Genetic Analysis of Cowpea Chlorotic Mottle and Brome Mosaic Viruses

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SUMMARY

We have assigned mutations in cowpea chlorotic mottle virus and the related brome mosaic virus to specific RNA components. Coat protein changes, as well as associated ‘maturation faults’ and systemic symptom changes, are located on RNA species 3; local and primary lesion alterations are found on either RNA species 2 or 3. Changes in nucleoprotein component ratio are associated either with the coat protein changes on RNA species 3 or with primary lesion alterations on species 2. Local lesion temperature sensitivity is found on RNA species 1.

INTRODUCTION

Cowpea chlorotic mottle virus (CCMV) and brome mosaic virus (BMV) are related (Scott & Slack, 1971; Bancroft, 1972) and each requires three complementary, physically distinct pieces of RNA to initiate infection and also contains a fourth smaller RNA component which is not required for infection (Lane & Kaesberg, 1971; Bancroft & Flack, 1972). These pieces with mol. wts. of roughly 1.1, 1.0, 0.8 and 0.3 million are numbered 1, 2, 3 and 4, respectively. We have isolated a number of mutants of these viruses and describe how we have assigned the mutations to specific RNA species.

METHODS

The mutants, with the exception of BMV F, BMV V2 and BMV V5, which arose naturally, were obtained by treating virus RNA with nitrous acid (Siegel, 1960) at pH 4.8 to a survival level between $10^{-2}$ and $10^{-3}$. All single lesions were passed through three serial single lesion transfers on Chenopodium hybridum L. The CCMV mutants (ts and mild) which grew on cowpea (Vigna unguiculata (L.) Walp. var. Blackeye) were purified as before (Bancroft et al. 1972) and separated into their three density components by preparative sedimentation in CsCl (Bancroft & Flack, 1972). RNA was extracted from the virus with phenol, and RNA species were separated by electrophoresis on 2.6 % polyacrylamide gels (Loening, 1967) for 4 h at 4 mA/5 mm diameter tube. Bands containing RNA were excised and extracted with phenol (Bancroft, 1971).

The BMV primary lesion mutants were grown in and purified from Chenopodium quinoa since it is infected more readily and shows symptom differences more readily than barley. BMV F, BMV V2 and BMV V5 were grown in and purified from barley (Hordeum vulgare (L.) var. Moore).

Mutations were assigned to RNA components using one or more of three tests. The most rigorous of these tests we will term the ‘hybridization test’. It consists of constructing
‘artificial hybrids’ between two strains by combining two required RNA species, which we will refer to as the homogenetic species, of one strain, with the third required RNA species, which we will refer to as the heterogenetic species, from another strain. Inocula were prepared by adjusting the gel electrophoretically purified components to about 2 μg/ml. Infectivities of complete mixtures were compared on opposite half leaves to those of mixtures of the two homogenetic species (Table 1). The comparison was also made at a two- to threefold dilution. Where the complete mixture gave many lesions and the mixture of two homogenetic species gave few or none, the lesions produced by the complete mixture were considered to contain a hybrid virus. Since lack of infectivity of the heterogenetic species is not the best criterion of purity, in most cases we tested its infectivity in combination with biologically active preparations of each one of its other two complementary components respectively. Where a hybrid with a mutant heterogenetic species showed the mutant phenotype, the mutant heterogenetic RNA species contained the mutant gene. To confirm this assignment the reciprocal hybrid was constructed using the corresponding RNA species from wild-type as the heterogenetic species and mutant RNAs as homogenetic species. In all cases the reciprocal hybrid exhibited the wild-type phenotype, confirming the location of the gene.

Six different hybrid strains can be constructed from two parental strains. In some cases the complete set was constructed, but in most cases it was possible to guess which RNA species contained the mutant gene and to construct only the two hybrids where this was the heterogenetic species.

Unfortunately some mutants (notably those CCMV mutants which gave small lesions on Chenopodium hybridum) grew poorly and produced insufficient material for the hybridization test. For these mutants a ‘supplementation test’ was applied. Approximately 2 g of C. hybridum leaves infected with a small lesion mutant were extracted with phenol and the vol. of the aqueous phase was adjusted to about 2 ml with 0.1 M-NaCl. The dilution at which no lesions appeared was ascertained and then the three purified wild-type RNA species at about 2 μg/ml were added separately to three samples of the diluted extracts so that no lesions appeared with either the wild-type RNA species or the extract alone, but 20 to 40 lesions appeared on comparable half leaves inoculated with the suitable mixture. Such conditions are possible because lesion number is related to the product of the three RNA concentrations, and thus by increasing the level of any one component, infectivity can be raised above the dilution end point.

Each of the three mixtures was inoculated onto Chenopodium hybridum half leaves with appropriate controls on opposite half leaves. Only where the wild-type RNA species was homologous to the mutated species did it supplement the inoculum to produce wild-type lesions. The remaining two RNA species could not supplement the mutant and caused production of small lesions only.

While the classical genetic complementation, involving interactions between gene products, has not been observed with BMV or CCMV, their genetic material exhibits complementation in that interaction between three components is required for infection. Similarly, mixtures of two abnormal strains of BMV or CCMV can produce normal virus if the two strains between them have a normal complement of RNA components. We have applied the latter form of complementation test to ‘abnormal lesion’ mutants. A Chenopodium hybridum leaf, heavily infected with a mutant, was ground in a mortar and the extract was diluted with tapwater until it was faintly green. Extracts from two mutant infections were combined for the complementation test and inoculated on to C. hybridum half leaves while opposite half leaves were inoculated with extracts of the individual mutants to serve as controls. The
Table 1. *Lesion numbers caused by complete as compared to incomplete mixtures of various RNA species from wild-type CCMV and the ts and mild mutants*

<table>
<thead>
<tr>
<th>Mutant mixture</th>
<th>ts</th>
<th>mild</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1 + 3 + 3^*$</td>
<td>75†</td>
<td>25</td>
</tr>
<tr>
<td>$1 + 2$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$1 + 2 + 3$</td>
<td>94</td>
<td>35</td>
</tr>
<tr>
<td>$3$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$1 + 2 + 3$</td>
<td>34</td>
<td>20</td>
</tr>
<tr>
<td>$1 + 2$</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>$1 + 2 + 3$</td>
<td>18</td>
<td>38</td>
</tr>
<tr>
<td>$2 + 3$</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* The numbers refer to RNA species 1, 2 and 3 with mol. wts. of $1 \cdot 10^6$, $1 \cdot 0$ and $0.8 \times 10^6$, respectively. Italicized numbers designate mutant RNA species.
† Local lesions on two half-leaves of Chenopodium hybridium. Five lesions from each complete mixture were subcultured, purified and tested for properties.

Table 2. *Phenotypes associated with different RNA species from various mutants of CCMV and BMV*

<table>
<thead>
<tr>
<th>Mutant RNA Species</th>
<th>Component ratio</th>
<th>Systemic symptoms</th>
<th>Temperature sensitivity</th>
<th>Specific infectivity</th>
<th>Coat protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCMV (ts)*</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>CCMV (mild)</td>
<td></td>
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<tr>
<td>CCMV (MCza)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCMV (MCzd)</td>
<td>Temperature sensitivity</td>
<td>Local lesions</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>BMV (MBzb)</td>
<td></td>
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<tr>
<td>BMV (MB2a)</td>
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<tr>
<td>BMV (MB4a)</td>
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<tr>
<td>BMV (MB4b)</td>
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<td></td>
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<tr>
<td>BMV (V2, V5)‡‡</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMV (F)</td>
<td></td>
<td></td>
<td></td>
<td>Systemic symptoms</td>
<td></td>
</tr>
</tbody>
</table>

Inoculation series was performed at several dilutions to assure a suitable response. Where the two mutations were in different RNA species the mixture contained the complete wild-type genome and wild-type lesions appeared (Fig. 2). Some mutant lesions occur of course in the complementing mixtures because some infections are initiated by the mutated RNA species. Where the two mutations were in the same RNA species only small lesions appeared.
RESULTS

The mutants and inheritance experiments are described.

**CCMV ts**

This mutant was chosen from nitrous acid survivors because of the *in vitro* thermal lability of its coat. Its properties have been described in detail (Bancroft et al. 1972). It differs from the type strain as follows: (a) decreased symptoms at high temperature; (b) decreased yield at high temperature; (c) decreased specific infectivity; (d) decreased thermal stability of coat protein; (e) altered nucleoprotein component ratio in CsCl.

CCMV *ts* was compared to *wt* by the hybridization test (Table 1). Progeny from five lesions of genotype (*wt* + 2 *wt* - 3 *ts*) were subcultured on cowpea and all contained the *ts* markers listed in Table 2, whereas those from five lesions of genotype (*wt* + 2 *ts* + *wt*) were all wild-type. RNA species 3 therefore specifies coat protein, systemic symptoms and the associated phenotypes listed in Table 2.

**CCMV mild**

Like CCMV *ts*, this mutant was chosen from nitrous acid survivors because of the *in vitro* thermal lability of its coat. It differs from wild-type as follows: (a) milder systemic symptoms (Fig. 1A); (b) smaller and less necrotic local lesions on *Chenopodium hybridum* (Fig. 1B); (c) one-half denaturation temperature less by 3 °C by the method of Bancroft et al. (1972).

CCMV *mild* was compared to *wt* by the hybridization test (Table 1). Lesions containing virus of genotype (*wt* + 2 *wt* + 3 *mi*) were of wild-type morphology while those containing virus of genotype (*wt* + 2 *mi* + 3 *wt*) were of mutant morphology, indicating that RNA species 2 specifies local lesion type (Table 2). Progeny of the former hybrid, however, had thermally unstable coat protein and gave mild symptoms on cowpea while progeny of the latter hybrid had normal coat protein and gave normal symptoms on cowpea (Table 2). Thus RNA species 3 again specifies systemic symptoms and coat protein. The divided genome of CCMV has allowed us to resolve this double mutation into its constituent mutations by constructing the two hybrids which carry respectively the mutant local lesion morphology and the mutant systemic symptom-coat protein type.

**CCMV MC2a**

This mutant was selected because it produced small lesions on *Chenopodium hybridum* (Fig. 1C). It neither produces visible lesions on soybean (Glycine max (L.) Merr. var. Lindarin) nor multiplies detectably in cowpea.

In supplementation tests of CCMV *MC2a*, only *wt* RNA species 2 caused production of *wt* lesions. Virus from the normal lesions, however, neither infected nor produced lesions on cowpea. Though the small lesion mutation is on RNA 2, CCMV *MC2a* contains at least one additional mutation in another RNA species which prevents it from growing on cowpea.

**CCMV MC2d**

This mutant was selected on the same basis as *MC2a* and has similar properties, but unlike *MC2a* it produces only 10 % as many lesions on *Chenopodium hybridum* at 32 °C as it does at 22 °C.

The lesion type of CCMV *MC2d* was, like that of *MC2a*, supplemented only by *wt* RNA 2 (Table 2). Virus from the normal lesions arising in these experiments, as well as virus from
Genetic analysis of CCMV and BMV

Fig. 1. Symptoms caused by mutant and wild-type CCMV and BMV. (A) CCMV mild (left) and CCMV wild-type on cowpea 13 days after inoculation; (B) CCMV mild (left) and CCMV wild-type on Chenopodium hybridum 5 days after inoculation; (C) CCMV MC2a (left) and CCMV wild-type on C. hybridum 4 days after inoculation; (D) BMV wild-type (left) and BMV MB1b on C. hybridum 5 days after inoculation; (E) BMV and (F) BMV MB1b primary lesions on paired C. quinoa leaves 7 days after inoculation; (G) BMV (left) and BMV F on C. hybridum 5 days after inoculation; (H, I) BMV and BMV F primary lesions on paired C. quinoa leaves 6 days after inoculation.
small lesions arising from a mixture of mutant with wt RNA 3, gave, as did authentic MC2d, only 10 to 15% as many lesions at 32 °C as at 22 °C. In another supplementation test, virus originating from a mixture of mutant extract with wt RNA 1 gave 60% as many lesions at 32 °C as at 22 °C. Both CCMV wt and CCMV ts gave 80% as many lesions at 32 °C as at 22 °C. It is clear that RNA 1 of CCMV MC2d contains a temperature-sensitivity mutation, though it is not certain that this accounts quantitatively for the temperature sensitivity.

**BMV, MB1b, MB2a, MB4a, MB4b**

These mutants were selected because they produced small local lesions with necrotic centres on Chenopodium hybridum (Fig. 1 D). The primary symptoms on C. quinoa (Fig. 1 F) were milder than those of wt (Fig. 1 E). MB1b was indistinguishable from wt BMV in regard to ratio of lesions produced at 22 °C relative to 32 °C, electrophoretic mobility in polyacrylamide gels and component ratio in CsCl.

Hybrids with RNA species 1 and 2 from small lesion mutants of the MB series and wt RNA species 3 all gave normal BMV lesions on Chenopodium hybridum and wild-type primary reactions on C. quinoa. The reciprocal hybrid, made only with MB1b, gave small lesions on C. hybridum and a mutant reaction on C. quinoa. It is clear that the small lesion mutations of these strains are on RNA species 3. Inocula containing mixtures of these small lesion strains (complementation test) in no case produced wild-type lesions.

**BMV V2, V5**

The former, which has been described by Lane & Kaesberg (1971), is a slowly migrating, naturally occurring electrophoretic variant of wt BMV and the latter is a still more slowly migrating, naturally occurring electrophoretic variant of the former. Both variants contain decreased amounts of RNA species 3 and 4. Their local and systemic symptoms are indistinguishable from wt.

Hybridization experiments between BMV V2 and BMV wt have already been described (Lane & Kaesberg, 1971). When all hybrids were grown on and harvested from barley under parallel conditions, the three hybrids with wild-type coat protein all contained higher proportions of RNA species 3 and 4 than did the hybrids containing the variant coat protein. It is clear that RNA species 3 determines both coat protein and component ratio. The two hybrids containing RNA 3 as the heterogenetic species were constructed between wt and V5. As with BMV V2, species 3 determined the coat protein.

**BMV F**

This naturally occurring mutant, which was selected because it exhibited rapidly migrating nucleoprotein components on polyacrylamide gels, gave non-necrotic spreading lesions on Chenopodium hybridum (Fig. 1 G) and diffuse primary lesions on C. quinoa (Fig. 1 I). An unusual feature of this variant is that roughly 15% of its particles are smaller than normal. A detailed study of the physical properties of this mutant will be the subject of another publication.

A hybrid of BMV F with genotype (1 wt + 2 wt + 3F) gave both wild-type lesion morphology and normal particles, while the reciprocal hybrid of genotype (1 F + 2 F + 3 wt) gave mutant lesions and abnormal particles. Therefore the ‘F’ phenotype is not associated with the coat protein gene on RNA species 3. A complementation test between BMV F and BMV MB4a, which is mutated on RNA species 3, gave wild-type lesions (Fig. 2) again showing that the ‘F’ phenotype is not associated with RNA species 3. The hybrid of genotype
Genetic analysis of CCMV and BMV

Fig. 2. Complementation test between BMV F and BMV MB4a 7 days after inoculation. The left side of each Chenopodium hybridum leaf was inoculated with a mixture of the two mutants. The right side was inoculated with (A) MB4a, (B) wild-type BMV and (C) BMV F. Note that lesion morphology resembles wild-type with the mixture, but not with the individual mutants.

(1 \( F+2 \) wt + 3 \( F \)) gave wild-type lesions and normal particles. This indicates that RNA species 2 specifies the 'F' phenotype. We have so far been unable to construct the reciprocal hybrid because BMV F is deficient in RNA species 2.

DISCUSSION

Earlier studies of BMV (Lane & Kaesberg, 1971) and CCMV (Bancroft & Flack, 1972) showed that each of three RNA components contributes to the infectivity of the virus. Chemical studies of BMV RNA (Shih, Lane & Kaesberg, 1972) indicate that each of these three BMV RNA components contains at least some unique nucleotide sequences. This paper shows that each of the three ‘required’ RNA species of CCMV RNA, as well as at least two of the ‘required’ RNA species of BMV, contributes unique genetic information to the infection. The genetic contribution of the RNA components confirms the division of the genome and indicates that the components do not play a transient role in the establishment of infection, as does the smallest RNA species of alfalfa mosaic virus (Dingjan-Versteegh, Van Vloten-Doting & Jaspars, 1972).

One is tempted to estimate the number of genes in BMV and CCMV. Since each has three RNA species, each must have at least three genes. If each gene codes for 20000 daltons of protein the viruses might have as many as 15 genes. Studies of the RNA bacteriophages (Horiuchi, Webster & Matsuhashi, 1971) and a recent study of the proteins produced during tobacco mosaic virus infection (Sakai & Takebe, 1972) indicate that the ‘average protein’ is much larger than 20000 daltons and that 4 to 7 genes each may be a better estimate for CCMV and BMV.

To demonstrate more than the minimum number of genes, one must show that two mutations in the same RNA species are in distinct genes. Genetic recombination, other than the exchange of components in divided genome viruses, has not been demonstrated in plant viruses, and the complementation systems that were so useful for the RNA phages (Gussin, 1966) do not presently exist for plant viruses.

Earlier experiments (Bancroft et al. 1972) indicated that the temperature sensitivity and low specific infectivity of CCMV ts were both consequences of a mutated coat protein. The genetic coincidence of these phenotypes on RNA species 3 agrees with this conclusion.

A possible case of multiple genes on a single RNA component is RNA species 3 of BMV, where the coat protein mutants and the small lesion mutants cause different syndromes. At present the only method for assigning these mutant classes to separate genes is to sequence BMV coat protein and to demonstrate that the small lesion mutations do not coincide with coat mutations.
Another genetic problem is the association of apparently unrelated phenotypes such as coat protein, systemic symptoms and RNA component ratio on a single RNA species. Coat protein mutations of BMV can almost certainly cause alterations in RNA component ratio. Three independently isolated, naturally occurring coat protein variants, which are unlikely to be multiple mutants, all have altered RNA component ratios (Lane, 1971). However, not all coat changes alter component ratio detectably, because CCMV mild is indistinguishable from CCMV wild-type in CsCl.

The genetic connexion between coat protein and systemic symptom variation is a general problem in plant virology. The majority of nitrous acid induced symptom mutants of tobacco mosaic virus (TMV) have unchanged amino acid compositions (Melchers, 1966). On the other hand, Jockusch & Jockusch (1968) have established a possible relationship between coat protein mutation and symptom variation in TMV. They noted that TMV mutants with temperature-sensitive coat proteins tend to exhibit yellow symptoms and that symptom severity correlates roughly with the amount of insoluble coat protein in the infected tissue, although one mutant (NiI8) is a notable exception.

The cohort protein and systemic symptom phenotypes of alfalfa mosaic virus (AMV) (Dingjan-Versteegh et al. 1972) and raspberry ringspot virus (Harrison, Murant & Mayo, 1972) occur together on the smallest genetically stable RNA component. The coat protein and systemic symptom phenotypes of CCMV likewise are on the smallest genetically stable RNA component (Table 2). The small size of the CCMV and AMV RNAs argues for a single gene controlling the two factors, but at least one of our CCMV coat protein mutants is a double mutant and we cannot rule out the possibility that both CCMV ts and CCMV mild are multiple mutants. The properties of revertants of another CCMV mutant indicate that the coat protein gene can affect systemic symptoms. This mutant is largely unable to infect, unless kept under reducing conditions, because of an arginyl to cysteinyl replacement in the coat protein (Bancroft et al. 1971). When the mutant was inoculated under non-reducing conditions, 9 of the 13 subcultured lesions were apparently reverted in both coat protein and systemic symptoms since they had high specific infectivity and wild-type symptoms rather than the low specific infectivity and mild symptoms of the mutant. The correlation between reversion of symptoms and reversion of the coat protein is strong evidence that a single mutation can effect both properties.

One cannot conclude from the above arguments that symptom changes and component ratio alterations are invariably coupled to coat protein mutations. The coat protein is the only gene product which can be directly characterized. All other properties of the virus such as symptoms and component ratio are dictated by the ‘overall virus metabolism’ and can conceivably be influenced by more than one virus gene. Indeed, we have shown with BMV that both primary symptoms and component ratio can be affected by at least two genes.

Hiebert & Bancroft (1969) have shown that coat protein and RNA are the only virus components necessary for the assembly of BMV in vitro. The discovery of an additional gene (BMV F) which influences assembly in vivo suggests that a search for additional factors which influence in vitro assembly may be profitable.

The collection, characterization and classification into linkage groups of mutants is aimed ultimately at establishing the relationship of biological properties to specific proteins. The development of systems for synchronously infecting plant protoplasts (Sakai & Takebe, 1972) should allow detailed study of virus metabolism, particularly since CCMV can be grown in tobacco protoplasts (F. Motoyoshi & J.B. Bancroft, unpublished results). The availability of well-characterized mutants will allow correlation of biological properties of the virus with cellular metabolism and ultimately with specific virus proteins. Even in the
absence of methods for studying the molecular biology of virus infection, combining genetic
data for several divided genome viruses will allow one to know which properties are common
to all plant viruses, which properties are unique to a particular virus, and which properties
occur together in genetic linkage groups.

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REFERENCES

BANCROFT, J. B. (1971). The significance of the multicomponent nature of cowpea chlorotic mottle virus
RNA. Virology 45, 830–834.
BANCROFT, J. B. (1972). A virus made from parts of the genomes of brome mosaic and cowpea chlorotic
BANCROFT, J. B. & FLACK, I. H. (1972). The behaviour of cowpea chlorotic mottle virus in CsCl. Journal of
21, 435–453.
by its smaller RNA. Journal of General Virology 17, 137–141.
296–311.
429–439.
Molecular and General Genetics 102, 204–209.
University of Wisconsin.
Biologv 232, 40–43.
LOENING, V. E. (1967). The fractionation of high molecular weight ribonucleic acid by polyacrylamide-gel
MELCHERS, G. (1966). Contributions of plant virus research to molecular genetics. Gregor Mendel Memorial
Symposium, Academia, Prague, 119–136.
SAKAI, F. & TAKEBE, J. (1972). A non-coat protein synthesized in tobacco mesophyll protoplasts infected by
tobacco mosaic virus. Molecular and General Genetics 118, 93–96.
Journal of Molecular Biology 64, 353–362.
156–157.

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