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In vivo Phenotypic Mixing between Two Strains of Tobacco Mosaic Virus

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Infective particles have been reconstituted *in vitro* from the disaggregated protein and RNA of several plant viruses and virus strains. The protein and nucleic acid need not be from the same virus strain, or indeed not even from serologically related viruses (Hiebert, Bancroft & Bracker, 1968; Lebeurier, Wurtz & Hirth, 1969; Breck & Gordon, 1970). Thus the RNA from brome mosaic, broad-bean mottle or cowpea chlorotic mottle viruses has been combined with the protein of any of the three viruses and the RNA of potato virus X with protein of tobacco mosaic virus (TMV). With strains of TMV, the more differences there are in amino acid composition or the more distant the serological relationship, the smaller is the yield of infective particles and the less stable they are (Fraenkel-Conrat & Singer, 1957; Holoubek, 1962).

There are many known cases of phenotypic mixing of animal and of bacterial viruses but only one reported for plant viruses, between two serologically unrelated isolates of barley yellow dwarf virus (Rochow, 1970), although claims of phenotypic mixing between strains of TMV have been made (Schaskolskaya *et al.* 1968; Sarkar, 1969). Barley yellow dwarf virus is a circulative virus transmitted only by aphids. The two isolates are normally transmitted by different species of aphids but in a mixed infection one of the aphid species can transmit both isolates because the protein of the isolate it transmits can coat the RNA of both.

We now report another example of phenotypic mixing, this one between PM2 (Siegel, Zaitlin & Sehgal, 1962) and the Nigerian cowpea virus (Bawden, 1958), two strains of TMV that are distantly related serologically. PM2, a mutant obtained by treating the type strain with nitrous acid, produces protein that fails to bind the infective RNA and the two remain separate in infected plants. Its protein is closely related serologically to that of the TMV-type strain. PM_2 multiplies in tobacco over the whole range 20 to 35° and produces bright yellow lesions in the inoculated leaves. The cowpea isolate (CV) is distantly related to the type strain (Bawden, 1958) and infects leguminous plants systemically. In inoculated leaves of tobacco plants var. White Burley, kept at 35°, CV multiplies well, and sap attains a serological titre of 1/256 but causes no symptoms; at 20° it multiplies very little but produces a few necrotic lesions. In tobacco var. Samsun it reaches the same concentration at either 20° or 35° as in White Burley at 35°, again without causing symptoms.

Phenotypic mixing occurred when the two viruses were inoculated either to young White Burley plants, which had then to be kept at 35°, or to young Samsun plants, which could be kept at 35 or 20°. The leaves were first inoculated with PM2 with the aid of carborundum. For experiments at 35°, the plants were kept at 20° for 3 hr after inoculation, then transferred to 35°. Next day the same leaves were inoculated with CV without carborundum. Inoculating the two viruses simultaneously decreased the number of yellow lesions produced by PM2, an effect avoided by inoculating PM2 a day before CV (Fig. 1). Serological tests show that PM2 approximately halves the concentration of CV. A week after inoculating with PM2 the sap was extracted from the inoculated leaves and aged for 3 days at 20° to degrade the PM2 RNA not coated by protein. 0·02% sodium azide was added to the extracted sap to prevent bacterial growth during ageing. Tobacco var. White Burley inoculated with the aged sap

produced numerous yellow local lesions, but no such lesions were produced by similarly treated saps from plants inoculated with either PM2 or CV alone (Fig. 2). The few necrotic lesions produced by CV were easily distinguished from PM2 lesions and could be eliminated by inoculating older tobacco plants, in which CV does not cause necrotic lesions.

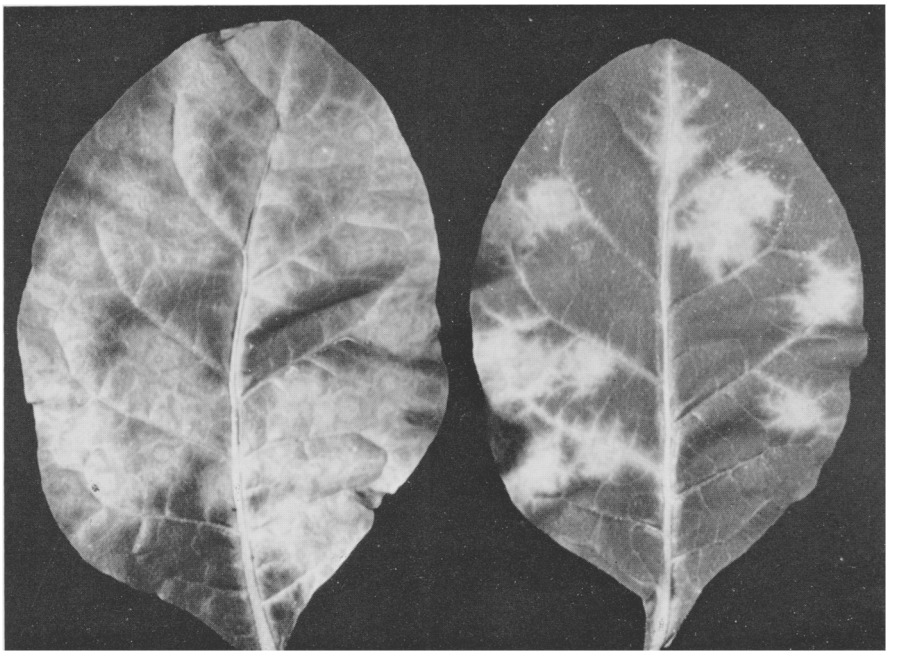

Fig. 1. Tobacco leaves var. White Burley inoculated a week before with the mixture of PM2 and CV. The leaf on the left was inoculated with PM2 24 hr before CV, the leaf on the right with both viruses at the same time.

To confirm that the yellow lesions were produced by PM2 RNA that had been stabilized by CV, sap was extracted from the test plants, aged as above, and inoculated to Samsun or White Burley tobacco plants. No lesions appeared because PM2 had been multiplying in conditions in which CV could not.

To find whether or not the PM2 RNA retained its usual behaviour after being mixed with CV, RNA was extracted by the phenol method from White Burley leaves inoculated with the 'phenotypic mixture' and showing numerous yellow lesions. (Extracted RNA of TMV has less than 0·1 % of the original infectivity of the virus, so the small amount of CV produced at 20° would be expected to be diluted too much to be infective). The extracted RNA was inoculated on to White Burley tobacco and seven of the few yellow lesions produced were cut out and used separately to inoculate young White Burley plants. These plants were kept for a week at 35° when the sap from them was extracted separately, aged for 3 days and inoculated, with carborundum, to tobacco plants. These plants remained uninfected showing that when no longer multiplying with CV the PM2 was again unstable at 35° as well as at 20°.

To confirm that the stable particles with the pathogenicity of PM2 produced in doubly infected plants were coated with CV protein, sap was mixed with various antisera and the infectivity of the unprecipitated particles assayed. Sap from plants infected with both PM2

and CV was clarified by centrifuging at 10,000 rev./min., diluted 1/10 and mixed with equal volumes of antisera diluted 1/200. The mixtures were left overnight, centrifuged at 10,000 rev./min., inoculated to Samsun plants, and infectivity assayed by counting the yellow lesions

Fig. 2. A tobacco leaf var. Samsun inoculated with aged saps, on the left from a plant infected with PM 2 alone and on the right from a plant infected with the mixture.

Table 1. *Relative infectivity of RNA extracted from fresh leaf tissue and aged saps from tobacco plants infected with the PM 2 and CV mixture*

Treatment	Dilution factor	Mean number of lesions/ half tobacco leaf
Fresh leaf	Undiluted	54
Fresh leaf	$\frac{1}{3}$	14
Fresh leaf	$\frac{1}{9}$	2
Aged sap	Undiluted	14
Aged sap	$\frac{1}{3}$	2

produced. With antiserum to brome mosaic virus, the average number of lesions per half leaf was 150, with antiserum to TMV 138, and with antiserum to CV only 33. Brome mosaic virus is unrelated to the TMV group of viruses. Antisera to CV and to type TMV contain few common antibodies and, when diluted to 1/200, are specific for their respective antigens.

To find the proportion of PM2 RNA that was coated with CV protein, a Samsun plant inoculated with both viruses was kept a week at 20° when 0·5 g. of leaf was extracted in 2 ml. M15 phosphate buffer, 2 ml. of water-saturated phenol, and 0·6 ml. of 3·8 % Bentonite in water. After centrifugation traces of phenol in the water phase were removed by shaking with ether and the RNA preparation stored at −15°. Sap from a comparable leaf sample from the same plant was aged for 3 days with 0·02 % sodium azide added, and the RNA then extracted as from the leaf sample. The two RNA preparations were then inoculated at a range of dilutions to tobacco var. White Burley to estimate their relative infectivities. The RNA from the fresh leaf tissue was three times as infective as that from the aged sap (Table 1).

The individuals of the two pairs of plant viruses shown to produce phenotypic mixtures *in vivo* are either serologically unrelated (the isolates of barley yellow dwarf virus) or are distantly related (CV and PM2). From the results of reconstituting TMV *in vitro* it might be expected that phenotypic mixing would be more probable with closely related strains, but a prerequisite to the forming of mixed particles is that the two viruses multiply in close proximity, and when two closely related strains are inoculated together it is usual for one to interfere with the other. However, even if closely related strains were not competitive, their similar properties and behaviour would make it difficult, if not impossible, to demonstrate serologically or otherwise that phenotypic mixing had occurred.

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