which the event by which they became
associated occurred on numerous independent occasions and involved RNA-2 species from
different sources is easy to reconcile with the data.

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electron microscopy, and Ann Jenkins for technical assistance.

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