Proc. Indian natn. Sci. Acad. B47 No. 5 pp 694–699 (1981)

Isolation of Auxotrophic Mutants in Somatic Cell Cultures of *Nicotiana tabacum*

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(Received 21 July 1981)

Auxotrophic mutants provide useful material for genetical studies. Isolation of auxotrophs in higher plants has been seriously hampered by lack of availability of suitable techniques. The BUdR enrichment technique of Puck and Kao (1967) followed by Carlson (1970) and Zyrd (1976) has not been found successful by other workers (Aviv & Galun 1977). A number of auxotrophs have been reported following other techniques (Savage et al. 1979). In the present study, five auxotrophs were isolated from the EMS-treated haploid cell cultures of *Nicotiana tabacum* (var. Wisc. 38) by using selective media for screening the mutants. The auxotrophs isolated were: (1) biotin$^-$ (2) thiamine$^-$ (3) pyridoxine$^-$ (4) Nicotinic acid$^-$ and (5) folic acid$^-$. Whole plants were regenerated from the variants and studied cyotologically.

Key words : *Nicotiana tabacum*, **Haploid cells, EMS, Induced mutations, Auxotrophic mutants, Vitamin B Group**

Introduction

Auxotrophic mutants in higher plants are of special interest as they provide ideal material for physiological and genetical studies. They provide useful genetic markers for the isolation of the somatic hybrids through complementation. The first report of isolation of auxotrophic mutants in cell cultures of a higher plant came from Carlson in 1970 who isolated six leaky mutants in cell cultures of a haploid *Nicotiana tabacum* by applying the BUdR enrichment technique of Puck and Kao (1967). Reports of auxotrophic mutants in higher plants are few. The BUdR enrichment technique has, subsequently, not been found to be applicable by other workers (Aviv & Galun 1977 Savage et al. 1979). Müller and Grafe (1975) isolated auxotrophs for nitrate reductase by selecting for chlorate resistance.

A number of thiamine HCl auxotrophs have been reported (Widholm 1977) in *Arabidopsis* and *Lycopersicon*. Savage et al. (1979) have reported a calcium pantothenate auxotrophic cell-line in *Datura innoxia* using a technique which they have called as the "non-selective screening procedure".

The present study was aimed at isolating auxotrophic mutants in haploid cell cultures

of *N. tabacum* (var. Wisconsin 38) using techniques other than the BUdR enrichment with a view to developing genetic markers for studies in somatic cell fusion.

Material and Methods

Fully expanded leaves of the androgenic haploids of *Nicotiana tabacum* (var. Wisconsin 38) growing in the glass-house were used. Protoplasts were isolated by treating peeled leaf segments with an enzyme mixture containing 1% macerozyme and 2 % cellulase dissloved in 12% mannitol at *p*H 5.5 and incubated for 3 hr at 30°C in dark. The washed protoplasts were plated in liquid medium of Ohyama and Nitsch (1972) in petri dishes of 5.0 cm diameter. The cultures were initially incubated in dark at 25°C.

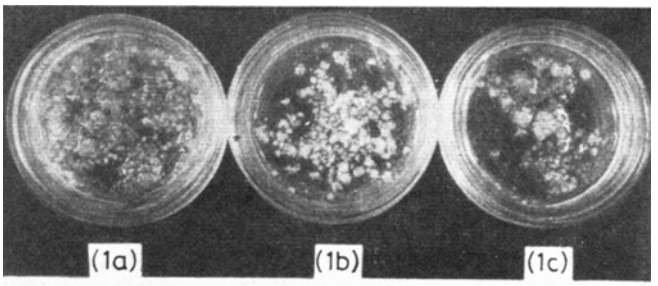
As the protoplasts formed colonies, the cells were periodically examined cytologically using Kao's (1975) carbol fuchsin staining procedure. Micro-colonies obtained from the protoplasts were pooled together and treated with Ethylmethane sulfonate (EMS) doses of 0.025%, 0.03% and 0.1% for 6 hr in shake cultures. The mutagen solution was removed by straining the suspension through a fine muslin cloth. The cells were resuspended in the liquid Ohyama and Nitsch's (1972) medium, hereafter referred to as CM medium. Washing of the cells was done by straining the suspension through the fine cloth and resuspending in the medium. The process was repeated thrice and finally cells were suspended in the same medium and distributed in a number of petri plates. (CM consists of all the ingredients of ON medium including thiamine HCl, biotin, pyridoxine HCl, folic acid and nicotinic acid). After the colonies had grown bigger in size (about 4.0 mm in diameter) they were transferred individually in tubes containing solid Ohyama and Nitsch's medium without vitamins, hereafter referred to as minimal medium or MM. The colonies were occasionally observed for growth. Some of the colonies did not show any growth while others grew normally. After six weeks the colonies which had practically not grown on MM were transferred to CM agar medium individually in separate tubes. When these colonies grew into callus masses in about 8-10 weeks each callus mass was divided into 10 small portions and transferred individually to tubes containing five types of media, each medium having four vitamin 'B' components and lacking in one. The five media were as follows:

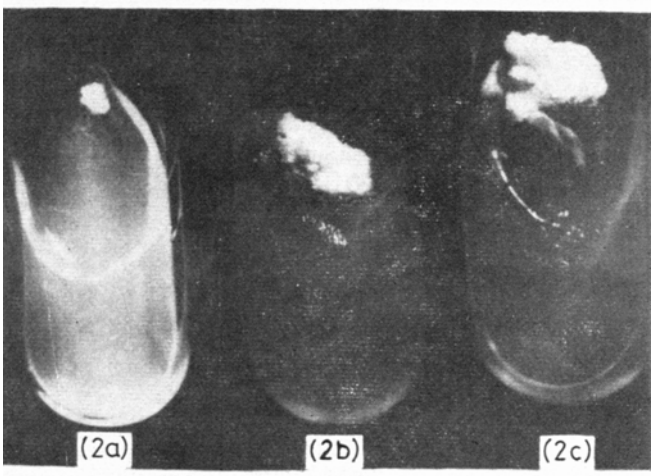
Complete medium: (1) lacking in thiamine HCl (i.e., thia$^-$), (2) devoid of biotin (i.e., boi$^-$), (3) devoid of pyridoxine HCl (i.e., pyr$^-$), (4) lacking in nicotinic acid (i.e., NA$^-$) and (5) lacking in folic acid (i.e., FA$^-$). Growth pattern of the callus pieces on these different types of media was followed. Colonies which did not show growth even after 8 weeks of incubation were picked up and further tested for the vitamin requirement by plating pieces from them on MM and MM + required vitamin and comparing growth pattern of the calli on the supplemented and unsupplemented media. Growth of the variants was compared with the growth of the untreated calli growing on MM.

The process of screening was repeated for 10 sub-cultures. The plantlets differentiated during the course of sub-culture were cytologically examined by the leaf tip squash technique of Burns (1964).

Experimental Results

Colonies treated with 0.05% EMS were chosen to screen for mutations, since, lethality in this case was around 40% — out of 340 treated colonies 212 had survived (figure 1). On transferring these colonies to the MM, 14 colonies showed complete arrest of growth whereas others continued to grow well on this medium (figure 2). Since MM





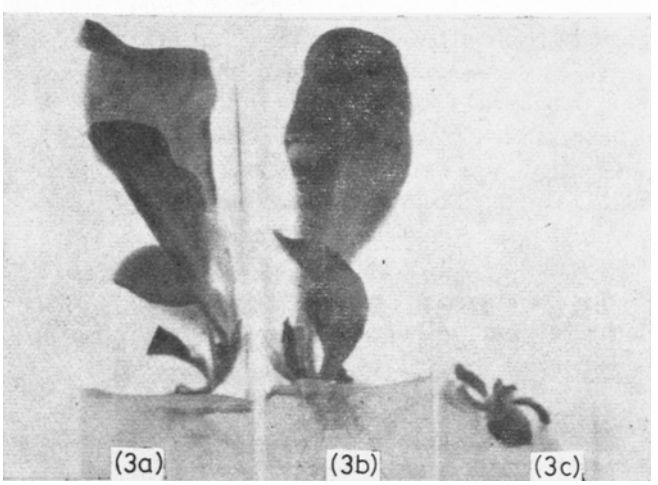


Figure 1 Survival of colonies after four weeks of treatment with EMS. (a) Untreated, (b) Treated with 0.5% EMS, (c) Treated with 0.1% EMS

Figure 2 Growth of auxotrophic callus on minimal medium (MM). (a) Variant callus on MM, (b) Variant callus on complete medium, (c Untreated control callus on MM

Figure 3 Auxotrophic plantlet. (a) Control plantlet on MM, (b) Auxotroph on supplemented medium, (c) Auxotroph on MM

lacked the five components of vitamin 'B' complex group it was evident that the 14 non-growing colonies were comprised of cells which had acquired auxotrophy for one or more of the five vitamin components. On transferring to CM these 14 colonies resumed growth, further confirming the vitamin auxotrophy of these cells. After these colonies had grown into a sufficiently big callus mass, each one of them was divided into a number of small portions and cultured individually on five types of media, the medium in each case lacking one of the vitamin components. Callus pieces were also transferred on the CM.

On comparing growth of the callus pieces on deficient media with that on CM and control calli growing on CM, 14 calli were found to show little or no growth on different deficient media. This indicated that each of the 14 callus pieces had at least one auxotrophy for the specific vitamin component, the deficiency of which arrested its growth. In this experiment, we were thus able to identify at least one auxotroph in each variant.

Callus pieces from each of the slow growing calli were sub-cultured on MM, and MM+ the suspected specific vitamin requirement and their growth compared with the control calli on MM. In this way multiple auxotrophs were screened out. Calli which had multiple auxotrophy did not show comparable growth on media having a single vitamin supplement and were, therefore, not taken into account.

In this manner 9 calli possibly showing single auxotrophy were isolated. Of these, 3 required thiamine, 2 biotin, one folic acid, 2 nicotinic acid and one pyridoxine. During the course of sub-cultures the calli got differentiated and thus plantlets with different kinds of vitamin auxotrophies were obtained. Growth of the variants was very much stunted on the MM but on the supplemented medium growth was as good as in the case of control, as observed three weeks after subculture (figure 3).

Differences in growth pattern within the variants were also observed. Of the three thia- variants two differentiated readily into complete plantlets, while the third grew very slowly and took much longer time to differentiate.

Out of the two bio$^-$ variants one showed slightly better growth on MM than the other. The leaves of these variants were more elongated and narrower. Differences in growth between the two NA$^-$ variants were not very apparent as they showed almost similar pattern of growth. In pyr$^-$ variant, apart from growth inhibition on MM, slight change in leaf morphology was observed. The leaves of the variant were broader than the normal plant.

Ploidy status of the auxotrophic variants

Cytological examination of the auxotrophs revealed that out of 9 variants 6 were haploids and the other 3 diploids. Among the haploids were the 3 thia$^-$, 1 bio$^-$, 1 NA$^-$, and 1 FA$^-$ while 1 bio$^-$, 1 pyr$^-$ and 1 NA$^-$ diploids.

Discussion

Isolation of auxotrophic mutants in plant cells is rather difficult, primarily because of non-availability of a suitable selection technique. The BUdR enrichment technique of Puck and Kao (1967) used by Carlson (1970) has not been found to be workable by other workers (Aviv & Galun 1977, Savage et al. 1979). Moreover, the complexity of the genome of a higher plant prevents easy detection of such mutations. Auxotrophy produced as a result of mutation may not result in inhibition of growth on MM, since, the required substance or another similar substance may still be synthesised by other genes elsewhere in the genome though in lesser quantities (Carlson 1970, 1973). However, auxotrophic mutants could be isolated by comparing growth of calli from mutagenized cells with that of controls on the MM. To avoid growth differences for reasons other than auxotrophy, use of media having specified requirement besides MM followed by repeated screening for a number of cell-generations could be of help. Only those cells which consistently exhibit mutant character should be taken as possible mutants and others rejected.

Isolation of a number of auxotrophic mutants in haploid cell-cultures of *N. tabacum* in the present study indicates that it is possible to isolate auxotrophic mutants following this technique. Savage et al. (1979) have followed a more or less similar technique and isolated a calcium pantothenate auxotrophic cell line in haploid cells of *Datura innoxia.* They have called the technique as 'non-selctive' screening procedure.

Widholm (1977, 1978) reported the frequent occurrence of thiamine auxotrophy in experiments with *Arabidopsis* and *Lycopersicon.* Our studies seem to substantiate this observation, since in our experiments the number of thiamine auxotrophs is highest. The reason for this could perhaps be that the response of tissues to thiamine in vitro is very much marked so that the growth differences arising as a result of thiamine deficiency get easily detected.

In our experiments, differences in the growth and morphogenesis between similar variants could be due to multiple mutations. The other mutation could be another auxotrophy as well. During the course of screening, occasionally a suspected auxotroph showed normal growth on the MM. This could have happened for the following reasons: (1) the initial inhibition of growth on MM might have been due to reasons other than a mutation for auxotrophy, (2) unmutated cells growing in association with the mutated cells could have overtaken mutated cells in growth and thus finally exhibited normal growth in course of time, and (3) there could be back

mutations resulting in revertant cells, which appears to be unlikely, because, it would involve simultaneous back mutation in a number of cells. Of the other two possibilities either could be responsible for normal growth of the 'mutant' callus. However, in the present study all normally growing 'mutant' calli were rejected.

Ploidy status of the variants

Cells in culture are known to exhibit instability in their chromosome numbers (Carlson 1973, Iyer & Raina 1972, Smith 1974, Street 1974, Mathews & Vasil 1975, Widholm 1978). Cytological examination of the variants revealed that six out of nine variants were haploids as also the control plants. Occurrence of haploids in high frequency indicates that change of ploidy status of the cells started quite late so that at the time when differentiation commenced majority of the cells were haploid. This could be due to frequent sub-cultures which were necessitated for testing the mutant nature of the cells. Diploidy in some variants could be due either to the presence of diploid cells in the initial cultures or subsequent duplication occurring during culture. The change of ploidy in cultured cells is generally attributed to endomitotic divisions of the cells (Iyer & Raina 1972). In our other experiments we have observed occurrence of diploids and triploids apart from haploids in *Datura innoxia* from anther culture in very early stages of embryoid formation (Gupta & Smith unpublished). Collins et al. (1972) reported the occurrence of polyploid and aneuploid cells in haploid plant tissues. Thus occurrence of polyploids and aneuploids in early stages suggests formation of embryoids from pre-existing polyploid cells. Collins et al. (1972) have suggested the possibility of tapetal cells taking part in embryoid formation. However, polyploid cells have been found to occur in haploid cell cultures in the present study. The possible mode of origin of such cells could be endomitosis.

The fact that a wide range of auxotrophic variants with haploid chromosome complement could be isolated indicates that auxotrophic mutants can be isolated in plant cell cultures using techniques described in this paper.

Acknowledgement

The authors express their thanks to Dr B R Murty, Project Director, Nuclear Research Laboratory, Indian Agricultural Research Institute for his interest in this study.

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