Regeneration of Virus-Free Plants From Dark-Green Islands of Tobacco Mosaic Virus-Infected Tobacco Leaves

Harry H. Murakishi and Peter S. Carlson

Professor, Department of Botany and Plant Pathology; and Professor, Department of Crop and Soil Sciences, respectively, Michigan State University, East Lansing 48824.

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ABSTRACT

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About 50% of plantlets regenerated from dark-green islands of tobacco leaves systemically infected with tobacco mosaic virus were virus-free. An obvious advantage of using these virus-free areas is that they are visually distinguishable and can be rapidly excised and plated to initiate regeneration. Leaf sap from such virus-free plantlets apparently contains a factor which is inhibitory to TMV infection. Our findings lend support to the hypothesis of Atkinson and Matthews that green islands are delimited by an as yet unidentified diffusible agent.

Current methods for recovering virus-free plants from infected material focus on the use of meristem culture (4, 7). This method takes advantage of the fact that with certain virus-host combinations, healthy plants can be regenerated from a small, virus-free zone at the extreme tips of shoot apices (5). Advances in culture and regeneration of plants from cells and tissues in vitro now offer the potential of recovering normal plants from other plant parts as well, including leaf tissue and protoplasts (10). Recently, Shepard (13) was able to regenerate virus-free tobacco plants after mass isolation of a large population of mesophyll protoplasts from plants systemically infected with potato virus X. Healthy tobacco plants also have been regenerated from callus cells previously infected with virus (3). These results, combined with the findings that well-defined areas known as "dark-green islands" of tobacco leaves systemically infected with tobacco mosaic virus (TMV) contain little or no virus (1, 2), prompted us to develop a novel method of obtaining virus-free plants. Specifically, we have demonstrated that virus-free plants can be recovered by regenerating leaf tissue excised from these visually recognizable, disease-free areas. A brief report has been presented (9).

Leaves of *Nicotiana tabacum* 'White Burley', inoculated with a Vulgare strain of TMV developed characteristic mosaic symptoms described as dark-green islands surrounded by a yellow-green background (1). The leaves were surface-sterilized with 95% ethanol, air-dried, and then explants were taken from the leaves. Small (approx. 1 mm^2) pieces were removed from the central region of the dark-green islands and placed on Linsmaier and Skoog's (LS) culture medium containing 3 mg/liter indole-3-acetic acid (IAA) and 0.3 mg/liter kinetin which stimulated cellular proliferation (8). Similar explants were initiated from yellow-green tissue of infected leaves and from normal, healthy leaves of noninfected plants. After several weeks, the tissue pieces were transferred to LS medium containing 0.3 mg/liter IAA and 10.0 mg/liter (δ,δ-dimethylallylamino)-purine which induced the formation of shoots and subsequently to one containing 1 mg/liter IAA which induced the formation of roots. Plantlets were recovered from 84% of dark-green island tissue originally plated. Similarly, plantlets were regenerated from 90% of both yellow-green areas and from healthy leaf tissue. When regenerated plantlets were assayed for the presence of TMV using leaves of Xanthi-nc tobacco and of *Phaseolus vulgaris* 'Pinto', plantlets derived from the yellow-green regions all displayed crystalline viral inclusions and indexed positively for TMV. Plantlets derived from healthy leaves were uniformly inclusion and virus-free. Of 24 plantlets regenerated from dark-green islands, 11 showed obvious mosaic symptoms and crystalline viral inclusions. Indexing these plantlets confirmed that they were all TMV-infected. The remaining 13 plantlets were symptomless and were found to be TMV-free after indexing. Phenol extraction (12) of these virus-free plants failed to show the presence of any defective virus RNA as did extraction with the high-pH, Tris-phosphate buffer-bentonite method (6, 11). The TMV-free plants derived from dark-green islands were successfully transplanted to soil and remained virus-free to maturity. Hence, it is possible to regenerate virus-free plants from TMV-infected tobacco leaves. We would expect that these techniques would be useful for deriving virus-free plants in a wide range of materials which display similar dark-green islands in virus-induced mosaic and for which the necessary in vitro manipulations have been defined. Our work confirms the previous findings (1) that many cells within dark-green islands have little or no virus. This fact makes possible the regeneration of virus-free plants.

None of the virus-free plants recovered in this work is genetic mutations to virus resistance, since all could be easily infected with TMV. There was, however, evidence for a transitory inhibition of viral multiplication in leaves of virus-free plantlets derived from dark-green island leaf tissue. This phenomenon was not observed in leaves of plantlets regenerated from healthy tissue. The inhibition was most striking in the first few leaves produced by the

TABLE 1. Comparative tobacco mosaic virus (TMV) multiplication in detached leaves of tobacco plants regenerated from dark-green islands (G) from TMV-infected plants and from healthy tissue (H)

Time from initiation (months)	Total lesions TMV Strains								
	Vulgare			U-1			U-5		
	G	H	Inhibition (%)	G	H	Inhibition (%)	G	H	Inhibition (%)
2	0	344	100	0	180	100	0	246	100
2-1/2	137	226	39	116	93	. . .	48	127	62
3-1/2	537	562	. . .	. . .	. . .	. . .	504	500	. . .

[a]Assayed on eight half-leaves of pinto bean with 1-50 dilution of tissue samples.

regenerated plantlet. When three strains of TMV (Vulgare, U-1, and U-5) were used to inoculate leaves from plantlets regenerated two months previously from dark-green islands, a 100% inhibition in virus multiplication was noted (Table 1). In leaves from older plantlets, the degree of inhibition was less (39-62%) and the effect was finally lost (no inhibition) in more mature plants.

The inhibition could also be demonstrated in sap extracted from leaves of plantlets regenerated 2.5 months previously from dark-green islands. Sap was obtained by grinding leaf tissue in 0.1 M phosphate buffer pH 7 (1/10, w/v), mixed with 12 μg/ml of TMV, and assayed on leaves of Pinto bean. Opposite leaves were treated identically except that sap from a similar-aged control plantlet was utilized. A 34% reduction in lesion number was noted with both the Vulgare and U-1 strains, whereas a 68% inhibition was observed for the U-5 strain. After the more mature regenerated plants were transplanted in soil, sap was extracted, mixed with virus, and again tested, but no inhibitory effect was evident. This inhibitory effect which appears to be temporary in nature could be related to the "as yet unidentified diffusable agent" postulated by Atkinson and Matthews (1). The inhibitory effect in the dark-green islands of intact tobacco plants seems to last for the life of the plant (1). In our tests, however, when dark-green islands were removed from the leaves and grown separately, the inhibitory effect in leaves of regenerated plantlets was of a transitory nature. This suggests that the infected tissue surrounding dark-green islands may be essential to the production of the hypothesized virus-inhibiting agent. Progressive loss of inhibition in new leaves of regenerated plantlets could be explained by dilution of the factor.

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