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Use of Amino Acid Analogue-resistant Cell Lines for Selection of *Nicotiana sylvestris* Somatic Cell Hybrids*

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Summary. *Nicotiana sylvestris* cell lines resistant to the amino acid analogues S-2-aminoethyl-cysteine (AEC^R), or 5-methyl-tryptophan ($5MT^R$), were isolated in suspension culture. Assuming these resistances to be dominant, we have attempted to determine if such variant cell lines can be used to select double resistant somatic cell hybrids. A total of 1.8×10^4 control calli from mixed AEC^R and $5MT^R$ protoplasts, and AEC^R and $5MT^R$ homokaryotic fusions were placed on double analogue selection, but none survived. Eight somatic hybrid calli (0.8%), able to grow without inhibition on the double analogue selection medium, were obtained after AEC^R + $5MT^R$ protoplast fusion. These were further determined as hybrids on the basis of resistance level, chromosome number, and chlorophyll content, all characteristics differing in the parental cell lines.

Key words: *Nicotiana sylvestris* – Somatic cell hybridization – Amino acid analogue – Resistance

Introduction

A variety of selection systems have been used for the isolation of somatic cell hybrids and hybrid plants. The growth of hybrid cells on a medium where the parents were unable to sustain growth made possible the selection of hybrid plants of *Nicotiana glauca* + *N. langsdorffii* (Carlson et al. 1972; Smith et al. 1976; Chupeau et al. 1978), and of *Petunia hybrida* + *P. parodii* (Power et al. 1976).

Complementing chlorophyll-deficient mutants have been used to select green, wild-type, intraspecific hybrids of *Nicotiana tabacum* (Melchers and Labib 1974; Gleba et al. 1975) and *Datura innoxia* (Schieder 1977), and interspecific hybrids of *Nicotiana tabacum* + *N. sylvestris* (Melchers 1977). A variation of this method using one chlorophyll-deficient parent and the other green but unable to regenerate, has produced hybrids of *Petunia hybrida* + *P. parodii* (Cocking et al. 1977), *Datura innoxia* + *D. stramonium* and *Datura innoxia* + *D. discolor* (Schieder 1978a). A similar method was used for isolating somatic hybrids of *Nicotiana tabacum* + *N. knightiana* (Maliga et al. 1978), and of *Daucus carota* + *D. capillifolius* (Dudits et al. 1977), by employing a secondary screening for shoot initiation and morphological markers, respectively.

Complementary auxotrophic mutants have been used to produce autotrophic wild-type hybrids after fusion of their protoplasts, e.g., in *Sphaerocarpos donnelli* (Schieder 1974), *Physcomitrella patens* (Grimsley et al. 1977a,b) and *Nicotiana tabacum* (Glimelius et al. 1978).

Somatic hybrid plants were also isolated after fusion of protoplasts of a kanamycin resistant cell line of *Nicotiana sylvestris* and sensitive *N. knightiana*. The parents lacked the potential to form shoots under the experimental conditions used although morphogenic potential was restored in the somatic hybrids (Maliga et al. 1977).

A number of variant cell lines resistant to the amino acid analogues, 5-methyl-tryptophan (Widholm 1972, 1974), or S-2-aminoethyl-cysteine (Chaleff and Carlson 1975; Widholm 1976) have been isolated previously. Assuming these resistances to be dominant, we have attempted to determine if such variant cell lines can be used to select double resistant somatic cell hybrids. To test this hypothesis an intraspecific somatic hybridization of *Nicotiana sylvestris* was attempted using variant cell lines resistant to 5-methyltryptophan (5MT) and S-2-aminoethyl-cysteine (AEC).

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Methods and Materials

Petioles of diploid *Nicotiana sylvestris* Speg. & Comes. were used for callus initiation. Both callus and suspension cultures were grown using Linsmaier and Skoog's (1965) medium containing 0.4 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.03 mg/l kinetin. Suspension cultures of cell lines used in this study were maintained in 250 ml Erlenmeyer flasks on a gyrotory shaker (120 rpm). Ten ml of cell suspension was diluted with 40 ml of fresh medium at four-day intervals. Both callus and suspension cultures were incubated at 25°C in the dark.

Growth studies on suspension cultures were made using 0.8 g fresh weight inoculum in 40 ml of fresh medium, grown for 10 days. Four replicates were made of each treatment. Fresh weight increases were determined after collecting cells on a Millipore absorbant filter pad by vacuum filtration. Cell counts were made using the method of Henshaw et al. (1966).

Growth of normal *Nicotiana sylvestris* suspensions was totally inhibited by either 10 μg/ml of AEC (Calbiochem) or 5MT (Sigma) when the growth period was 10 days. Spontaneous resistant variants were isolated by incubating for 6-8 weeks, at which time a few small cell clumps appeared and proliferated. The cell lines selected for use (AEC^R or $5MT^R$) grew at normal rates when reinoculated into 10 μg/ml of their respective analogues, and were, thereafter, maintained at a higher cell density in 50 μg/ml of the analogue.

Protoplasts of the AEC^R and $5MT^R$ cell lines were isolated and cultured in the medium of Nagy and Maliga (1976), containing 250 mg/l glucose, 0.4 mg/l 2,4-D, 0.03 mg/l kinetin, and 0.4M sucrose as osmoticum. Cells of a four-day old suspension were collected on a 100 μm stainless stell filter and added to 10 ml of protoplast medium: 6% Cellulysin (Calbiochem), 4% Driselase (Kyowa Hakko), 1% Macerozyme (Yakult Biochemical), pH 5.6, to give a final volume of 20 ml per 50 ml Erlenmeyer flask, and incubated at 30°C for 5-6 hrs at 80 strokes/min in a water-bath shaker. Protoplasts were filtered through 100 μm and 50 μm stainless steel filters, and washed four times in the culture medium by centrifugation at 300 × g for 3 min. The floating protoplasts were collected and the number adjusted to ca 10^6/ml and mixed with an equal volume of 1.2% agar medium at 40°C to give final agar and protoplast concentrations of 0.6% and 5 × 10^5/ml, respectively. One ml of this suspension was overlayed on 5 ml of agar medium (1%) in a 60 × 15 mm Falcon plastic petri dish, sealed with Parafilm, and incubated at 28°C in continuous light of 500 lux.

For fusion and control experiments AEC^R and $5MT^R$ protoplast were mixed 1:1 after washing. Fusion was induced by a modification of the method of Kao (1977). To 400 μl of protoplast suspension in a plastic petri dish was added 600 μl of polyethylene glycol solution (PEG), as described by Kao (1977). A 22 × 22 mm glass cover slip was placed on top of the droplet so that the floating protoplasts adhered to its underside. After 25 min, 600 μl of a high calcium-high pH solution (50 mM $CaCl_2 \cdot 2H_2O$, 50 mM Na-glycine buffer, 0.4 M sucrose) was added slowly. After 15 min, 1 ml of the protoplast culture medium was added. The protoplasts were gently washed an additional 5 times, collected and washed twice by centrifugation, adjusted to 10^6/ml and plated. Controls included (a) plating 1:1 mixed AEC^R and $5MT^R$ protoplasts, and (b) fusing AEC^R + AEC^R protoplasts and $5MT^R$ + $5MT^R$ protoplasts separately, washing, and then mixing 1:1 before plating. After 30-60 days when the protoplasts had formed small calli, the top soft agar layer containing the calli was cut into 1 cm wide strips and transferred to the selection medium with reduced osmoticum (0.2 M sucrose) containing 200 μg/ml AEC and 30 μg/ml 5MT. Somatic hybrid calli were transferred to the selection medium only once, and were thereafter grown in the absence of analogues. Selection was made in ca 2000 lux continuous light.

Resistance levels of parental and hybrid calli were determined by transferring small callus pieces (30 mg) to agar medium (5 ml per 60 × 15 mm petri dish), incubating for 14 days, and recording the fresh weight increase. The values given represent the means of 16 replicates. Chlorophyll determinations were made using the method of Arnon (1949), after 14 days' growth.

Chromosome counts were made by squashing rapidly growing callus or suspension cells in 45% acetocarmine.

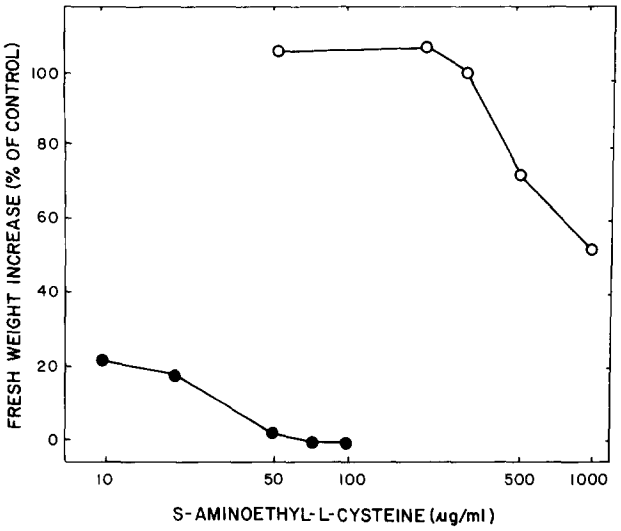

Fig. 1. Effect of S-2-aminoethyl-cysteine on the growth of AEC^R(o) and $5MT^R$(•) cells. Values are the increase of fresh weight given as a percentage of the same line grown in the absence of the analogue

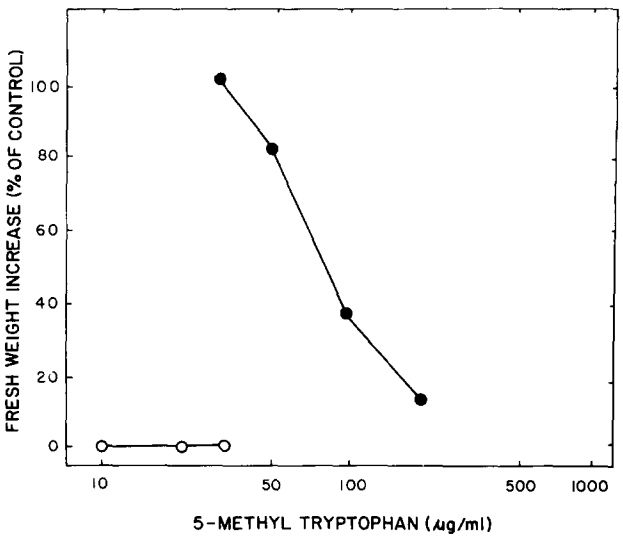

Fig. 2. Effect of 5-methyl-tryptophan on the growth of $5MT^R$(•) and AEC^R(o) cells. Values are the increase in fresh weight given as a percentage of the same line grown in the absence of the analogue

Results

1 Amino Acid Analogue-Resistant Cell Lines

Preliminary experiments demonstrated that the growth of suspension cultures was inhibited by 10 μg/ml of AEC. A total population of 6×10^6 cells was placed under selection and after 6 weeks a few small cell clumps proliferated in one of the three flasks. When tested for resistance, this cell line (AEC^R) showed normal growth in 200 μg/ml AEC (Fig. 1). The AEC^R cell line also showed other distinctive characteristics. Suspensions grown in the dark are bright yellow, in contrast to the normal dull white of the wild-type, and cell aggregates are more compact, and comprised of round cells. All these phenotypes were stable after cloning by protoplast culture.

A $5MT^R$ cell line was selected in 10 μg/ml 5MT from 10^7 cells, appearing 4 weeks after inoculation. Cells proliferated in one of the four flasks and were tested for resistance (Fig. 2). Growth of the $5MT^R$ cell line was not inhibited by 30 μg/ml 5MT, but was 60% inhibited in 100 μg/ml. This cell line demonstrates the wild-type colour and cell aggregate morphology, with a portion of the cells growing in chains. These characteristics – resistance, and lack of pigmentation – were stable through protoplast culture.

Normal sensitivity to 5MT is exhibited by the AEC^R cell line, but the $5MT^R$ line shows more resistance to AEC (Fig. 1), and requires 75 μg/ml for total inhibition. The AEC^R and $5MT^R$ cell lines used in this study are not auxin autotrophic (unpublished observations). Attempts to regenerate shoots from the two-year old AEC^R and $5MT^R$ cell lines using the RMO and RMB media of Maliga et al. (1977) have been unsuccessful.

2 Selection of Cell Hybrids

Approximately 40-70% of the AEC^R protoplasts divided by 10 days after plating and subsequently formed calli. However, $5MT^R$ protoplasts rarely formed calli, and only 1-5% did so when mixed with AEC^R protoplasts in control or fusion experiments. This could be determined due to the differences between $5MT^R$ and AEC^R protoplast callus morphology and pigmentation.

Calli from control experiments died when placed on the double-analogue selection medium, after 35-60 days' unchallenged growth (Table 1). In order to eliminate the possibility that larger calli might overcome the selection, some of the controls were grown for a longer period than fusion experiments, prior to selection. Plating density in control experiments was such that AEC^R and $5MT^R$ calli were observed immediately adjacent to each other, but even calli in this situation died at the same time as other control calli when placed on the selective medium. The results in Table 1 and this observation eliminates the possibility of any cross feeding providing 'double resistance'.

Of the 975 calli derived from three separate fusion experiments, only 8 calli (0.8%) were able to grow without inhibition on the double-analogue selection medium (Table 1). These calli were designated H1 through H8, and were characterized further to determine whether they were somatic hybrids. Growth of H1, H3 and H5, versus lack of growth of AEC^R and $5MT^R$ calli, is illustrated in Figure 3. All presumptive hybrid calli had a light green pigmentation, intermediate between the dark green AEC^R and the white $5MT^R$ cell lines (total chlorophyll mg/g fresh weight: AEC^R = 0.213, H2 = 0.106, $5MT^R$ = 0.0). Three of the selected calli (H1, H3 and H5) were further

Table 1. Fusion and control experiments with AEC^R and $5MT^R$ protoplast-derived calli

	Period of culture prior to selection (days)	Number of colonies screened	Number of calli selected in a double-analogue medium
Control Experiments			
Mixed AEC^R and $5MT^R$ protoplasts	60	5×10^3	0
Mixed AEC^R and $5MT^R$ protoplasts	45	10^4	0
AEC^R and $5MT^R$ protoplasts fused separately then mixed 1:1	35	3×10^3	0
Fusion Experiments			
AEC^R + $5MT^R$ protoplasts			
1.	35	656	6
2.	35	236	1
3.	35	83	1

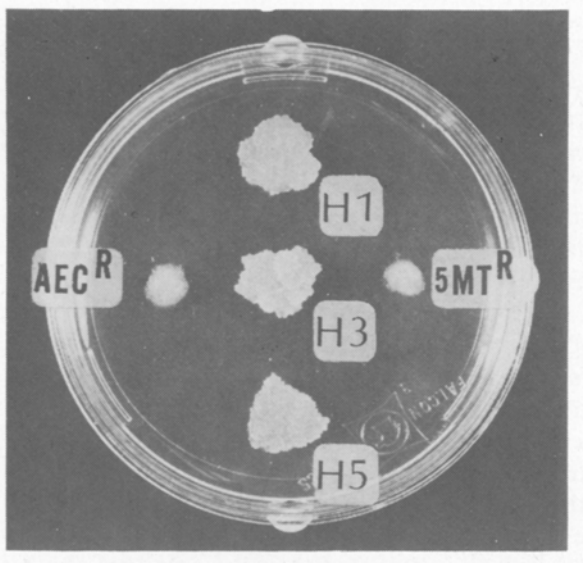

Fig. 3. Growth of parental calli (AEC^R and $5MT^R$) and hybrids H1, H3 and H5 on 200 μg/ml AEC and 30 μg/ml 5MT, after 14 days

studied to determine their levels of sensitivity to AEC and 5MT, compared with the parental cell lines. All three demonstrated AEC resistance curves very similar to the AEC^R parent; H3 and H5 are shown in Figure 4. The H1, and H3 calli have a 5MT resistance similar to that of $5MT^R$ at 30 μg/ml, but show a distinctly greater sensitivity at higher analogue levels (Fig. 5). A similar level of sensitivity was shown by H5 calli.

3 *Chromosome Numbers*

The parental cell lines used in this study, AEC^R and $5MT^R$, have distinct and non-overlaping differences in chromosome number. Suspension cells of the AEC^R line have 48 chromosomes (2n = 2x = 24), with no detectable variation in number (Table 2). In contrast, $5MT^R$ suspension cells have a 54-178 range in number, with a mode of 96. This variation is not continuous, only 6 different numbers being observed, i.e., 54 (16%), 72 (23%), 80 (10%), 96 (30%), 127 (13%), and 178 (3%). Chromosome numbers of some of the somatic cell hybrids, H1, H2, H3 and H5, were determined in callus cells 3-4 months after isolation. Three hybrids, H1 (216), H3 (223) and H5 (192), had numbers greater than either parental line, and the other, H2 (175), a number not detected in either parental type. A metaphase plate with 223 chromosomes is shown in Figure 6. Because of the difficulty in obtain-

Table 2. Chromosome numbers of parental and hybrid cell lines

Cell line	Number of cells observed	Chromosome number	
		Mode	Range
AEC^R	30	48	none
$5MT^R$	30	96	54-178
H1	3	216	*
H2	1	175	*
H3	1	223	*
H5	6	192	*

* = Number of cells observed insufficient to preclude range, see text

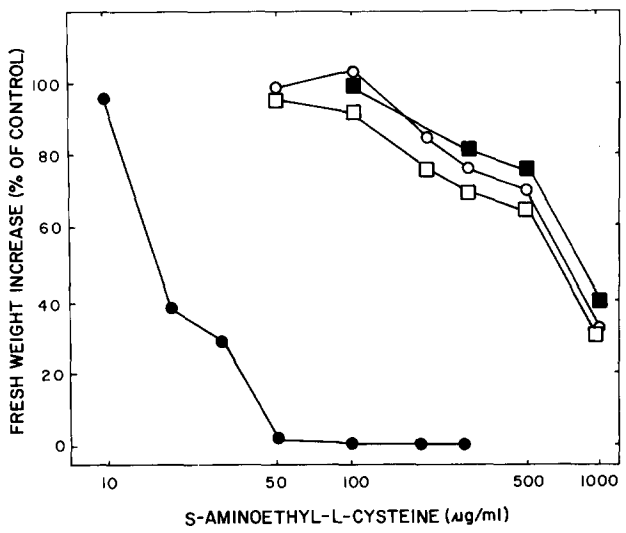

Fig. 4. Effect of S-2-aminoethyl-cysteine on the growth of AEC^R(○), $5MT^R$(●), and hybrid H3 (□) and H5 (■) cells. Values are the increase in fresh weight given as a percentage of the same line grown in the absence of the analogue

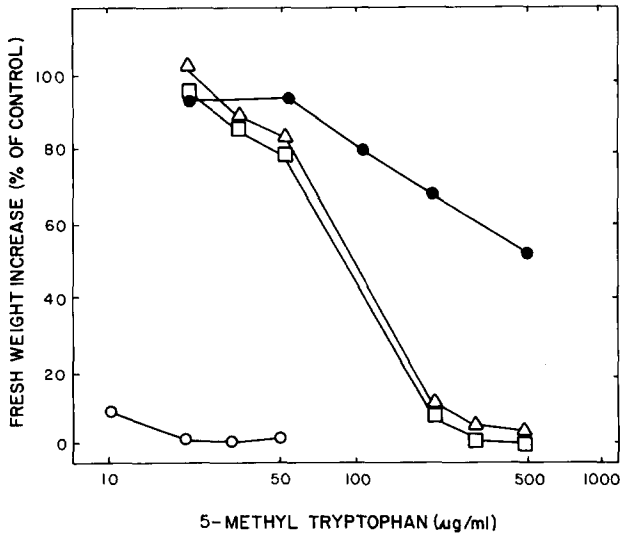

Fig. 5. Effect of 5-methyl-tryptophan on the growth of $5MT^R$(●), AEC^R(○), and hybrid H3 (□) and H1 (▲) cells. Values are the increase in fresh weight given as a percentage of the same line grown in the absence of the analogue

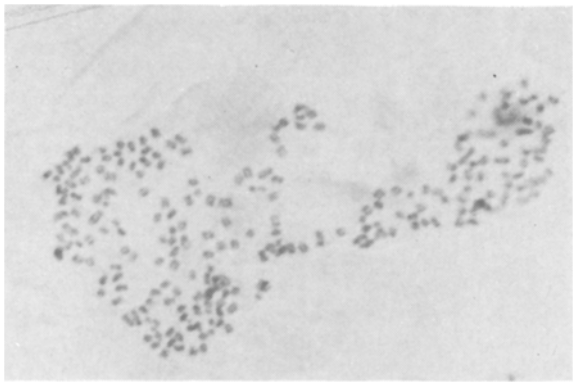

Fig. 6. Mitosis in a callus cell of H3. X1250

ing a large number of counts of such high chromosome numbers it has not been possible to determine whether or not variation exists in the cell hybrids.

Discussion

Previously, intraspecific hybridization in *Nicotiana tabacum* and *Datura innoxia* has been demonstrated definitively by segregation of complementary recessive mutations in the progeny (Melchers and Labib 1974; Schieder 1978b). Such segregation analysis is not applicable in our investigation due to the absence of plant regeneration. The conclusion that the 8 selected lines are somatic hybrids relies on their resistance to both AEC and 5MT (Figs. 4, 5). Resistance of H1, H3 and H5 to AEC, is very similar to that of the AEC^R cell line. Similar demonstrations in mammalian somatic cell genetics (Harris 1973; Baker et al. 1974) have been interpreted as evidence of dominance of the resistance phenotype. Resistance of these hybrid calli to 5MT is at an intermediate level between that of the $5MT^R$ and AEC^R cell lines, and could be characterized as semi-dominant expression.

High chromosome numbers in the selected lines (Table 2), and intermediate levels of pigmentation seem to support the somatic hybrid origin of the double-resistant isolates. Of the four hybrids examined, three (H1, H3 and H5) had numbers greater and one (H2) a number within the range of the $5MT^R$ line. These chromosome numbers can be explained in terms of additions of AEC^R and $5MT^R$ parents, and their subsequent modifications, known to occur in tissue culture cells (Sunderland 1973).

The main alternative explanation for selecting double resistant cells is the existence of double resistance within one of the parental cell lines. Therefore, a relatively large number (1.5×10^4) of calli from mixed AEC^R and $5MT^R$ protoplasts were examined in order to test and subsequently disprove this possibility. Appearance of new resistants at such a high frequency (0.8%) is very unlikely, and would be contrary to our own experience and other data in the literature (Widholm 1977; Maliga 1978). Also, the possibility that increase in ploidy level due to homokaryotic fusion might change the resistance expression was similarly eliminated by fusing the two parental cell lines separately, washing, and then mixing the protoplasts.

Evidence presented here suggests that dominant amino acid analogue-resistant cell lines can be used to select intraspecific somatic hybrids. Whether this selection system is applicable to interspecific or intergeneric somatic hybridization remains to be determined.

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