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The development of a negative selection system for the isolation of plant temperature-sensitive auxin auxotrophs

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Summary. A protocol has been developed for the negative selection of plant auxotrophs using the nucleoside analogues BUdR and FUdR. The protocol was optimised using nitrogen-starved protoplast-derived cells of *Nicotiana plumbaginifolia* to simulate auxotrophy. The present results represent a significant improvement over previous reports in that: 1) The background of colonies escaping BUdR/FUdR kill is low and reproducible. 2) The protocol was improved to the point where background survival was 0.03% for non-starved cultures and 0.09% for auxin-starved cultures. 3) It was shown that UV irradiation decreases BUdR sensitivity of dividing cells and that this is overcome by increased exposure to BUdR. 4) Application of the method to auxin-starved haploid protoplast-derived cell suspensions resulted, for the first time, in the selection of temperature-sensitive (ts) auxin auxotrophs. 5) It could be demonstrated, for the first time, that the method in practice enriches for auxotrophs, in this case by a factor of 10 for auxin auxotrophs and at least 60 for ts auxin auxotrophs.

Key words: Auxin – Auxotrophs – BUdR – Enrichment – Temperature sensitivity

Introduction

One strategy for the isolation of auxotrophic and other conditional-lethal mutants from plant cell cultures, for which there are no replica plating methods, is negative selection. Toxic compounds are introduced into cell populations cultured under conditions where wild-type cells remain metabolically active and are killed, but the mutants become quiescent and survive. When such cultures are freed of the drug and cultured further, the fraction of mutants amongst the surviving colonies should be enriched and the mutants easier to identify by colony testing.

Several preliminary studies have shown that BUdR and/or FUdR can be used to discriminate between dividing and non-dividing plant cells (Zyrd 1976; Strauss et al. 1978; Shillito et al. 1981). Reconstruction experiments with BUdR (Zryd 1976; Shillito et al. 1981) and another enrichment agent, arsenate (Horsch and King 1985), applied to several different plant cell variants, suggested that enrichment factors of $\times 20-60$ were theoretically possible.

BUdR and arsenate were subsequently applied in attempts to isolate plant conditional-lethal mutants in vitro (Malmberg 1979; Horsch and King 1983; Shimamoto and King 1983; Negrutiu et al. 1985). However, although variants were isolated, in no case was it evident that enrichment occurred. The yields of variants per colony tested in these experiments are essentially not different to the yields obtained by "total isolation", i.e. by testing colonies without prior enrichment (Savage et al. 1979; Gebhardt et al. 1981; Sidorov et al. 1981; Blonstein et al. 1988 a, b). To determine whether an enrichment protocol has served its purpose, it is necessary to know the frequency of variants in the population before treatment. Without this information, calculations of "enrichment factors", for example by Negrutiu et al. (1985), are erroneous.

The reasons for the discrepancy between reconstruction experiments and the actual application of en-

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Abbreviations: BUdR: 5-bromodeoxyuridine; FUdR: 5-fluorodeoxyuridine; MNNG: N-methyl-N'-nitro-N-nitrosoguanidine; NAA: 1-naphthaleneacetic acid; BAP: 6-benzylamino-purine; CFE: colony forming efficiency; PE: plating efficiency; ts: temperature sensitive

richment agents for mutant isolation is not clear. In some of the experiments described above, a selection protocol with a particular enrichment agent was optimised using one type of variant. It is by no means certain that the same protocol would be efficient in enriching for other variants with different metabolic defects. For example, an agent acting through DNA replication (like BUdR) might enrich for different variants than an agent acting through protein synthesis (like an amino-acid analogue). King (1985) has shown that different plant cell auxotrophs react quite differently to the same enrichment agents.

Nitrogen-starved plant cell cultures cease DNA replication within hours and become immune to BUdR exposure (Strauss et al. 1978). Auxin starvation of an auxin-auxotroph isolated by total isolation (Blonstein et al. 1988 b) also rapidly results in cessation of replication and in protection from BUdR (Fracheboud and King, unpublished results). The present paper describes the optimisation of a BUdR/FUdR enrichment protocol for haploid protoplast-derived suspensions of *Nicotiana plumbaginifolia* using the nitrogen-starvation model, and the subsequent isolation of auxin auxotrophs and temperature-sensitive (ts) auxin auxotrophs following enrichment of 10→60 fold. This is the first report of the selection of ts auxin auxotrophs in plants. Together with auxin resistant mutants and other auxin auxotrophic variation previously isolated, ts auxin auxotrophs will strengthen the genetic approach to the outstanding problems of auxin biology in plants.

Materials and methods

Haploid plants

A haploid shoot culture of *N. plumbaginifolia*, kindly supplied by J.-P. Bourgin, CNRA, Versailles, France, was maintained in sterile conditions by subculture of axillary shoots on B medium (Bourgin et al. 1979) without hormones and illumination with Osram L30W/36-2 Natura lamps at ca. 1500 lux during an 11 h light period ("standard light conditions"). The ploidy of shoots was checked before use by counting guard cell chloroplast number (haploid = 3.80 ± 0.05; diploid = 6.21 ± 0.12).

Culture media

Filter sterilized K3 medium (Kao et al. 1974) was used for protoplast culture with the following modifications: K3G – sucrose 0.4 M, NAA 16.2 μM, BAP 4.4 μM; K3H – glucose 0.4 M, NAA 16.2 μM, BAP 4.4 μM; K3D – glucose 0.2 M, NAA 1.1 μM, BAP 0.88 μM. Autoclaved RMC medium, used for callus stock cultures and for colony plating, is a modification of the RM medium described by Bourgin et al. (1979) with NAA 2.7 μM, BAP 2.2 μM. K3 medium without nitrogen was prepared by omitting KNO_3, NH_4NO_3 and $(NH_4)_2SO_4$, and adding KCl at 1.34 mM.

Hormone solutions

BAP was dissolved in 2 M KOH and diluted with water to give a stock solution of 0.22 mM. NAA was dissolved in ethanol and diluted with water to make a stock solution of 0.25 mM (maximum ethanol in the medium 0.001% v/v). Stock solutions were stored at 4 °C.

Protoplast isolation and culture

The procedure for protoplast isolation from haploid shoots was as described by Blonstein et al. (1988 a) except that enzyme treatment and initial culture were carried out in liquid media K3G and K3H, respectively. The yield varied between 1 and 15×10^6 protoplasts per g leaf tissue. The protoplasts were cultured at a density of 4×10^4 ml^{-1} in 5 ml aliquots at 26 °C, for the first 4–7 days in the dark and thereafter in standard light conditions. Under these conditions the CFE was ca. 2%.

UV mutagenesis

Protoplasts were irradiated immediately after isolation with a dose of 1000 ergs mm^{-2} at a wavelength of 254 nm using the procedure described by Blonstein et al. (1988 a). This treatment reduced the CFE by ca. 70%.

Culture dilution and medium changes

Cultures were diluted 10 fold by transferring suspensions of developing colonies to centrifuge tubes, spinning down the cells (ca. $500 \times g$), resuspending in K3D medium and replating. Transfer of cultures to auxin- or nitrogen-deficient media for prestarvation was performed in the same way.

BUdR and FUdR

Fresh aqueous solutions of BUdR and FUdR (both from Fluka) were prepared for each experiment at ×100 the final medium concentration. After treatment, cultures were washed free of the analogues by centrifugation and resuspension twice in RMC medium containing nitrogen, auxin and 40 μM thymidine, plated in the same medium solidified with agar, using the three-layer plating technique described by Strauss and King (1981), and cultured further at 26 °C.

CFE and PE determination

To determine the effects of mutagenesis on protoplast division (CFE), and of BUdR/FUdR treatments on the survival of treated cells (PE), colonies were washed and plated as above. CFE was calculated from the number of dividing colonies present on plates of treated cells compared to control plates without mutagen treatment. PE was calculated from the number of dying and dividing colonies on each plate (Strauss and King 1981).

Callus cultures

Stock cultures were subcultured at four-week intervals on RMC medium at 26 °C in standard light conditions.

Basic enrichment protocol

In the initial experiments on BUdR/FUdR enrichment (Fig. 1 and Table 1) the following timetable was followed:

Day 0 – protoplast isolation and mutagenesis
Day 14 – culture dilution 1 : 10 and transfer to 33 °C for acclimatisation
Day 17 – 1) experiments with no pre-starvation: transfer to fresh medium with or without nitrogen and addition of BUdR or FUdR; 2) pre-starvation experiments: transfer to fresh medium with or without nitrogen
Day 19 – 1) experiments with no pre-starvation: removal of BUdR/FUdR and plating; 2) pre-starvation experiments: addition of BUdR or FUdR

Day 21 – Removal of BUdR/FUdR from pre-starvation experiments and plating
Day 28–35 – PE determination.

Results and discussion

Sensitivity to BUdR or FUdR

Changes, in the PE of protoplast-derived micro-colonies after transient (48 h) exposure to increasing concentrations of BUdR and FUdR are shown in Fig. 1. The analogues are both toxic at high concentrations, the cells being more sensitive to FUdR than BUdR by one order of magnitude.

Transfer of dividing colonies to minus-nitrogen medium at 33 °C was used to simulate the cessation of growth of a temperature-sensitive auxotroph. The analogue sensitivity of nitrogen-starved cells decreases by two orders of magnitude, both when starvation is imposed simultaneously with analogue treatment (Fig. 1 A, B) and when cells are pre-starved for 48 h prior to analogue addition (Fig. 1 C, D). The results for the non-starved controls in Fig. 1 C and D suggest that the extra two-day incubation at 33 °C itself led to decreased analogue sensitivity.

Separation between the response of dividing and non-dividing cells most acceptable for a potential mutant isolation protocol would be that seen with BUdR concentration of 10^{-5} M to 10^{-4} M after pre-starvation (Fig. 1 D). However, under these conditions the background of surviving non-starved cells is too high (3–5%) for efficient enrichment of auxotrophs. In a typical mutant isolation experiment using this protocol, several thousand wildtype colonies would escape BUdR killing and hamper mutant identification. Treatment with 2×10^{-5} M BUdR for 48 h without pre-starvation (Fig. 1 B) was chosen for further investigation, using the argument that a 50% loss of mutant colonies with zero background is preferable to 100% retention of mutants in a high background.

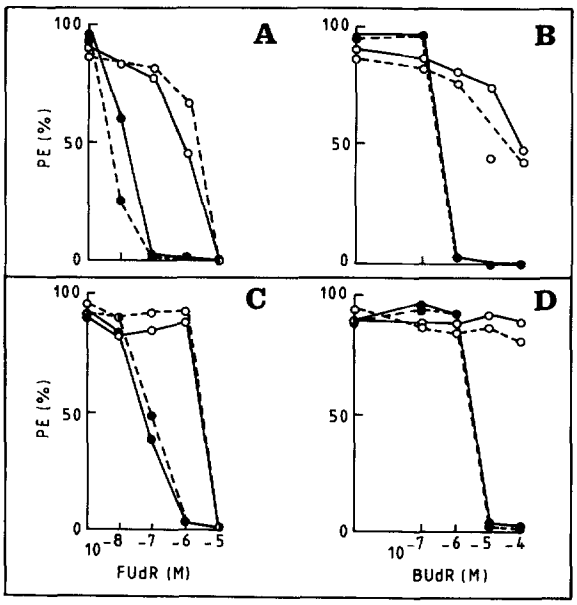

Fig. 1. The effects of BUdR or FUdR treatment on the plating efficiency of protoplast-derived colonies with or without nitrogen prestarvation. The cultures all received three days pretreatment at 33 °C before *either* transfer to fresh medium at 33 °C with (●) or without (○) nitrogen and immediate addition of FUdR (*A*) or BUdR (*B*) (no prestarvation), *or* transfer to fresh medium at 33 °C with (●) or without (○) nitrogen for two days before addition of FUdR (*C*) or BUdR (*D*) (prestarvation). The analogues were removed after 48 h in all cases and the cultures replated in nitrogen-containing medium. The data in each case are from two parallel, replicate experiments

Reproducibility

In the experiments shown in Fig. 1, two replicate series of cultures derived from the same batch of protoplasts responded very similarly to the analogue treatments, showing that the protocol itself is reproducible. Shimamoto and King (1983) reported considerable variation in the BUdR sensitivity between different batches of leaf protoplasts of *H. muticus*, and large variations are commonly seen in yield, CFE and growth rates between protoplast preparations made at different times and from different individual plants. To test the reproducibility of the basic protocol with leaf protoplasts of *N. plumbaginifolia*, the effect of 2×10^{-5} M BUdR for 48 h without pre-starvation was tested on six independent protoplast preparations (Table 1).

The variation observed between these preparations, both in the killing of dividing cells and in the protection of starved cells, is relatively small and acceptable. However, whilst the predicted protection level of ca. 50% afforded by starvation was achieved, the mean background survival of cells that continued to divide was ca. 1% and not 0% as expected from the results given in Fig. 1 B.

UV treatment decreases BUdR sensitivity

A further unexpected result of the experiments shown in Table 1 was that the mean background of dividing colonies surviving BUdR exposure was higher in populations irradiated with UV. This observation has subsequently been confirmed in other experiments, where it was also shown that another mutagen, MNNG, had no effect on background BUdR survival (Yvan Fracheboud, unpublished). The reason for the UV effect is not known, but it may be that prolonged UV-induced excision repair of DNA or elevation of DNA precursor pools reduces

Table 1. The sensitivity to BUdR of independent haploid *N. plumbaginifolia* protoplast preparations with and without prior UV treatment. The UV dose was 1000 ergs mm^{-2} and the BUdR treatment was for 48 h at 2×10^{-5} M incubated at 33 °C

Experiment no.	Protoplast yield 10^6/g	CFE %	UV kill %	BUdR survival (%)			
				No UV		After UV	
				+N	−N	+N	−N
12	1.2	1.8	39	0	40	3.0	51
14	7.1	2.9	54	0	6	0	50
15	1.9	0.6	60	4.0	41	15.0	33
17	7.4	5.2	51	0	18	7.0	31
18	9.8	7.2	nd	1.7	44	20.0	53
19	11.2	[a] 8.0	76	0	59	5.0	64
		[b] 4.0	nd	0	68	nd	nd
Mean	6.4	4.2	56.0	0.8	39.4	8.3	47.0
SEM	1.6	1.0	6.1	0.5	8.2	3.1	5.2

[a] Standard protoplast density (4×10^4 ml^{-1}) in 5 ml culture
[b] Increased protoplast density (12×10^4 ml^{-1}) in 10 ml culture
nd: no data

the number fo BUdR residues stably intercalated. This phenomenon would clearly be a problem for mutant isolation using UV as mutagen, but as there are significant practical advantages in the use of UV, further steps were undertaken to reduce the background.

Treatments reducing the background

There are many variables in the protocol and many of them were tested. The following combined changes were most effective in reducing the background without reducing protection:

1) shortening the exposure to 33 °C; 2) including pre-starvation; 3) prolonging the exposure to BUdR; 4) using higher BUdR concentrations; 5) applying BUdR and FUdR together.

The revised protocol

The following protocol was accepted as the basis for mutant isolation experiments:

Day 0 – protoplast isolation and mutagenesis
Day 7–12 – culture dilution 1:2 to pre-starvation medium and transfer to 33 °C
Day 9–14 – addition of analogues
Day 12–17 – removal of analogues and plating at 26 °C.

This protocol using BUdR (10^{-5}–10^{-4} M) plus FUdR (1–5.10^{-6} M) applied after UV irradiation gave 67–98% protection to nitrogen-starved cells and a background of ca. 0.03%. Searching for auxin auxotrophs under these conditions from a starting population of 20×10^6 protoplasts, approximately 50 colonies would theoretically survive the analogue treatment, of which six would be auxotrophs, given the auxin auxotroph frequency of 10^{-4} shown by Blonstein et al. (1988 b) using the total isolation method. The theoretical enrichment of auxotrophs in the population using this BUdR negative selection principle would, therefore, be 1000 fold. However, losses of auxotrophs due to crossfeeding and variable BUdR kill would be expected to reduce yields. The frequency of ts auxin auxotrophs to be expected was not known at the beginning of these experiments (but see below).

Auxin starvation

The revised protocol was tested in parallel on both nitrogen- and auxin-starved cultures (Fig. 2). As expected, killing on control non-starved plates (Fig. 2C) was high; in the experiment illustrated 0.03% of treated colonies survived. Nitrogen-starved populations (Fig. 2B) were afforded substantial protection (67% survival), whereas auxin-starved populations (Fig. 2A) were almost entirely eliminated (0.09% survival). The few colonies arising on the latter plates are clearly of great interest.

Mutant isolation

The optimised protocol with various minor modifications has since been applied in our laboratory to many independent protoplast preparations starved of auxin at 33 °C in attempts to isolate ts auxin auxotrophs. To date,

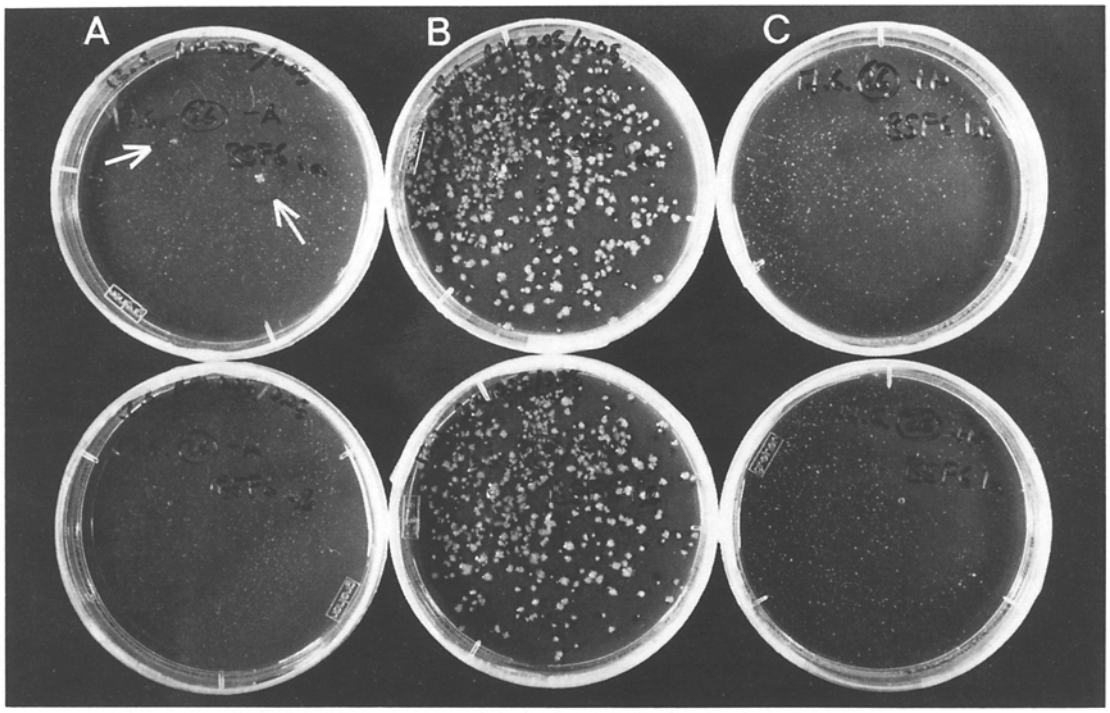

Fig. 2. Colony growth after application of the optimised protocol to cultures prestarved of auxin or nitrogen. After two days prestarvation of auxin (*A*) or nitrogen (*B*), or culture in complete medium (*C*), cells were treated for three days with BUdR (10^{-5} M) plus FUdR (10^{-6} M), then washed and plated in complete medium. Colonies growing on 'A' plates were picked, grown up and tested for ts auxin auxotrophy

at least 13 auxin auxotrophs, and 7 ts auxin auxotrophs have been obtained after testing a total of 13,700 colonies surviving BUdR/FUdR treatment (Martin Suter and Yvan Fracheboud, papers in preparation). This is an underestimate of the variants produced as not all auxin auxotrophs were followed up and only the most stable lines are included. Taking the data for total isolation of auxin auxotrophs (Blonstein et al. 1988 b), where five auxotrophs were found after testing 56,000 *N. plumbaginifolia* colonies, the enrichment factor for auxin auxotrophs after BUdR selection in our present experiments appears to be at least $\times 10$. A single ts auxin auxotroph was found by Gebhardt et al. (1981) after individually testing about 60,000 colonies of *Hyoscyamus muticus*, but no such variants were found amongst the 56,000 colonies tested by Blonstein et al. (1988 b). Thus, the enrichment for the ts auxin auxotrophs now obtained by negative selection must be at least $\times 60$. The frequency of such auxotrophs in the enriched population was ca. 5×10^{-4}, which would suggest an apparent mutation frequency of ca. 8×10^{-6} per cell.

The variant cell cultures, and plants now regenerated from them, should allow new, interesting questions to be asked about auxin metabolism and the possible role of auxin in plant growth and development.

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