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Selection of a *Nicotiana plumbaginifolia* universal hybridizer and its use in intergeneric somatic hybrid formation

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Summary. A *Nicotiana plumbaginifolia* cell strain carrying a positive (dominant) trait, resistance to azetidine-2-carboxylate (A2C), was selected in strain NX1 which lacked nitrate reductase activity (a negative or recessive trait). This universal hybridizer strain, denoted NXA^r, was fused with dextran to a *Daucus carota* strain, PR, which carried glyphosate (GLP) resistance. A large number of hybrids were selected in a medium with NO_3^- as the sole nitrogen source and A2C as inhibitor, conditions which prevent the growth of both parents. When the selected colonies were then tested for GLP resistance, 93% carried this trait. In addition the hybrid nature was indicated by additive chromosome numbers, both A2C and GLP resistance in suspension cultures, intermediate nitrate reductase activity and the presence of banding patterns for three isozymes which match those of the parents. Southern hybridization analysis using an enolpyruvylshikimic acid-3-phosphate synthase (EPSPS) probe, pMON 6145, also showed the presence of the gene from both parents in the hybrid strains based on restriction length polymorphisms. The PR strain contains increased levels of EPSPS which confers GLP^r due to gene amplification. Since the universal hybridizer can be used as a fusion partner with any wild-type line many protoplast fusion studies can be carried out easily.

Key words: Azetidine-2-carboxylate resistance – Glyphosate resistance – Gene amplification – *D. carota* – Protoplast fusion

Introduction

Protoplast fusion can allow somatic hybrid formation between any two plant species but the selection of hybrid cells from the population of unfused protoplasts is one of the major areas of difficulty. Many hybrid selection schemes are possible but these usually require two unique (selected) cell lines as fusion partners (Widholm 1982).

Fusion hybrid selection might be simplified by developing cell lines called "universal hybridizers" (Lo Schiavo et al. 1983) which would carry both a dominantly expressed trait like resistance and a recessive trait like auxotrophy. Such a line could be fused with any wild-type line not carrying resistance or auxotrophy and the hybrid would carry resistance obtained from the universal hybridizer line, and the ability to grow on minimal medium obtained from the wild type. Only the hybrid would grow on the minimal medium with inhibitor since the universal hybridizer is auxotrophic and the wild type is sensitive to the inhibitor.

Such a system was developed by Lo Schiavo et al. (1983) who first selected carrot cells resistant to 8-azaguanine. This strain contained an altered hypoxanthine-phosphoribosyl transferase which was insensitive to 8-azaguanine and had a lower affinity for the natural substrate hypoxanthine. This altered enzyme made the strain sensitive to HAT (hypoxanthine, aminopterin, glycine and thymidine) medium. Resistance to α-amanitin was then selected for and the double mutants were fused with wild-type carrot protoplasts and hybrids selected on medium with HAT and α-amanitin. These authors describe this double mutant strain as a universal hybridizer.

Hamill et al. (1983) developed a similar double mutant system by sexual means by crossing a *Nicotiana tabacum* strain lacking nitrate reductase activity (nia-130) with a streptomycin-resistant strain (SRI). Mesophyll protoplasts of this double mutant were used by Pental et al. (1984) in fusion experiments with *N. rustica* suspension culture protoplasts and hybrids were selected by their ability to grow with nitrate as the nitrogen source in the presence of streptomycin.

In this paper we describe the selection of azetidine-2-carboxylate (A2C) resistant cell strains from an *N. plumbaginifolia* mutant line lacking nitrate reductase. This double mutant, NXA^r was then used as a universal hybridizer with a carrot strain carrying glyphosate (GLP) resistance as an independent marker. Several techniques were used to verify the hybrid nature of the selected colonies including GLP resistance, isozyme analysis and Southern hybridization analysis of the 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS) gene polymorphism. Evidence for the stable intertribal transfer of amplified genes through somatic hybridization is also presented.

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Abbreviations: A2C, azetidine-2-carboxylate; 2,4-D, 2,4-dichlorophenoxyacetic acid; EPSPS, 5-enolpyruvylshikimic acid-3-phosphate synthase; GLP, glyphosate; HAT, hypoxanthine, aminopterin, glycine and thymidine medium; IDH, isocitrate dehydrogenase; MDH, malate dehydrogenase; 5MT, 5-methyltryptophan; NBT, nitroblue tetrazolium; PGI, phosphoglucoisomerase; SDS, sodium dodecylsulfate

Materials and methods

Cell lines and culture conditons. A nitrate reductase deficient line, NX1, of *N. plumbaginifolia* Viviani isolated by Marton et al. (1982) and kindly provided by P. Maliga was cultured in AA liquid medium (Glimelius et al. 1978). The garden carrot root (*Daucus carota* L. cv. Danvers) strain PR resistant to GLP (Nafziger et al. 1984) was cultured in Murashige and Skoog (1962) medium containing 0.4 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). All suspension cultures were grown on a reciprocating shaker (80 rpm) at 27°–28° C with 7 days between transfer for NX1 and NXA^r and 5 days for PR.

Selection of A2C resistant lines. Suspension cultured NX1 cells (0.5 g fresh weight) were inoculated into flasks containing 50 ml of AA medium with various A2C concentrations and after 14 days were harvested by vacuum filtration on Miracloth filters for fresh weight determination. Selection for A2C resistance was carried out by inoculating 0.5 g fresh weight NX1 cells into 50 ml AA liquid medium containing 0.4 mM A2C. After incubating for 7 days the cells were mixed with an equal volume of AA medium with 1.2% Bacto agar in plastic petri plates. The colonies which appeared within 6 weeks were transferred to AA solid medium with progressively higher concentrations of A2C until 3 mM was reached. The selected strains were denoted NXA^r.

Protoplast preparation and fusion. Protoplasts were isolated from suspension cultures 3–4 days after subculturing by incubating in 2% cellulase (Onazuka R10, Kinki Yakult, Nishinomiya, Japan), 0.1% macerozyme (Kinki Yakult, Nishinomiya, Japan), 10% mannitol and 0.1% $CaCl_2 \cdot 2H_2O$. The cells were incubated on a platform shaker at 30 rpm for 14–16 h. Protoplasts were filtered through a 74 μm sieve and washed two times with 10% mannitol, 0.1% $CaCl_2 \cdot 2H_2O$ (pH 5.7) by centrifugation at 100 *g* for 5 min. The protoplasts were purified by floating in a 20% (w/v) sucrose solution by centrifugation at 100 *g* for 10 min in Babcock bottles. Protoplasts were brought to a density of 2×10^5/ml in 5% mannitol, 2% $CaCl_2 \cdot 2H_2O_5$ mixed in equal volumes and fused using the dextran method of Kameya et al. (1981).

Selection and characterization of the fusion hybrids. After fusion the protoplasts were rinsed and cultured at a density of 5×10^4/ml for 14 days in AA liquid culture medium containing the amino acid mixture at half concentration and 5% filter sterilized glucose. The parental protoplasts alone and a mixture of unfused protoplasts were cultured similarly as controls. After 14 days the cells were washed once with selective K3 medium (Nagy and Maliga 1976) in which only cells with nitrate reductase activity can grow (Muller and Grafe 1978; Marton et al. 1982). The cells were then plated in K3 medium (0.6% Bacto Agar) containing 3 mM A2C and were incubated at 27° C. After 6 to 8 weeks the colonies which formed were transferred to RMOP medium (Sidorov et al. 1981).

Chromosomes were counted using the modified carbol fuchsin staining method (Kao 1975) except that cells were hydrolyzed with 0.5 N HCl for 8 min instead of using the enzyme treatment. The in vivo nitrate reductase activity was assayed in 10-day-old calli by the method of Marton et al. (1982).

Electrophoresis was carried out in 14% starch gels using gel buffer containing 8.6 mM Tris and 5 mM L-histidine-HCl, pH 6.7 and electrode buffer containing 0.4 M Tris and 0.1 M citric acid $\cdot H_2O$, pH 8.0. The phosphoglucoisomerase (PGI) activity was measured by the method of Tanksley (1980) except that the 20 mg nitroblue tetrazolium (NBT) was not used. For isocitrate dehydrogenase (IDH) a staining method modified from that of Fine and Costello (1963) was used. The gel was stained using 200 mg of trisodium isocitrate in 20 ml 0.5 M Tris, pH 8.5 and 70 ml distilled water, plus 6.0 ml of 1.0 M $MgCl_2$, 2.0 ml 5 mg/ml NADP, 2.0 ml of NBT (10 mg/ml) and 2.0 ml phenazine methosulfate (1.0 mg/ml). Staining was conducted at room temperature in the dark overnight. The malate dehydrogenase (MDH) band staining was carried out according to a method modified from that of Brown et al. (1978). The MDH band was visualized in 100 ml of 0.1 M Tris, pH 7.5, adding 8 ml of D, L-malate buffer, pH 7.5, 5 ml of NAD^+ (10 mg/ml), 4 ml of NBT (10 mg/ml) and 4 ml phenazine methosulfate (1 mg/ml). The gel was incubated at room temperature in the dark overnight.

Free amino acids were extracted and analysed as described previously (Hauptmann and Widholm 1982).

Southern hybridization analysis. High molecular weight DNA was isolated from parental and hybrid cell suspension cultures and purified by CsCl density gradient centrifugation (Shure et al. 1983). DNAs were digested with *Eco*R1 or *Hin*dIII, separated on 0.8% agarose gels and transferred to nitrocellulose (Fedoroff et al. 1983). A *Bgl*II–*Sal*I fragment containing the 72 amino acid transit peptide and 444 amino acid coding region of petunia EPSPS was cloned into the *Sal*I–*Bam*HI multiple cloning site of pGEM – 2 riboprobe gemini transcription plasmid (D. Shaw, unpublished). The EPSPS DNA probe was made either by nick translation (Rigby et al. 1977) of total plasmid or by random oligo labeling of a *Sma*I–*Pvu*II gel-purified fragment (Feinberg and Volgelstein 1983).

Hybridization was performed under high (68° C) or low (55° C) stringency in 2 × SSPE (1 × SSPE = 1 mM Na_2EDTA, 8 mM NaOH, 10 mM NaH_2PO_4, 180 mM NaCl), 4 × Denhardt's (50 × Denhardt's = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 0.1% SDS and 100 μg/ml denatured salmon sperm DNA. Filters hybridized under high stringency were washed for 15 min, two times at room temperature with 0.3 × SSPE containing 0.1% SDS and once for 30 min at 68° C with 0.3 × SSPE with 0.1% SDS. Filters hybridized under low stringency were washed two times for 15 min each at room temperature and once for 30 min at 55° C in 2 × SSPE with 0.1% SDS. ^{32}P-labeled *Hin*dIII fragments of lambda DNA were used as size markers.

Results and discussion

Selection and characterization of A2C resistant NX1 lines

Initial conditions for the selection of A2C resistant cells were determined from the NX1 suspension culture growth curve obtained with different A2C concentrations. The growth of NX1 cells was inhibited about 70% by 0.1 mM A2C and 0.4 mM A2C caused complete inhibition (Fig. 1). Thus 0.4 mM A2C was used in the initial selection attempt where a total of 5×10^7 cells were inoculated into ten flasks.

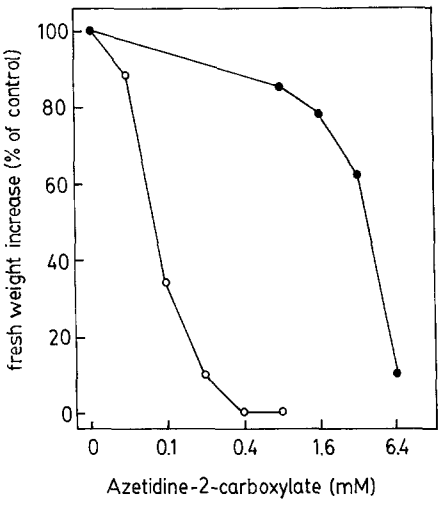

Fig. 1. Effect of azetidine-2-carboxylate (A2C) on the growth of parental NX1 (o) and selected NXA^r (•) cells grown for 14 days in liquid medium. Four flasks were weighed for each point

After 7 days the cells were plated in the same medium diluted twofold to give 2 mM A2C and 27 colonies formed after 6 weeks giving a resistance frequency of 5.4×10^{-7}. When these colonies were placed on progressively higher A2C concentrations 12 of the original clones survived on 3 mM A2C.

One of these selected strains, denoted NXA^r, when grown as a suspension, required 6.4 mM A2C for near complete growth inhibition while the NX1 cells were completely inhibited by 0.4 mM A2C (Fig. 1). Amino acid analysis of three different resistant cell lines revealed an increase in the levels of several free amino acids over those of the parental cells including proline, the amino acid to which A2C is an analog (data not shown). These increases in the pool size of proline and some other amino acids in the A2C resistant cell lines are in agreement with the previous results with A2C resistant carrot cells (Widholm 1976; Cella et al. 1982).

Protoplast fusion and hybrid selection

Protoplasts produced from both NXA^r and PR cells were grown in several media to determine the conditions needed for hybrid selection (Table 1). Colonies formed from PR protoplasts in K3 medium but not in the presence of 3 mM A2C, indicating that this concentration of A2C was high enough to inhibit the growth of PR cells. There were no colonies observed from NXA^r protoplasts in K3 medium confirming the fact that NXA^r cells cannot utilize non-reduced nitrogen in the medium. The NXA^r protoplasts can grow with reduced nitrogen and 3 mM A2C, however. Thus, NXA^r and PR can be selected against by growing in medium containing 3 mM A2C and nitrate as the sole nitrogen source.

Dextran induced fusion of PR and NXA^r protoplasts gave rise to colonies in NO_3^- medium (K3) containing 3 mM A2C (Table 1). There was no growth of the controls which consisted of a mixture of unfused parental protoplasts, demonstrating that no cross feeding occurred between the two species. The 528 hybrid colonies formed from a total of 2×10^6 protoplasts of each species which were incubated in a total of 24 plates. The frequency of fusion

Table 1. Colony formation from parental cells, unfused cell mixtures and fused protoplasts in growth and selection media

Cell line	Medium	No. of cells per plate $\times 10^3$	No. of colonies formed	
			per plate	per 10^3 cells
PR	AA+10 GLP	1.1	53	48
	K3	0.8	49	61
	K3+3 mM A2C	3.7	0	0
NXA^r	AA+3 mM A2C	0.8	48	60
	K3+3 mM A2C	4.2	0	0
	AA+10 mM GLP	5.1	0	0
NXA^r+PR (unfused)	AA	0.8	51	64
	AA+3 mM A2C	0.9	28	31
	AA+10 mM A2C	0.7	19	27
	K3+3 mM A2C	5.6	0	0
NXA^r+PR (fused)	K3+3 mM A2C	8.5	22	3

The number of growing cells and cell aggregates derived from protoplasts after fusion was determined immediately after plating (14 days after fusion) and the number of colonies formed were counted after 6 weeks. GLP, glyphosate; A2C, azetidine-2-carboxylate

hybrid selection was about 4.6% when the number formed was corrected for the plating efficiency obtained with the mixture of unfused protoplasts in AA medium. If the value was not corrected for plating efficiency the frequency would be about 0.3%.

Characterization of the fusion hybrids

To help confirm that the selected colonies were indeed hybrids, the colonies were plated on a NO_3^- medium with both A2C and GLP. In this test, 491 of the 528 colonies grew (93%; Fig. 2) so were therefore carrying GLP resistance, presumably from the PR fusion partner even though this was not selected for in the original hybrid selection procedure. The PR callus did not grow on this medium (with A2C and GLP) because it was inhibited by A2C and the NXA^r callus was inhibited by GLP (Fig. 2).

Three of the selected hybrid lines (PN-1, PN-2, PN-3) were further tested as suspension cultures and were more resistant to GLP and A2C than the respective sensitive parental strains but were less resistant than the respective resistant cell strains (Figs. 3, 4). The hybrid cell lines PN-1 to PN-5 also contained intermediate levels of nitrate reductase activity in relation to the parents (63%–91% of the PR activity) where PR contained activity while NXA^r contained none (Table 2). The mean chromosome numbers of four hybrid lines, PN-1 to PN-4 were very close to that expected by adding the parental chromosome numbers together (Table 3). Since PR contained a mean number of 27.5 and NXA^r 19.8, the hybrid additive number should be near 47 and the measured range was from 45–47.

Starch gel isozyme analysis of PGI, IDH and MDH also indicated that the selected fusion hybrids PN-1 to PN-6 were indeed hybrids. In the case of PGI (Fig. 5) the six hybrid lines produced three activity bands with the top one corresponding to one from *N. plumbaginifolia* and the mid-

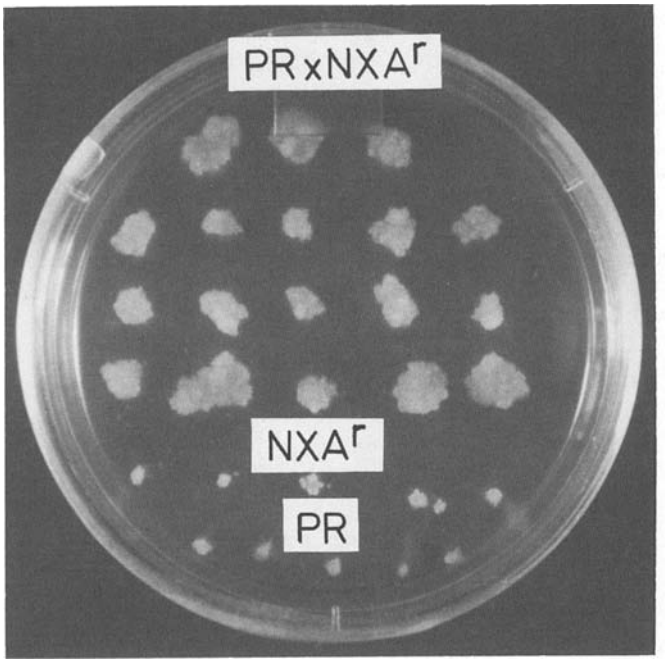

Fig. 2. Growth of PR × NXA^r hybrid colonies and NXA^r and PR callus on RMOP (NO_3^-) medium containing 3 mM azetidine-2-carboxylate (A2C) and 10 mM glyphosate (GLP) after 25 days incubation

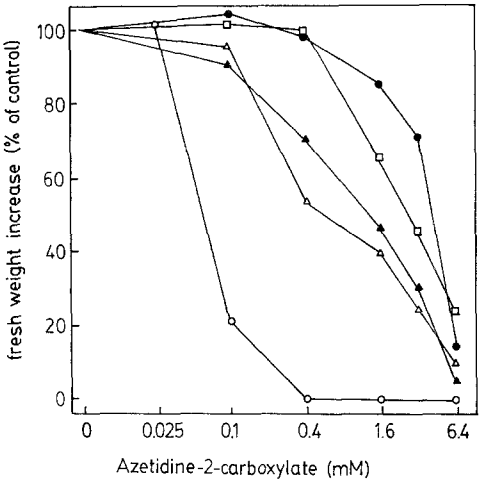

Fig. 3. Effect of azetidine-2-carboxylate (A2C) on the growth of parental PR (○) and NXA^r (●) and hybrid PN-1 (△), PN-2 (▲) and PN-3 (□) cells. The cells were grown for 14 days in liquid medium with duplicate flasks

dle one to one from carrot. The lower activity band appears to be common to both species.

In the case of IDH isozymes the hybrids have two bands corresponding to one each from each parent (Fig. 6). With MDH the hybrids are identical in banding patterns (Fig. 7) and have three lower bands in common with both parents. The top band and fainter second band correspond to those from carrot while the third band corresponds to one from *N. plumbaginifolia*.

Southern analysis of *Eco*RI-digested DNA of parental strains and hybrids PN-1 to PN-10 showed weak levels of hybridization with EPSPS probe pMON 6145 under highly stringent conditions (68° C; Fig. 8A). Although exact copy

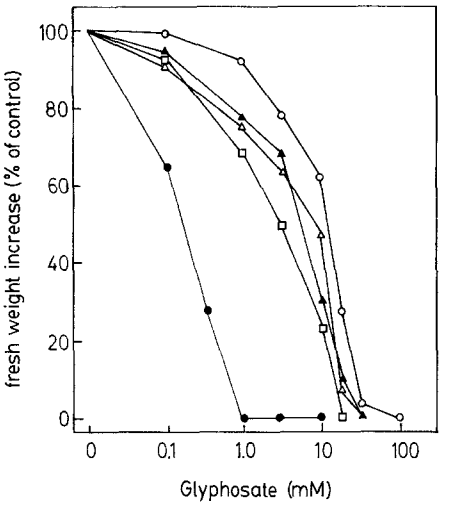

Fig. 4. Effect of glyphosate (GLP) on the growth of parental PR (○) and NXA^r (●) and hybrid PN-1 (△), PN-2 (▲) and PN-3 (□) cells. The cells were grown for 14 days in liquid medium with duplicate flasks

Table 2. Nitrate reductase activity in PR, NXA^r and five hybrid (PN) cell lines

Cell line	Nitrate reductase activity (% of control)
PR	100[a]
NXA^r	0
PN-1	76
PN-2	84
PN-3	82
PN-4	91
PN-5	63

[a] The PR nitrate reductase activity was 170 nmol NO_2^- formed per 100 mg callus per hour. Values are the means from three independent experiments

Table 3. Chromosome numbers of *Daucus carota* PR ($n=9$), *Nicotiana plumbaginifolia* NXA^r ($n=10$) and four somatic hybrid (PN) cell lines

Chromosome number range	Number of cells in each range					
	PR	NXA^r	PN-1	PN-2	PN-3	PN-4
10–16	0	4				
17–23	3	24		1		2
24–30	23	3	1	1	2	1
31–37	3		1	2	1	3
38–44	1		2	4	2	3
45–51			10	14	9	12
52–58			1	4	3	2
59–65				3	1	1
Total cells	30	31	15	29	18	24
Average chromosome no.	27.5	19.8	45.2	46.8	46.1	44.2
Expected chromosome no. in hybrid cells			47.3	47.3	47.3	47.3

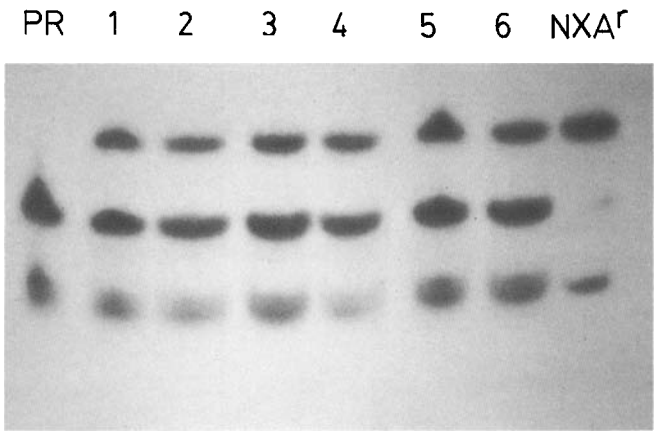

Fig. 5. Starch gel electrophoresis of 6-phosphoglucoisomerase (PGI) activity from parental PR and NXA^r and hybrid PN-1, PN-2, PN-3, PN-4, PN-5 and PN-6 cells run from top to bottom towards the anode

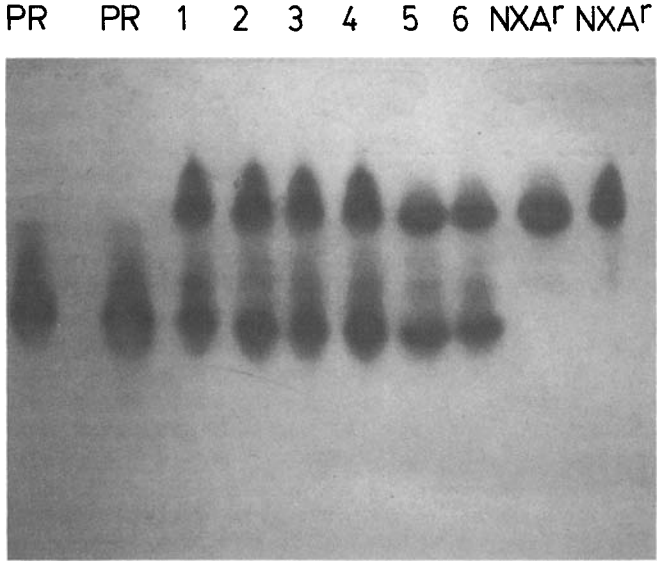

Fig. 6. Starch gel electrophoresis of isocitrate dehydrogenase (IDH) activity from parental PR and NXA^r and hybrid PN-1, PN-2, PN-3, PN-4, PN-5 and PN-6 cells run from top to bottom towards the anode

numbers could not be determined since a non-homologous petunia probe was used, the level of hybridization is consistent with a low copy number of EPSPS since the 1C copy number reconstruction using a petunia cDNA was at least five times greater in intensity (data not presented). A species specific band characteristic of NXA^r occurred at approximately 2.9 kb while PR contained a band at approximately 3.5 kb. No unique bands were found in *Hin*dIII digests. PN-1 to PN-10 contained an additive banding pattern of both parental strains further confirming their hybrid nature.

When reprobed with pMON 6145 using less stringent hybridization conditions (55° C; Fig. 8B), higher degrees of hybridization were seen in the GLP resistant carrot parent PR and hybrid strains but not in the *N. plumbaginifolia* parent NXA^r. Stronger hybridization is expected between the petunia and *Nicotiana* EPSPS relative to petunia and carrot since petunia and *Nicotiana* are in the same family. However, the lower stringency hybridization condition

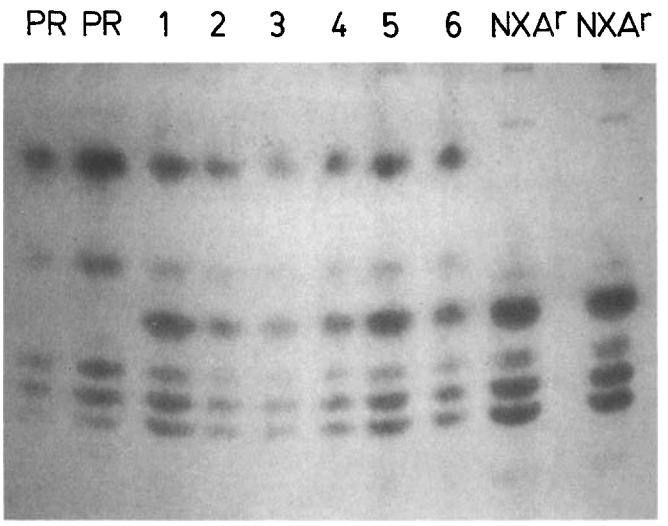

Fig. 7. Starch gel electrophresis of malate dehydrogenase (MDH) activity from parental PR and NXA^r and hybrid PN-1, PN-2, PN-3, PN-4, PN-5 and PN-6 cells run from top to bottom towards the anode

shows enhanced hybridization to the GLP tolerant carrot line suggesting amplification of the EPSPS coding sequences. This differential hybridization further proves that hybrids have been obtained since PN-1 to PN-10 contain the stronger hybridization pattern of carrot while the NXA^r fragment remains of similar intensity under the low stringency conditions. Amplification of EPSPS in petunia has been shown to confer GLP tolerance (Shah et al. 1986) and the mechanism appears to be the same in carrot due to the higher levels of hybridization under low stringency as compared with *N. plumbaginifolia* and due to the fact that the strain contains ca. 12 times the normal level of EPSPS enzyme activity (Nafziger et al. 1984).

A number of additional bands appeared in the hybrids with varying intensities. This could indicate extensive chromosomal rearrangement as seen in other carrot + *Nicotiana* hybrids (R.M. Hauptmann, K. Paka and J.M. Widholm, unpublished) or variability in the arrangement of the amplified EPSPS genes. The high intensity bands seen in the hybrids are probably not due to non-specific hybridization since they are not seen in wild-type carrot when probed under identical conditions. This variability is also seen in different suspension cultures of amplified EPSPS – GLP tolerant PR strains and these are being further characterized.

These experiments indicate that the selection scheme for protoplast fusion hybrids utilizing the universal hybridizer NXA^r-2, carrying nitrate reductase deficiency and A2C resistance, can efficiently be fused with dextran to another cell strain (in this case the carrot strain PR) and the hybrids be selected on NO_3^- medium with A2C. Confirmation of the hybrid nature of the selected colonies was accomplished by showing the presence of the unselected marked GLP, the presence of the additive number of chromosomes, the presence of isozyme bands from both parents with three different enzymes and the presence of restriction endonuclease fragments from both parents showing homology to an EPSPS probe.

The Southern hybridization experiments revealed that the PR strain did have an increased EPSPS gene copy number which is consistent with the strain containing ca.

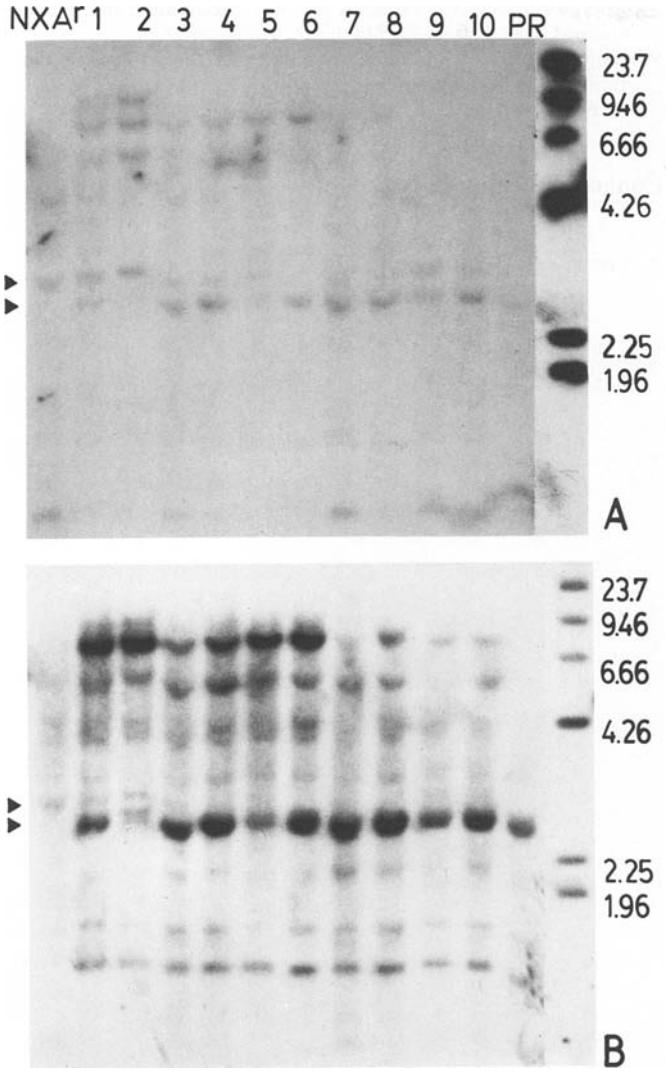

Fig. 8A, B. Southern hybridization analysis of parental PR and NXA^r and hybrid PN-1 to PN-10 cell DNA using a petunia 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS) cDNA clone (pMON 6145) as probe. High molecular weight DNA was isolated from parental and hybrid cell suspension cultures. DNA (15 μg) of each was digested with *Eco*R1, separated on agarose gels, transferred to nitrocellulose, and probed with petunia EPSPS cDNA as described in Materials and methods. Hybridization was performed under high (68° C; **A**) or low (55° C; **B**) stringency. ^{32}P-labeled *Hin*dIII fragments of lambda DNA were used as size markers. *Arrows* indicate bands which show restriction length polymorphism

12 times the normal level of EPSPS enzyme activity (Nafziger et al. 1984). Studies with GLP resistant petunia strains (Shah et al. 1986) have shown that the increased EPSPS activity was due to amplification of the EPSPS gene. The only other example of selected gene amplification in plants is the case of phosphinothricin resistance with alfalfa cells where the target enzyme glutamine synthetase activity was increased by 3- to 7-fold due to a 4- to 11-fold amplification of a glutamine synthetase gene (Donn et al. 1984).

Universal hybridizer cell strains can be used in many protoplast fusion studies since this one line can be fused with any other wild-type strain. Such studies would include species compatibility, gene expression, organelle segregation, organelle DNA recombination and chromosome loss.

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References

Brown AHD, Nevo E, Zohary D, Dagan O (1978) Genetic variation in natural populations of wild barley (*Hordeum spontaneum*). Genetica 49:97–108

Cella R, Parisi B, Nielsen E (1982) Characterization of a carrot cell line resistant to azetidine-2-carboxylic acid. Plant Sci Lett 24:125–135

Donn G, Tischer E, Smith JA, Goodman HM (1984) Herbicide-resistant alfalfa cells: an example of gene amplification in plants. J Mol Appl Genet 2:621–635

Fedoroff NJ, Mauvais J, Chaleff D (1983) Molecular studies on mutations at the shrunken locus in maize caused by the controlling element Ds. J Mol Appl Genet 2:11–29

Feinberg AP, Volgelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13

Fine IH, Costello LA (1963) The use of starch electrophoresis in dehydrogenase studies. Methods Enzymol VI:958–972

Glimelius K, Eriksson T, Grafe R, Muller AJ (1978) Somatic hybridization of nitrate-deficient mutants of *Nicotiana tabacum* by protoplast fusion. Physiol Plant 44:273–277

Hamill JD, Pental D, Cocking EC, Muller AJ (1983) Production of a nitrate reductase deficient streptomycin resistant mutant of *Nicotiana tabacum* for somatic hybridization studies. Heredity 50:197–200

Hauptmann RM, Widholm JM (1982) Cryostorage of cloned amino acid analog-resistant carrot and tobacco suspension cultures. Plant Physiol 70:30–34

Kameya T, Horn ME, Widholm JM (1981) Hybrid shoot formation from fused *Daucus carota* and *D. capillifolius* protoplasts. Z Pflanzenphysiol 104:459–466

Kao KO (1975) A chromosomal staining method for cultured cells. In: Gamborg OL, Wetter LR (eds) Plant tissue culture methods. National Research Council of Canada, Saskatoon, pp 63–64

Lo Schiavo F, Giovinazzo G, Terzi M (1983) 8-azaguanine resistant carrot cell mutants and their use as universal hybridizers. Mol Gen Genet 192:326–329

Marton L, Dung TM, Mendel RR, Maliga P (1982) Nitrate reductase deficient cell lines from haploid protoplast culture of *Nicotiana plumbaginifolia*. Mol Gen Genet 186:301–304

Muller AJ, Grafe R (1978) Isolation and characterization of cell lines of *Nicotiana tabacum* lacking nitrate reductase. Mol Gen Genet 161:67–76

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Nafziger ED, Widholm JM, Steinrucken HC, Killmer JL (1984) Selection and characterization of a carrot cell line tolerant to glyphosate. Plant Physiol 76:571–574

Nagy JI, Maliga P (1976) Callus induction and plant regeneration from mesophyll protoplasts of *Nicotiana sylvestris*. Z Pflanzenphysiol 78:453–455

Pental D, Hamill JD, Cocking EC (1984) Somatic hybridization using a double mutant of *Nicotiana tabacum*. Heredity 53:79–83

Rigby PWJ, Dieckmann M, Rhodes C, Berg P (1977) Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J Mol Biol 113:237–251

Shah DM, Horsch RB, Klee HJ, Kishore GM, Winter JA, Tumer NE, Hironaka CM, Sanders PR, Gasser CS, Aykent S, Siegel

NR, Rogers SG, Fraley RT (1986) Engineering herbicide tolerance in transgenic plants. Science 233:478–481
Shure M, Wessler S, Fedoroff N (1983) Molecular identification and isolation of the waxy locus in maize. Cell 35:225–233
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
Tanksley SD (1980) Pgi-1 a single gene in tomato responsible for a variable number of isozymes. Can J Genet Cytol 22:271–278
Widholm JM (1976) Selection and characterization of cultured carrot and tobacco cells resistant to lysine, methionine and proline analogs. Can J Bot 54:1523–1529
Widholm JM (1982) Selection of protoplast fusion hybrids. In: Fujiwara A (ed) Plant tissue culture 1982. Maruzen, Tokyo, pp 609–612

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