Serological Relationships among Tombusviruses

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SUMMARY

Serological differentiation indices (SDIs) based on serum titrations in agar-gel double diffusion tests were determined for all ten definitive tombusviruses known at present, namely artichoke mottled crinkle, carnation Italian ringspot, cymbidium ringspot, eggplant mottled crinkle, Moroccan pepper, pelargonium leaf curl, petunia asteroid mosaic, the BS3 and type strains of tomato bushy stunt, and tombus Neckar viruses. More than 2000 titrations using 222 antisera from 36 rabbits were done. The SDIs ranged from 1 to >9, and all intermediate values were found. Simple trigonometry and computer methods were used to classify the results. There was no correlation between the serological relatedness of the particles of these viruses and the homology of their genome nucleic acids previously assessed by hybridization tests. In this respect they resemble the tymoviruses but not the tobamoviruses. The electrophoretic migration of the particles of 12 tombusviruses was studied in 1% agarose containing 20 mM-phosphate buffer pH 7.0. Some serologically indistinguishable tombusvirus strains migrated at different rates.

INTRODUCTION

Tombusviruses (Martelli, 1981; Matthews, 1982) have isometric particles about 30 nm in diameter. Each particle contains the single-stranded RNA genome of the virus and 180 protein subunits that have a molecular weight of about 40000. Cells infected with tombusviruses contain characteristic multivesicular bodies in their cytoplasm.

The classification of viruses within the tombusvirus group has been discussed for many years (e.g. Martelli et al., 1977). In the Commonwealth Mycological Institute/Association of Applied Biologists Description of tomato bushy stunt virus (TBSV), which is the type member of the group, Martelli et al. (1971) listed as strains of TBSV all the other tombusviruses known at that time, namely artichoke mottled crinkle (AMCV), carnation Italian ringspot (CIRV), pelargonium leaf curl (PLCV) and petunia asteroid mosaic (PAMV) viruses. In contrast, the International Committee on Taxonomy of Viruses has always considered these viruses as distinct (Matthews, 1982). The latter classification was also followed by Martelli et al. (1977) and by Martelli (1981). Tombusviruses have been distinguished from one another by host range and serological studies, and several workers have reported that immunoelectrophoresis is useful for distinguishing between them (Bercks & Lovisolo, 1965; Martelli & Quacquarelli, 1966; Hollings & Stone, 1975; Makkouk et al., 1981; Koenig & Kunze, 1982; Koenig & Avgelis, 1983).

In this paper, we report a study of the serological relatedness of the particles of 10 tombusviruses, and a comparison between the rates of their electrophoretic migration. The results are discussed and compared with those of nucleic acid hybridization tests of tombusvirus genomes by Gallitelli et al. (1985).

METHODS

Viruses and their purification. This study included all the definitive tombusviruses (Matthews, 1982) namely AMCV, CIRV, cymbidium ringspot virus (CybRSV), eggplant mottled crinkle virus (EMCV), PLCV, PAMV,
The type and BS3 strains of TBSV, and also two newly recorded tombusviruses, Moroccan pepper virus (MPV; Fischer & Lockhart, 1977; Makkouk et al., 1981) and tombusvirus Neckar (TVN; Koenig & Lesemann, 1985).

The virus isolates were kindly provided by Drs H. U. Fischer (MPV), M. Hollings (CIRV, CybRSV and TBSV-type), O. Lovisolo (PLCV, PAMV and TBSV-BS3), K. M. Makkouk (EMCV) and G. P. Martelli (AMCV). In a limited number of tests, four possible members of the group, i.e. galinsoga mosaic, glycine mottle, saguaro cactus and turnip crinkle viruses showed no serological cross-reactivities with the definitive members of the group. They were therefore not included in further studies.

The definitive tombusviruses were propagated in either *Nicotiana benthamiana* or *N. clevelandii*, and were purified either at pH 7-2 by a chloroform/butanol method (Koenig & Kunze, 1982) followed by sucrose density gradient centrifugation, or in acidic buffers followed by centrifugation in caesium chloride density gradients as described by Gallitelli et al. (1985).

**Serology.** Rabbits were immunized by intramuscular injections of preparations of purified virus particles, emulsified in, for the first injection, Freund's complete adjuvant and for the second, a week later, Freund's incomplete adjuvant. The rabbits were bled at fortnightly intervals over a period of 1 to 2 years, and the sera were titrated every third or fourth bleeding. Double diffusion tests were done in a gel consisting of 0-85% Difco special Noble agar, 0-85% sodium chloride, 0-25% sodium azide and either 10 mM-Tris special buffer pH 8.0 or 20 mM-phosphate buffer pH 5.5. The reactant wells were 4 mm in diameter and 2-5 mm apart. Serial dilutions of antisera were tested against purified virus preparations that had been diluted to have a titre of 1:8. Slide precipitation tests were done as described by Bercks et al. (1972).

**Electrophoresis.** Virus particles (approx. 10 μg in 10 μl) were electrophoresed for 90 min at 120 V in a 1% agarose gel (Sigma type I: low electro-endosmosis) prepared in 20 mM-phosphate buffer pH 7.0. Gels were stained in a solution containing 0.0175% Coomassie Brilliant Blue and 5.3% TCA in a 320:80:28 (by vol.) mixture of water/methanol/acetic acid. They were destained in the same solution without stain and TCA.

**RESULTS**

**Serological tests**

Homologous and heterologous titres of antisera were determined in gel diffusion tests using an agar medium containing 10 mM-Tris-HCl buffer pH 8.0. Later we became aware of a paper by Krüse et al. (1982) who found that the particles of TBSV swell in certain buffers, such as alkaline Tris buffers, but not in acidic phosphate buffers. Therefore, in a number of tests we compared the titres obtained using gels containing either 10 mM-Tris-HCl buffer pH 8.0, or 20 mM-phosphate buffer pH 5.5, and obtained identical results with all 10 tombusviruses. In the presence of 20 mM-EDTA, however, the formation of precipitin lines was greatly inhibited in 10 mM-Tris-HCl buffer pH 8, but not in 20 mM-phosphate buffer pH 5.5. This effect was more pronounced in agar than in agarose gels, and was apparently caused by restricted diffusion; those lines which did form were very close to the antigen wells. In the slide precipitin test, 20 mM-EDTA caused little or no change in titre in either 10 mM-Tris-HCl buffer pH 8.0, or 20 mM-phosphate buffer pH 5.5.

The serological relationships of the particles of the 10 tombusviruses were assessed from the results of more than 2000 titrations using 222 antisera obtained from 36 rabbits to make all pairwise comparisons. Such a large number of tests was required in order to minimize the differences between antisera. The relatedness of each pair of viruses was expressed as an average 'serological differentiation index' (SDI), namely the mean number of twofold dilution steps separating the homologous and heterologous titres of the antisera prepared against one of the viruses (Van Regenmortel, 1975; Koenig, 1976). Fig. 1 shows a block diagram of the observed SDIs; the viruses are arranged in order of increasing average SDI values.

The SDI values of reciprocal titrations were significantly correlated (correlation coefficient 0.953, 44 degrees of freedom, P < 0.001); therefore, for most comparisons we used the average SDI of reciprocal tests (RT-SDI). The standard deviation for each RT-SDI was usually between 1 and 1-5 SDI units, and for the closely related pairs TBSV-type/TBSV-BS3 and AMCV/PAMV it was 0-7 and 0-6 respectively. This means that the most distant relationship that could be measured accurately had an SDI of about 9-0 provided that the homologous titre of the antiserum being tested was at least 1/2048, which was true of most of our antisera. Even more distant relationships were recorded as being > 9-0; often they were only detected with some of the antisera. The most closely related viruses, namely the TBSV strains and AMCV/PAMV.
Serological relationships among tombusviruses

Antisera

<table>
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<tr>
<th>Virus</th>
<th>TBSV-type</th>
<th>TBSV-BS3</th>
<th>AMCV</th>
<th>PAMV</th>
<th>PLCV</th>
<th>MPV</th>
<th>EMCV</th>
<th>CIRV</th>
<th>TVN</th>
<th>CybRSV</th>
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<td>CybRSV</td>
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Fig. 1. Serological relationships among the particles of ten tombusviruses. The serological differentiation indices are indicated by the number of white squares in each rectangle: a rectangle with only a half-filled square indicates that the SDI was above 9, and one with no filled squares that no serological reaction was detected. The second column from the left shows the number of rabbits immunized with particles of the virus/the number of serum samples tested.

were separated by RT-SDI values of 1-0. With some antisera homologous and heterologous titres seemed identical; however, when such viruses were placed in adjacent wells, the homologous virus always gave a spur at the merging point of the precipitin lines.

Fig. 2 illustrates the RT-SDI relationships of the viruses as distances in a two-dimensional diagram. This diagram was devised by simple trigonometry with emphasis given to the most consistent results. It is, of course, an approximation as the relationships, which are multidimensional, are represented in two dimensions, and this resulted in some cases in slight differences between the relative distances in the diagram and the relative RT-SDIs on which they were based. In such cases the 'diagrammatic' value is given in parentheses.

The RT-SDI data were also analysed by unweighted computation methods. Fig. 3 shows a dendrogram calculated from the RT-SDI values (Gibbs & Fenner, 1984); centroid strategy was used so that the position of each branching point in the dendrogram indicates the mean RT-SDIs linking the viruses connected through that bifurcation.

The RT-SDI data for the eight most closely related viruses were also analysed by the principal coordinates method (Gower, 1966), and the diagram in Fig. 4 illustrates the first three dimensions of that ordination.

The different methods of displaying the relationships of these viruses gave congruent classifications. They showed that TBSV-type, TBSV-BS3, AMCV, PAMV, PLCV, MPV and EMCV are interrelated with SDI values ranging from 1 to 6, and are well represented by the first three dimensions of the ordination, which contained 87% of the information. CIRV, TVN and CybRSV are more distantly related not only to the other seven viruses but also to one another, although the precise relationships of the last two are uncertain as they failed to react with some antisera or had SDI values > 9.0. However, removing CIRV from the ordination only increased the information content of the first three dimensions to 89%, which indicates that the ordination in Fig. 4 is not dominated by RT-SDIs associated with CIRV, its most disparate member.

Electrophoretic migration in agarose gels

Fig. 5 shows the pattern obtained when the particles of 12 tombusvirus isolates were electrophoresed in 1% agarose gel containing 20 mM-phosphate buffer pH 7.0. It can be seen that there is no correlation between the relative electrophoretic migration of the particles of the
Fig. 2. Diagram illustrating a classification of ten tombusviruses with distances representing the mean serological differentiation indices of reciprocal tests (RT-SDI). RT-SDI values have been rounded to the nearest 0.25, and when, in order to represent the relationships in two dimensions, the 'observed' and 'diagrammatic' (in parentheses) RT-SDI values differ, the two values are shown. CIRV, TVN and CybRSV are only distantly related to one another and to the other tombusviruses which form a central cluster. In a multi-dimensional system these three viruses would have to be arranged in planes above and below that of the central cluster. The arrows indicate the average distance of these viruses from the central cluster as a whole, but not from individual viruses.

Fig. 3. Dendrogram calculated from the RT-SDI relationships of ten tombusviruses by centroid sorting strategy (Gibbs & Fenner, 1984).
Serological relationships among tombusviruses

Fig. 4. Diagram illustrating a principal coordinates classification (Gower, 1966) of eight tombusviruses calculated from their RT-SDI relationships: the first three dimensions of the ordination are illustrated.

![Diagram](image)

**TBSV-type**  
**TBSV-BS3**  
**TBSV-Peru**  
**PAMV**  
**PAMV-cherry**  
**AMCV**  
**PLCV**  
**MPV**  
**EMCV**  
**CIRV**  
**TVN**  
**CybRSV**

Fig. 5. Photograph of a stained gel showing the relative electrophoretic migration of the particles of 12 tombusvirus isolates in 1% agarose gel containing 20 mM-phosphate buffer pH 7.0; anode left, cathode right.

different tombusviruses and their serological relatedness. Most isolates migrated towards the anode only the type strain of TBSV and CybRSV migrated towards the cathode. Furthermore, serologically indistinguishable viruses such as TBSV-BS3 and TBSV-Peru (Koenig & Avgelis, 1983) or PAMV and PAMV-cherry (Koenig & Kunze, 1982) migrated at different rates.
Fig. 6. Graph comparing estimates of the serological relatedness and genome homologies (Gallitelli et al., 1985) among seven tombusviruses. The serological relationships are given as RT-SDIs, and the genome homologies as mean of reciprocal cDNA/RNA hybridization estimates for all viruses except petunia asteroid mosaic virus. The cDNA for the RNA of the latter virus was not tested. A, Artichoke mottled crinkle virus; Ci, carnation Italian ringspot virus; Cy, cymbidium ringspot virus; E, eggplant mottled crinkle virus; Pa, petunia asteroid mosaic virus; PI, pelargonium leaf curl virus; T, type strain of tomato bushy stunt virus.

**DISCUSSION**

Until recently, the diagnosis of unknown viruses was confirmed, or the relatedness of similar viruses was compared, by a serological test of some sort. However, nowadays comparisons of genomes by nucleic acid hybridization techniques are frequently used for the same purposes. Thus, it is of practical importance to know how well the results of these two types of tests agree with one another, and in particular how well their assessments of relatedness correlate.

For tombusviruses, our serological results may be compared with those of Gallitelli et al. (1985), who assessed the relatedness of the genomes of seven of the viruses by nucleic acid hybridization tests. As in the present study, they found all grades of relationship between the viruses with no obvious clustering. However, as shown in Fig. 6, there is no correlation between our estimates of serological relatedness and their estimates of genome homology. CybRSV, for instance, is serologically the most distantly related tombusvirus (Fig. 1 and 2). We found cross-reactions with other tombusviruses only with a few antisera, and Hollings et al. (1977), who used a smaller number of antisera, found no cross-reactions at all. Nucleic acid hybridization tests, however, did not indicate an especially distant relationship between CybRSV and other tombusviruses. PAMV and AMCV, on the other hand, are closely related serologically (Fig. 1 and 2) and Hollings & Stone (1975), with some antisera, failed even to
observe spur formation between the two viruses. Their genome homology, nevertheless, was only 23\% (Gallitelli et al., 1985).

These observations suggest that during evolution different parts of the tombusvirus genome must have been conserved to a different extent. With some viruses, like the pair AMCV/PAMV, the part of the genome encoding the antigenic determinants of the particles has been strongly conserved, but other parts of the genome must have changed considerably. With CybRSV, however, the part of the genome encoding antigenic determinants of the particles has apparently been conserved to a lesser extent than the other parts of the genome. From melting data, Gallitelli et al. (1985) also concluded that the regions of RNA homology may be different for different tombusviruses.

For tobamoviruses, Gibbs (1980) showed that there was a clear correlation between the SDI values obtained by Van Regenmortel (1975) and the similarities among the amino acid sequences or compositions of their particle proteins. Furthermore, there are significant correlations between similarities in particle protein sequence and the genome homology estimates found by Van de Walle & Siegel (1982) and J. Blok & A. Mackenzie (personal communication); the respective correlation coefficients were 0.786, 8 degrees of freedom, $P < 0.01$ to 0.001 and 0.614, 5 degrees of freedom, $P$ about 0.15. Unfortunately, neither set of estimates involved a sufficient number of the tobamoviruses that Van Regenmortel studied to allow a direct comparison of SDI values and genome homology to be made.

Tymoviruses are like tombusviruses in that there is no correlation between SDI values (Koenig, 1976) and estimates of genome homology (A. Blok, A. J. Gibbs & A. Mackenzie, unpublished results) or similarities between coat protein compositions (Paul et al., 1980).

Our observations on the electrophoretic migration of tombusvirus particles differ somewhat from those reported by Hollings & Stone (1975), who reported that AMCV, CIRV and PLCV migrated towards the cathode. This difference may be because we used a medium with a lower pH, and agarose rather than agar to reduce electro-endosmosis. Hollings & Stone (1975) found with PLCV that serologically indistinguishable isolates may differ in their electrophoretic migration. We found that this was also true for isolates of TBSV-BS3 and PAMV. Particles of TBSV have been reported not to swell in neutral phosphate buffer in the absence of EDTA (Krüse et al., 1982). However, since the conditions leading to a swelling of particles may differ with different tombusviruses, we cannot exclude the possibility that with some viruses differences in electrophoretic migration may be due to differences in size rather than charge.

Because of the lack of correlation between tombusvirus relationships assessed by serological, nucleic acid homology and electrophoretic migration tests, we suggest that for the time being it may be best to continue to use separate names for those viruses which have been listed by Matthews (1982) and for the newly described EMCV, MPV and TVN.

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REFERENCES


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