Theor Appl Genet (1986) 72, 11–14

Direct selection of cybrids by streptomycin and valine resistance in tobacco

J. P. Bourgin, C. Missonier and J. Goujaud

Laboratoire de Biologie Cellulaire, INRA, F-78000 Versailles, France

Received July 9, 1985; Accepted September 12, 1985
Communicated by P. Maliga

Summary. Direct selection of cybrids by simultaneous selection for "donor" chloroplasts and for the "recipient" nuclei is described. Mesophyll protoplasts of two tobacco (*Nicotiana tabacum*) mutants, SR1 (streptomycin resistant) and Val^r-2 (valine resistant), were fused by polyethylene glycol treatment. Streptomycin resistance in the SR1 mutant is a maternally inherited chloroplast trait while valine resistance is a Mendelian (nuclear) digenic recessive character. The fused protoplast population was cultured and colonies were selected for resistance to valine (1 mM) and streptomycin (343 μM). The efficiency of selection has been confirmed in three clones by demonstrating seed transmission of both streptomycin and valine resistances. In one subclone both streptomycin resistant and sensitive plants were obtained indicating that the streptomycin sensitive chloroplasts had not been totally eliminated by growth on the selective medium.

Key words: Cybrid – Streptomycin resistance – Valine resistance – Tobacco

Introduction

Protoplast fusion and subsequent segregation of nuclei and chloroplasts in the heterokaryons lead to the one-step transfer of chloroplasts from one parental line to another (Belliard et al. 1978; Glimelius and Bonnett 1981). This procedure results in cytoplasmic hybrids (or cybrids) with chloroplasts (and/or mitochondria) from the "donor" and a nucleus from the "recipient". If the nuclei fuse, somatic hybrids are obtained.

Elimination of the donor nuclei in the heterokaryons has been facilitated by irradiation of donor protoplasts with X- or gamma-rays before fusion (Zelcer et al. 1978; Sidorov et al. 1981; Menczel et al. 1982). Another alternative to prevent hybrid formation is the use of enucleated protoplasts or cytoplasts (Maliga et al. 1982). However, cytoplast preparations contain protoplasts and nuclei in X-irradiated protoplasts can be reactivated following fusion with untreated cells. In experiments designed to follow the fate of chloroplasts or to select for their transfer, these techniques were combined with the use of a donor line carrying a selectable cytoplasmic trait of resistance to streptomycin (Maliga et al. 1975) or lincomycin (Cséplö and Maliga 1982; Medgyesy et al. 1980; Menczel et al. 1983, Fluhr et al. 1983; Cséplö et al. 1984). Screening for antibiotic resistance does not, however, discriminate between cybrids and somatic (nuclear) hybrids. Consequently, in most instances, somatic hybrid clones were also obtained in addition to cybrids (e.g. Menczel et al. 1982).

In this paper we describe direct selection for cybrids by simultaneous selection for streptomycin resistant "donor" chloroplasts and for "recipient" nuclei displaying valine resistance, a recessive marker.

Materials and methods

Plant material

SR1, a streptomycin resistant line of *Nicotiana tabacum* cv. 'Petit Havana' was obtained by Maliga et al. (1973). Val^r-2, a valine resistant line derived from the wild type haplo-diploidized line XHFD8 of *N. tabacum* cv. 'Xanthi' nc was obtained in our laboratory (Bourgin 1978).

Protoplast preparation and fusion

Mesophyll protoplasts were prepared from the leaves of greenhouse-grown plants according to the method of Chupeau et al. (1978), except that the protoplasts were washed in the WO.6 salt medium of Meyer and Abel (1975). Protoplast fusion was induced by polyethylene glycol (PEG) essentially as described by Chupeau et al. (1978), with the following modifications: the PEG 6000 solution (20% (w/v)) was prepared in 0.245 M $CaCl_2$ and the pH was adjusted to 7.0 before autoclaving (Chupeau, personal communication). The fusion

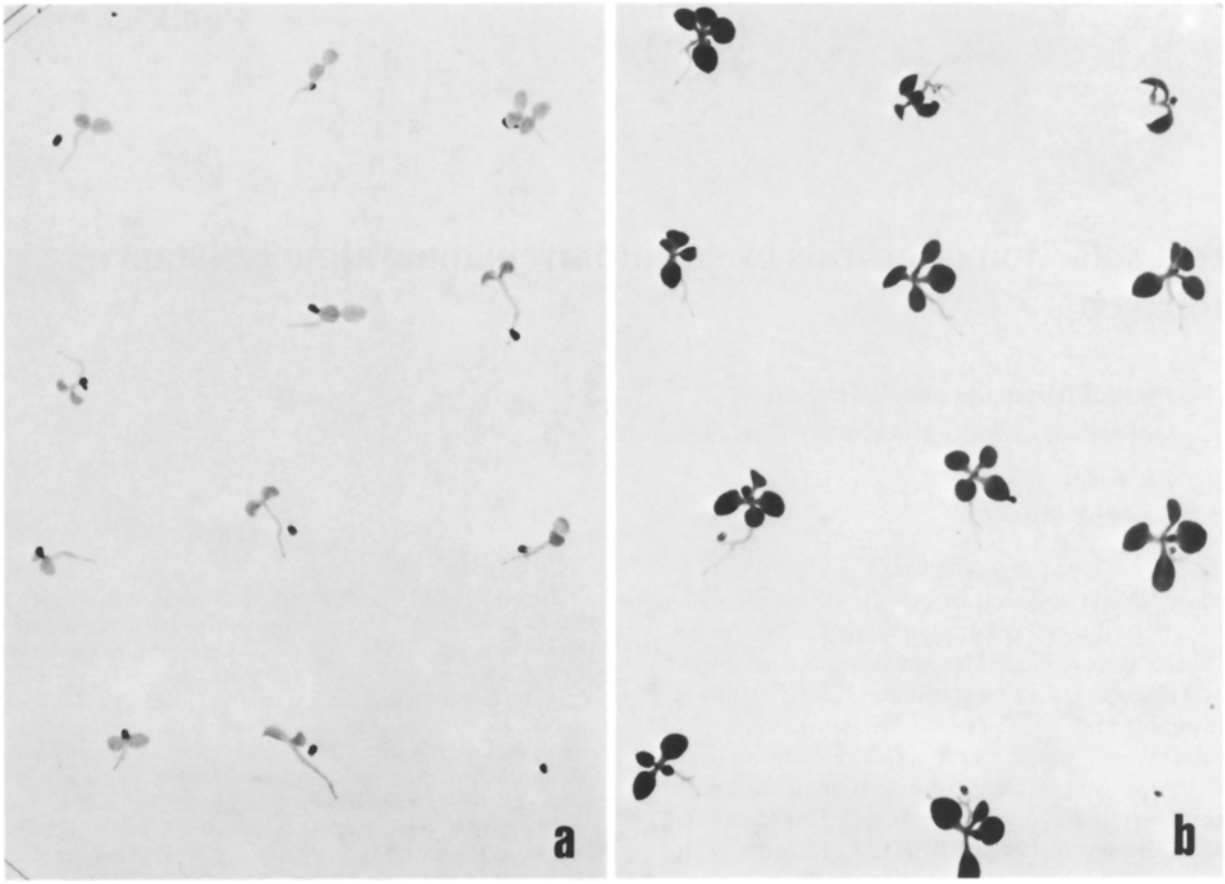

Fig. 1. Test for streptomycin resistance of seedlings obtained through self-fertilization of a plant of the Val^r-2 streptomycin-sensitive parental line (**a**) and of the A1-10 cybrid clone (**b**)

mixture was diluted after 30 min with 10 ml of medium TO, the medium in which the protoplasts were subsequently cultured.

Plant regeneration

Plants were regenerated from the selected colonies by inducing shoot formation on medium R4 and the resulting shoots were rooted on medium B (Bourgin et al. 1979).

Tests for resistance

Seeds of the various lines were germinated on medium B containing respectively 2 mM valine (0.234 g/l) or 0.69 mM streptomycin sulphate (1 g/l). These concentrations allow normal development of resistant seedlings whereas development of valine sensitive seedlings is arrested after germination (Bourgin 1978) and streptomycin sensitive seedlings are bleached (e.g. Medgyesy et al. 1980).

Streptomycin and valine resistance of protoplast-derived cells was determined by the ability to form colonies in medium T8 (100 cell/ml) supplemented with various concentrations of valine or streptomycin sulphate (Bourgin et al. 1980; Caboche 1980).

Results

Selection of clones resistant to streptomycin and valine

After 10 days of culture, protoplast-derived microcolonies were subcultured at an initial density of about 200 colony/ml in medium T8 supplemented with appropriate volumes of filter-sterilized stock solutions of valine and streptomycin sulfate. Two combinations of streptomycin and valine concentrations were devised from preliminary experiments (Table 1). Out of approximately 3×10^3 original microcolonies, 4 colonies were recovered 3 months later from plates of series A containing 0.5 mM valine and 343 µM streptomycin sulphate, whereas no colony was recovered from plates of series B containing the same concentration of streptomycin sulfate but 1 mM valine. One colony did not give rise to transferable shoots even after repeated subculture on medium R4. Plants were regenerated from the 3 other colonies (labelled A1 to A3).

Table 1. Effect of valine and streptomycin on the plating efficiency of protoplast-derived cells from the cybrid clone A1-10 and from the parental lines

Line		Relative plating efficiency (% of control)		
		SR1	Val^r-2	A1-10
Supplement: Control		100[a]	100[b]	100[c]
Valine (μM)	30	42	100	80
	100	0	111	84
	300	0	111	87
	600	0	91	76
	1,000	0	75	79
Streptomycin sulphate (μM)	34	32	81	77
	69	37	47	46
	172	24	6	21
	343	14	0	19
	687	0	0	0
Plating efficiency (% of cells forming colonies in the absence of selection pressure; mean of 3 replicates ±SD) (1,200 cells plated per series)		a: 13±2	b: 29±3	c: 25±4

Table 2. Inheritance of streptomycin and valine resistance in plants regenerated from the selected clones. Labeling of the plants derived from the selected clones (e.g. A1-1): the letter indicates the series in which the colony was obtained; the first numeral the code attributed to the colony from which the plant derived; the second numeral the code attributed to different shoots derived from the same colony

Cross	No. of seedlings			
	Sensitive to streptomycin	Resistant to streptomycin	Sensitive to valine	Resistant to valine
SR1 selfed	0	40	41	0
Val^r-2 selfed	45	0	0	46
SR1 × Val^r-2	0	48	50	0
A1-1 selfed	18	0	0	44
A1-3 selfed	0	42	0	39
A1-5 selfed	0	50	0	40
A1-6 selfed	0	47	0	35
A1-9 selfed	0	39	0	45
A1-10 selfed	0	37	0	44
A2-1 selfed	0	30	0	26
A2-2 selfed	0	17	0	35
A2-4 selfed	0	30	0	29
A3-1 selfed	0	35	0	34
A1-10 × Val^r-2	0	40	0	47
Val^r-2 × A1-10	42	0	0	45

Classification of the selected clones by the resistance of their progeny

Resistance of the clones was tested on progeny seedlings obtained from self-fertilization of regenerated plants. One plant was grown to flowering from clone A3 and several (labelled by the name of the clone, followed by a second numeral, e.g. A1-1) from independently regenerated shoots in the case of clones A1 and A2. Results of the progeny tests (Table 2, Fig. 1) showed that 9 plants transmitted both valine and streptomycin resistance, whereas the subclone A1-1 transmitted only valine-resistance. An additional test of the randomly chosen doubly resistant clone A1-10 confirmed the maternal inheritance of the streptomycin resistance trait. It was also confirmed that this trait was transmitted to the next generation through self-fertilization for all the doubly resistant clones. Plants of all these clones displayed a phenotype characteristic of the 'Xanthi' cultivar, which was expected from their Val^r-2 nuclear background. Resistance to valine and to streptomycin of the doubly resistant clone A1-10 was confirmed by a test with protoplast-derived cells (Table 1).

Discussion

Reconstruction experiments were not carried out to assess the efficiency of the selective procedure on mixed cell populations. Calculations based on the plating efficiencies observed in the study on the resistance to valine and to streptomycin of protoplast-derived cells of the doubly resistant clone A1-10 (Table 1), however, allow one to estimate the plating efficiency of these cells in selective series A as about 4%. Accordingly, the frequency of recovery of selected clones (4) corresponds to about 3% of the potential colonies under these conditions (120). This is much higher than the frequency of spontaneous mutation to streptomycin resistance which one might expect. For instance, in the control experiment described in Table 1 the frequency of streptomycin resistant colonies from protoplast-derived cells of the Val^r-2 line is less than 1/360. The doubly resistant clones can thus be considered as cybrids obtained through protoplast fusion followed by nuclear and chloroplast segregation. Loss of the streptomycin resistance trait in the A1-1 subclone could be ascribed to a segregation in favor of the sensitive chloroplast type during the subsequent culture step without selection for streptomycin resistance.

The feasibility of selecting colonies from protoplast-derived cells of the Val^r-2 line has been demonstrated in earlier studies (Bourgin et al. 1980; Grandbastien et al. 1985). In contrast, screening for the streptomycin resistance trait has previously been carried out at a later stage, based on the lack of bleaching on a streptomycin-containing medium rather than on the lack of growth inhibition (e.g. Medgyesy et al.

1980; Fluhr et al. 1983; Menczel et al. 1983), although differential response to streptomycin of the growth of SR1 and wild type seedling-derived calli had been reported (Maliga et al. 1975). In the present work, we carried out in a single step the selection for both valine and streptomycin resistance by growth at low cell density on selective media. Our results show that under these conditions streptomycin resistance can confer a slight but selectable growth advantage over the wild type.

Under the conditions used no nuclear hybrids were recovered in spite of the fact that according to previous experiments (Bourgin et al. 1982) cells heterozygous for the mutant alleles involved in the Val^r-2 trait displayed a slight resistance to valine. This result suggests that, after a treatment inducing chromosome loss in heterozygous hybrid cells (Roth and Lark 1984), selection for valine resistance could be used in experiments designed to determine the chromosomal localization of the loci involved in this trait (Bourgin et al. 1985).

Seedlings of the new streptomycin resistant lines obtained on the 'Xanthi' Val^r-2 background are indisputably resistant to streptomycin, since they stay green in the presence of a drug concentration that bleaches their wild type counterpart. Their growth, however, seems to be more affected by streptomycin than that of seedlings of the original SR1 line on the 'Petit Havana' nuclear background (results not shown). This difference is most probably due to the nuclear background rather than to putative differences in the mitochondrial populations, since growth of seedlings with a SR1 cytoplasm and hybrid 'Xanthi'×'Petit Havana' nuclear genome display a level of resistance to streptomycin intermediate between that of SR1 seedlings of the original 'Petit Havana' line and that of cybrid seedlings (data not shown). Conversely, Val^r-2 protoplast-derived cells displayed a decreased sensitivity to low doses of streptomycin than SR1 cells (Table 1). These observations point to the probable involvement of nucleus-chloroplast interactions and the type of culture on the response to streptomycin.

Acknowledgements. We thank Drs. Mark Tepfer and Pal Maliga for helpful criticism of the manuscript.

References

Belliard G, Pelletier G, Vedel F, Quétier F (1978) Morphological characteristics and chloroplast DNA distribution in different cytoplasmic parasexual hybrids of *Nicotiana tabacum.* Mol Gen Genet 165:231–237

Bourgin JP (1978) Valine-resistant plants from in vitro selected tobacco cells. Mol Gen Genet 161:225–230

Bourgin JP, Chupeau Y, Missonier C (1979) Plant regeneration from mesophyll protoplasts of several *Nicotiana* species. Physiol Plant 45:288–292

Bourgin JP, Hommel MC, Missonier C (1980) Expression of resistance to valine in protoplast-derived cells of tobacco mutants. In: Sala F, Parisi B, Cella R, Ciferri O (eds) Plant cell cultures: results and perspectives. Elsevier/North Holland Biomedical Press, Amsterdam, pp 167–177

Bourgin JP, Chupeau MC, Missonier C (1982) Amino acid-resistant plants from tobacco cells selected in vitro. In: Earle ED, Demarly Y (eds) Variability in plants regenerated from tissue culture. Praeger, New York, pp 163–174

Bourgin JP, Goujaud J, Missonier C, Pethe C (1985) Valine resistance, a potential marker in plant cell genetics. 1. Distinction of two types of valine-resistant tobacco mutants isolated from protoplast-derived cells. Genetics 109:393–407

Caboche M (1980) Nutritional requirements of protoplast-derived, haploid tobacco cells grown at low cell densities in liquid medium. Planta 149:7–18

Chupeau Y, Missonier C, Hommel MC, Goujaud J (1978) Somatic hybrids of plants by fusion of protoplasts. Observations on the model system "*Nicotiana glauca – Nicotiana langsdorffii*". Mol Gen Genet 165:239–245

Cséplö A, Maliga P (1982) Lincomycin resistance, a new type of maternally inherited mutation in *Nicotiana plumbaginifolia.* Curr Genet 6:105–109

Cséplö A, Nagy F, Maliga P (1984) Interspecific protoplast fusion to rescue a cytoplasmic lincomycin resistance mutation into fertile *Nicotiana plumbaginifolia* plants. Mol Gen Genet 198:7–11

Fluhr R, Aviv D, Edelman M, Galun E (1983) Cybrids containing mixed and sorted-out chloroplasts following interspecific somatic fusions in Nicotiana. Theor Appl Genet 65:289–294

Glimelius K, Bonnett HT (1981) Somatic hybridization in *Nicotiana:* restoration of photoautotrophy to an albino mutant with defective plastids. Planta 153:497–503

Grandbastien MA, Bourgin JP, Caboche M (1985) Valine resistance, a potential marker in plant cell genetics. 2. Optimization of UV-mutagenesis and selection of valine-resistant colonies derived from tobacco mesophyll protoplasts. Genetics 109:409–425

Maliga P, Sz-Breznovits A, Márton L (1973) Streptomycin-resistant plants from callus culture of haploid tobacco. Nature New Biol 244:29–30

Maliga P, Sz-Breznovits A, Márton L, Joo F (1975) Non-Mendelian streptomycin-resistant tobacco mutant with altered chloroplasts and mitochondria. Nature 255:401–402

Maliga P, Lörz H, Lázár G, Nagy F (1982) Cytoplast-protoplast fusion for interspecific chloroplast transfer in *Nicotiana.* Mol Gen Genet 185:211–215

Medgyesy P, Menczel L, Maliga P (1980) The use of cytoplasmic streptomycin resistance: chloroplast transfer from *Nicotiana tabacum* into *Nicotiana sylvestris,* and isolation of their somatic hybrids. Mol Gen Genet 179:693–698

Menczel L, Galiba G, Nagy F, Maliga P (1982) Effect of radiation dosage on efficiency of chloroplast transfer by protoplast fusion in *Nicotiana.* Genetics 100:487–495

Menczel L, Nagy F, Lázár G, Maliga P (1983) Transfer of cytoplasmic male sterility by selection for streptomycin resistance after protoplast fusion in *Nicotiana.* Mol Gen Genet 189:365–369

Meyer Y, Abel WO (1975) Budding and cleavage division of tobacco mesophyll protoplasts in relation to pseudo-wall and wall formation. Planta 125:1–13

Roth EJ, Lark KG (1984) Isopropyl-N-(3-chlorophenyl) carbamate (CIPC) induced chromosomal loss in soybean: a new tool for plant somatic cell genetics. Theor Appl Genet 68:421–431

Sidorov VA, Menczel L, Nagy F, Maliga P (1981) Chloroplast transfer in *Nicotiana* based on metabolic complementation between irradiated and iodoacetate treated protoplasts. Planta 152:341–345

Zelcer A, Aviv D, Galun E (1978) Interspecific transfer of cytoplasmic male sterility by fusion between protoplasts of normal *Nicotiana sylvestris* and X-ray irradiated protoplasts of male-sterile *N. tabacum.* Z Pflanzenphysiol 90: 397–407