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Multiplication of tobacco mosaic virus in tobacco callus tissues and in vitro selection for viral disease resistance

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ABSTRACT

Tobacco mosaic virus-resistant tobacco was selected in vitro using callus tissues induced from axillary buds of systemically infected tobacco plants. Callus lines in which the virus was continuously multiplying were first isolated and redifferentiated into shoots. By the procedure, non-diseased, healthy shoots were successfully isolated from diseased shoots, which showed typical mosaic symptoms of the virus, and regenerated into intact plants. These regenerated plants showed resistance to virus inoculation, and selfed progeny of virus-resistant regenerants segregated the resistance and susceptibility according to the Mendelian system.

INTRODUCTION

Somaclonal variation involved in plant tissue cultures has been utilized for improving crop plants [Larkin and Scowcroft 1981]. One of the most effective applications of this methodology was the production of disease resistant plants. Especially, the selection of pathotoxin-resistant cells was useful for producing new cultivars resistant to diseases caused by the pathogens which produce these toxins as pathogenicity or virulence factors [Brettell and Ingram 1979]. However, there are many plant diseases, where these factors have not been clear, and therefore the selection for disease resistance has been difficult or, even if possible, not so effective. Viral diseases of plants are also included in this criterion.

Resistance for viral disease was isolated by Murakishi and Carlson [1982], who induced callus tissues from mutagenized haploid tobacco plants and selected tobacco mosaic virus (TMV)-resistant tobacco by inoculating regenerants with this virus. In general, it has been known that somaclonal variation can be frequently induced in plant tissue cultures even when mutagens are not used [Shepard et al. 1980, Evans and Sharp 1983]. Actually, the authors succeeded in isolating bacterial wilt-resistant tomato from non-mutagenized tomato callus cultures [Toyoda et al. 1989]. The authors have tried to devise effective selection methods for viral diseases in plants. In the present paper, the authors describe a relatively easy and efficient selection method for TMV resistance using tabacco callus lines infected with TMV.

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MATERIALS AND METHODS

TMV Inoculation

Thirty-day-old seedlings of tobacco (Nicotiana tabacum cv. Bright Yellow) developing four fully-expanded leaves were used for an inoculation with TMV (strain OM). Lowest-positioned leaves of tobacco were gently rubbed with an inoculum solution (1 mg TMV and 0.2 mg abrasive, 600 mesh carborundum, in 1 ml of water) and rinsed with water. Inoculated plants were grown in a green house until the systemic mosaic symptoms of TMV appeared.

Callus Culture

Axillary buds of inoculated plants were harvested, surface-sterilized, and cultured on Murashige-Skoog (MS) [1962] medium supplemented with 0.5 μg/ml 2,4-dichlorophenoxyacetic acid and 0.01 μg/ml kinetin, adjusted to pH 5.6, and solidified with 0.8 % agar (A-medium). Callus tissues were subcultured for several passages, and the friable callus tissues were excised and gently shaken in liquid MS medium (without agar). Cell suspensions were filtered with a stainless steel sieve (pore size; 250 μm in diameter). Filtrates containing 3- to 5-cell-aggregates were mixed with an equal volume of A-medium (1.6 % agar) kept at 49–50 ℃, poured into a Petri dish, and incubated at 26 ℃ for 1 month under a constant illumination of 4,000 lux.

Plant Regeneration

Shoot formation from callus tissues was achieved by the method previously reported [Toyoda et al. 1985a]; Callus tissues derived from the aggregates were transferred to B-medium where plant hormones of A-medium were replaced with

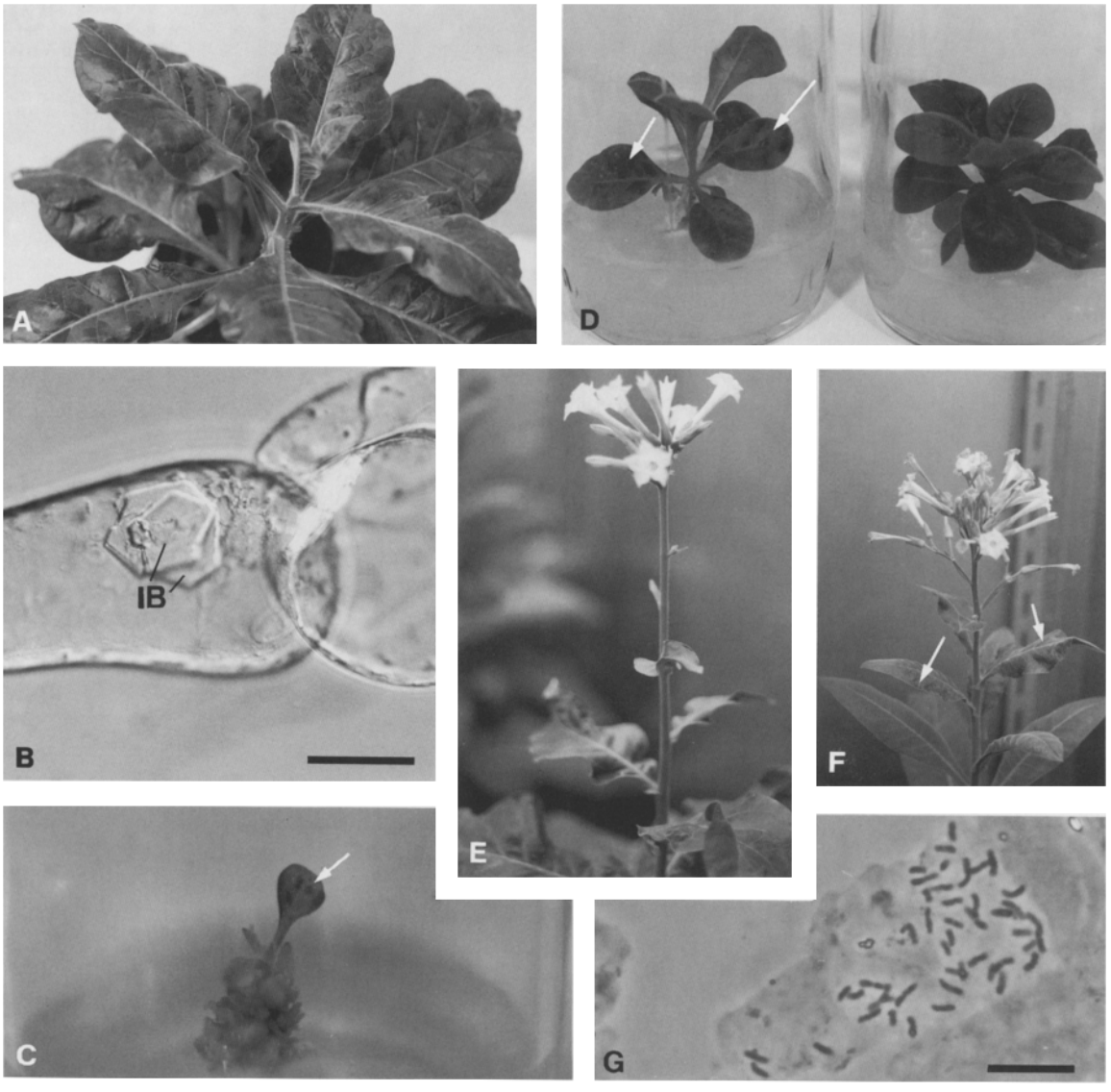

Fig. 1. Selection for TMV resistance from tobacco (N. tabacum cv. Bright Yellow) callus lines (CMT-1) containing high levels of TMV.
A; Tobacco plant showing severe mosaic symptoms of TMV in whole leaves (1 month after inoculation), B; Inclusion body (IB) of TMV formed in callus 3-cell-aggregate of tobacco, C; Mosaic symptom (arrow)-showing leaflet of shoot differentiated from IB-carrying aggregate (CMT-1) (7 days after transfer to the medium for shoot formation), D; Mosaic symptom (arrows)-showing (left) and symptomless, healthy (right) shoots (20 days after transfer), E; Highly resistant regenerant, CMT-1R03, (3 months after transplantation to soil), F; Moderately resistant regenerant, showing the symptom in upper, young leaves (arrows) (3 months after transplantation), G; Metaphase chromosomes of root tip cell of highly resistant regenerant, CMT-1R03. Normal numbers (2n = 48) of chromosomes were counted. Bar represents 20 μm.

0.05 μg/ml 3-indoleacetic acid and 1 μg/ml kinetin, and cultured for 20-30 days. The shoots formed were transferred to C-medium (hormone-free MS medium) for root initiation, after green leaflets were well developed.

Estimation of TMV Amounts

TMV amounts in callus tissues or plant leaves were estimated using a quantitative immunoelectrophoresis ('Rocket' method) previously described [Toyoda et al. 1983]; Sample materials were homogenized with 10 parts of water, and homogenates were clarified by centrifugation (3,000xg, 20 min). Supernatants were applied to an electrophoresis gel containing fluorescein isothiocyanate-conjugated anti-TMV-antibody and electrophoresed. The amount of TMV in each sample was determined from the migration distance as compared with the distances noted with known concentrations of TMV.

RESULTS AND DISCUSSION

Cytological Observation

In the present study, callus tissues were induced from axillary buds of tobacco systemically expressing mosaic symptoms of TMV, as shown in Fig. 1A. Callus tissues used in the present study were friable and cell-aggregates were easily released by gently shaking the tissues with liquid A-medium. The aggregates were collected by filtration, embedded in solid A-medium and observed by an Olympus differential phase contrast microscope. Fig. 1B shows typical inclusion bodies (IB) of TMV formed in the aggregates. IB was detected in about 30 % of observed aggregates, which consisted of 3-4 cells. The aggregates carrying IB were specified by their locations on the grid lines scored on bottom surface of a Petri dish.

Selection of Callus Lines Containing High TMV Levels

After 1 month of incubation, callus tissues originated from specified aggregates were separately transferred to fresh A-medium and subcultured for three passages at an interval of 14 days. Fifty calli lines were randomly picked and the amounts of TMV were estimated before and after subculturing. According to the patterns of change of TMV-amounts, callus lines were subdivided into two groups; i) in which higher level of TMV amounts (1.2-1.8 mg per gram fresh weight of callus tissues) were constantly maintained during subculturing (CMT callus lines), and ii) in which the amounts were gradually lowered when the tissues were repeatedly subcultured. In the following study, CMT callus lines were used for a selection of TMV resistance.

Plant Regeneration and Selection for TMV Resistance

CMT-1 callus line (highest amounts of TMV) was transferred to B-medium for shoot formation. In this line, almost all of differentiated shoots (49 of 50 shoots) developed small leaflets with typical mosaic symptom of TMV as shown in Fig. 1C. These results indicate that TMV was stably multiplied in callus tissues and efficiently translocated to leaflets regenerated from the tissues. Judging from these results, the authors considered that if TMV resistance is induced in callus cells, shoots differentiated from these cells would develop healthy leaflets, and that TMV-resistant plants would be easily and effectively isolated by selecting healthy shoots. In this experiment, CMT-1 callus tissues were subcultured for 6 months at an interval of 14 days in order to enrich the efficiency of somaclonal variation and multiply the variant cells. Fig. 1D shows diseased and healthy shoots obtained from the CMT-1 callus line. The authors selected healthy shoots (first step for selection), transferred to C-medium for root formation, and then transplanted to soil. After 30 days of cultivation, symptomless, healthy regenerants were selected as putative TMV-resistant plants (second step for selection). For confirming TMV resistance of these plants, they were inoculated with TMV (third step for selection). Table 1 shows the numbers of plants selected in each step. By the present selection, finally 3 highly resistant (no symptom) (CMT-1R) (Fig. 1E) and 33 moderately resistant (delayed symptom) (Fig. 1F) regenerants were successfully obtained from 105 shoots firstly selected.

Table 1. Selection of TMV-resistant plants from regenerants of tobacco callus lines, CMT-1

Selection steps[1)]	No. of plants	
	Diseased	Healthy
1st	967	105
2nd	14	91
3rd	58	3(33)[2)]

[1)] Shoots were differentiated from tobacco (N. tabacum cv. Bright Yellow) callus line (CMT-1), and symptomless healthy shoots were selected for TMV resistance (first step). Healthy shoots were grown up to intact plants and cultivated for further 1 month (second step). At the third step, selected regenerants were inoculated with TMV and grown for 3 months till seeds were harvested.

[2)] Regenerants showing delayed mosaic symptoms (at the flowering stage 3 months after inoculation).

For an analysis of chromosome numbers, root tips of TMV-resistant regenerants (CMT-1R03) were fixed, stained with 1 % acetocarmine, and squashed for microscopic observation according to the procedures of the previous report [Ogura 1976]. As shown in Fig. 1G, these plants showed the normal numbers of metaphase chromosomes (2n = 48).

For evaluating the effectiveness of this selection, the authors examined whether TMV resistance acquired in the regenerants would be passed to their progeny. In this experiments, therefore, selfed progeny (R2 plants) of highly resistant regenerants (CMT-1R03) were inoculated with TMV and the segregation of resistance and susceptibility was examined. Table 2 shows the numbers of both resistant and susceptible plants determined after inoculation with TMV. The data suggest that TMV resistance is probably due to a dominant single gene mutation, and this mutation was heterozygously induced in CMT-1 callus line. Further genetical analysis for TMV resistance will be described in a following paper.

In the present study, the authors also examined the multiplication and translocation of TMV inoculated into CMT-1R03 or control tobacco plants (noncultured R2 plants). Inoculated and non-inoculated leaves were harvested separately 1 month after inoculation, and used to estimate TMV concentrations. In control tobacco plants, all of 31 plants inoculated showed mosaic symptoms first in non-inoculated younger leaves 7-10 days and in whole leaves 20-25 days after inoculation. On the other hand, about 80 % of inoculated R2 plants (24 of 31 plants) did not show any symptom in either inoculated or non-inoculated leaves. In these symptomless R2 plants, the levels of TMV were considerably limited in inoculated leaves and, in non-inoculated ones, the levels were below the detectable

Table 2. Segregation of TMV resistance in selfed progeny of TMV-resistant lines (CMT-1R03)

Experiments	No. of selfed progeny inoculated with TMV		Ratio	χ^2	p
	Resistant	Susceptible			
1	29	14	3:1	1.30	0.62-0.88
2	39	9	3:1	1.00	0.63-0.87
3	44	18	3:1	0.58	0.64-0.86

limitation of this method (Fig. 2). These results indicate that resistant plants did not completely suppress the multiplication of virus in inoculated leaves, but inhibited the translocation of TMV from inoculated to non-inoculated leaves.

The authors reported the possible application of microinjection technique for the introduction of TMV into single cells [Toyoda et al. 1985b] or cell-aggregates [Toyoda et al. 1986] of callus tissues. Our preliminary results using this technique showed that callus cell-aggregates obtained from this resistant line permitted TMV to multiply in injected cells, but not to move to adjacent cells of the aggregates, whereas TMV moved and multiplied in both cells in controls. These data strongly support, at the cellular level, the similar mechanism for suppressing the cell-to-cell movement of TMV. Detail analysis for the resistance mechanisms will be reported in a following paper.

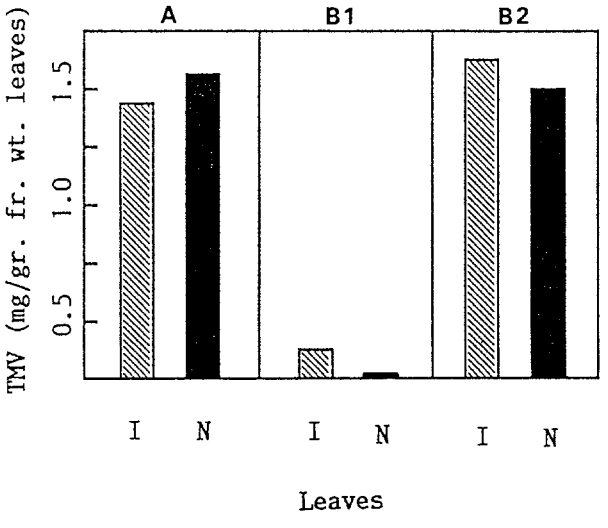

Fig. 2. Estimation of TMV amounts in control tobacco (A) and selfed progeny of TMV-resistant regenerant (CMT-1R03).
Each 31 plants were inoculated with TMV, and inoculated (I) and non-inoculated (N) leaves were harvested separately 1 month after inoculation and used to estimate TMV amounts by quantitative immunoelectrophoresis. TMV amounts in CMT-1R03 were shown separately in 24 symptomless (B1) and 7 symtom-appearing plants (B2).

One of the most important devices in the present system was to isolate the callus line which expressed TMV mosaic symptoms in leaflets when redifferentiated into shoots. This enabled us to easily and effectively isolate TMV-resistance mutation caused in callus cultures by selecting symptomless shoots, without any selection pressure. Thus, the present method may be widely applicable to the selection of resistance for other viral diseases causing mosaic symptoms in host plants.

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