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Selection of *Daucus carota* somatic hybrids using drug resistance markers and characterization of their mitochondrial genomes

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Summary. Protoplasts from different *Daucus carota* L. cell strains carrying resistance to glyphosate, 5-methyltryptophan, sodium selenate or selenocystine were fused in three combinations using dextran. Clones were selected for both of the resistances carried by the individual parental strains in medium with both inhibitors. No doubly resistant colonies formed from unfused controls or from protoplasts from each individual parental strain alone. Suspension cultures from the selected clones contained predominantly the additive chromosome numbers of the parental strains. Apparently the four resistances used are expressed dominantly in fusion hybrids. Analysis of mitochondrial DNA showed that recombination occurred in one fusion combination since the mitochondrial DNA in the hybrid cells was different from that of either parent as shown by restriction endonuclease fragment analysis. Mitochondrial DNA in the other two somatic hybrid combinations was parental. Thus, a dominant, nuclear resistance marker system has been developed to select efficiently for somatic hybrids in which mitochondrial DNA recombination can be studied.

Key words: Amino acid analog resistance – Herbicide resistance – Glyphosate – 5-methyltryptophan – Sodium selenate – Selenocystine

Abbreviations: 2,4-D = 2,4-dichlorophenoxyacetic acid; FW = fresh weight; Glp = Glyphosate; mt = mitochondrial; MX = Murashige and Skoog (1962) medium containing 0.4 mg/l 2,4-D; 5MT = 5-methyltryptophan; MXG = MX containing (5% Glucose); SC = selenocystine; SS = sodium selenate

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Introduction

Protoplast fusion can allow certain unique circumstances to occur which cannot happen with conventional genetic methods. These would include forming hybrids between sexually incompatible species and the mixing of organelles in most species where the organelles are strictly maternally inherited. Only if organelle mixing occurs can recombination occur between mt and plastid DNAs from different plants of the same or different species.

Novel compositions of mitochondrial genomes produced by protoplast fusion that differ from both parental mitochondrial genomes have been observed previously in *Nicotiana* (Belliard et al. 1979; Nagy et al. 1981, 1983; Galun et al. 1982), *Petunia* (Boeshore et al. 1983), Cruciferae (Chetrit et al. 1985) and *Daucus* sp. (Matthews and Widholm 1985). The presence of new and unique fragments in the somatic hybrids mtDNA restriction endonuclease fragment patterns of tobacco was first taken as evidence of parental mitochondrial fusion followed by mtDNA recombination in the hybrid cells (Belliard et al. 1979). Evidence that, in fact, mitochondria can fuse within the cell was given by Wildman et al. (1962) who obtained motion pictures of living plant cells, where particles interpreted as being mitochondria can be seen to fuse with one another, as well as with chloroplasts, and then to separate again.

Boeshore et al. (1983) pointed out that the new unique mtDNA restriction fragments found in *Petunia* protoplast fusion hybrids would not necessarily have to arise by recombination but could be due to the presence of DNA with a low copy number in the parents which would not be easily detectable. This DNA would then somehow be amplified in the fusion hybrid. Rothenberg et al. (1985) have, however, demon-

strated that recombination can occur in such *Petunia* fusion hybrids. In this case, a cloned region of the mtDNA from a *Petunia* somatic hybrid was shown to contain restriction fragments derived from each of the parental mitochondrial genomes.

In the studies reported here, resistance complementation was used to select protoplast fusion hybrids from three different combinations of *Daucus carota* cell strains each carrying mtDNAs which can be differentiated by restriction fragment analysis. In one of the combinations the selected hybrid cells contained mtDNA with different restriction endonuclease patterns from that of either parent.

Materials and methods

Plant material

The following cell strains of *Daucus carota* L. were used in these studies: PR resistant to Glp (30 mM) (Nafziger et al. 1984), HCl-3 resistant to 5MT (0.46 mM) (unpublished), C-1 resistant to SC (0.1 mM) (Furner and Sung 1983) and S232 resistant to SS (0.2 mM) (Furner and Sung 1982). The figures in brackets are the concentrations at which the hybrids were selected in these experiments. Suspension cultures of these lines were subcultured every 7 days in MX liquid medium (Murashige and Skoog [1962] medium plus 0.4 mg/l 2,4-D) under the conditions described by Nafziger et al. (1984).

Protoplast isolation

Protoplasts were isolated on the 4th day after subculturing by incubating one packed cell volume of cells with four volumes of the enzyme solution consisting of 2% cellulase (Onozuka R-10, Kinki Yakult, Nishinomiya, Japan). 0.1% Macerozyme R 10 (Kinki Yakult, Japan), 10% mannitol, 0.1% $CaCl_2 \cdot 2H_2O$, pH 5.8. The cells were incubated at 40 rpm on a platform shaker for 15 h in diffuse light. Protoplasts were filtered through 105 µm stainless steel sieves and washed two times by centrifugation for 5 min at 100×g in 10% mannitol and 0.1% $CaCl_2 \cdot 2H_2O$ solution. Protoplasts were purified by floating them on a 20% sucrose solution in Babcock bottles by centrifugation at 100×g for 10 min.

Protoplast fusion

The purified protoplasts were counted and mixed together in a 1:1 ratio at the densities per ml listed in Table 2. Following fusion using the dextran method of Kameya et al. (1981), the protoplasts were rinsed and were diluted 10X in liquid MXG medium. Control plates containing unfused mixtures with the number of protoplasts listed in Tables 1 and 2 in 2.5 ml MXG liquid medium were also included. After incubation at 27–28 °C for 14 days an equal volume of MX medium with 1% Bacto-agar and double the inhibitor concentrations listed above for each strain was added and the plates were incubated in the dark at 27–28 °C for 40 days. Growing colonies were placed on MX medium (0.5% agar) containing the same inhibitors for 30 days to retest the resistance. The hybrids resulting from this procedure were denoted PH1, PH2, etc. (PR×HCl-3 fusion), CH1, etc. (C-1×HCl-3 fusion) and PS1, etc. (PR×S232 fusion).

Chromosome counting

Chromosome counts were made following modified carbolfuchsin (Kao 1975) staining of colchicine treated (0.05% for 2 h) fixed (95% ethanol: acetic; 3:1), and hydrolyzed (0.2 N HCl for 5 min) cells.

Table 1. Colony formation from PR, HCl-3, C-1 and S232 protoplasts plated 14 days after preparation in the presence of several inhibitors. Colonies were counted 40 days after plating in MXG with the inhibitor concentrations shown

Cell strains (No. of units plated)	Colony forming media	No. of colonies formed
PR (Glp^r)	Glp (30 mM)	414
(2.12×10^5)	MXG	460
	5 MT (0.46 mM)	0
	5 MT (0.46 mM) + Glp (30 mM)	0
	SS (0.2 mM)	0
	SS (0.2 mM) + Glp (30 mM)	0
HCl-3 ($5\ MT^r$)	5 mT (0.46 mM)	450
(1.18×10^5)	MXG	480
	Glp (30 mM)	0
	Glp (30 mM) + 5 MT (0.46 mM)	0
	SC (0.1 mM)	0
	SC (0.1 mM) + 5 MT (0.46 mM)	0
C-1 (SC^r)	SC (0.1 mM)	320
(2×10^5)	MXG	360
	5 MT (0.46 mM)	0
	5 MT (0.46 mM) + SC (0.1 mM)	0
S232 (SS^r)	SS (0.2 mM)	448
(1×10^5)	MXG	630
	Glp (30 mM)	0
	SS (0.2 mM) + Glp (30 mM)	0

Table 2. Colony formation from unfused and fused mixtures of protoplasts plated 14 days after preparation in the presence of inhibitors. Colonies were counted 40 days after plating in MXG with the inhibitor concentrations shown

Cell strains (No. of units plated)	Colony forming media	No. of colonies formed
PR + HCl-3 (Unfused mixture) (1.65×10^5)	MXG	510
	Glp (30 mM)	312
	5 MT (0.46 mM)	307
	5 MT (0.46 mM) + Glp (30 mM)	0
PR + HCl-3 (After fusion) (1.65×10^6)	Glp (30 mM) + 5MT (0.46 mM)	144
C-1 + HCl-3 (Unfused mixture) (2.06×10^5)	MXG	548
	SC (0.1 mM)	428
	5 MT (0.46 mM)	536
	5 MT (0.46 mM) + SC (0.1 mM)	0
C-1 + HCl-3 (Fused) (2.06×10^6)	5 MT (0.46 mM) + SC (0.1 mM)	338
PR + S232 (Unfused mixture) (1×10^5)	MXG	486
	Glp (30 mM)	183
	SS (0.2 mM)	260
	Glp (30 mM) + SS (0.2 mM)	0
PR + S232 (Fused) (1×10^6)	SS (0.2 mM) + Glp (30 mM)	203

Growth measurements

Suspension cultured cells (250 mg FW) were inoculated into 50 ml liquid MX medium containing different inhibitor concentrations and incubated on a reciprocal shaker at 27–28 °C before FW measurement of the cells at four d intervals after collection on Miracloth (Nafziger et al. 1984).

mtDNA isolation

Mitochondrial DNA was isolated from suspension cultures of the various carrot cell lines by the procedure of DeBonte and Matthews (1984). Protoplasts were isolated from freshly harvested cells 4days after innoculation and then ruptured by one to three gentle passages through an 18 gauge needle. Nuclear, plastid and mitochondrial fractions were collected by differential centrifugation at 160, 3,000 or 12,000 × g for 15 min each, respectively. mtDNA recovered from lysed organelles was purified by CsCl-bisbenzimide gradient centrifugation. The DNA was precipitated with ethanol after the addition of 2 to 3 volumes of sterile distilled water at −20 °C.

mtDNA restriction endonuclease analysis

All restriction enzymes except EcoRI and Bg*l*II (Worthington Diagnostics, NJ) were purchased from Bethesda Research Labs and were used as directed by the supplier. Complete digestion of the mtDNAs (1–2 μg) was accomplished within 3 to 4 h at 37 °C.

The restriction endonuclease DNA fragments were separated by electrophoresis in 26 × 18.4 × 0.4 cm submerged agarose gels at 1.2–3.0 V/cm in 2X Tris-borate buffer (1X = 0.089 M Tris, 0.089 M boric acid and 2 mM EDTA, pH 8.0). The agarose concentrations used were 1.0%, 0.7% or 0.5% depending on the size of the fragments to be separated.

The gels were stained with 0.5 μg/ml ethidium bromide for 15 min, rinsed three times in water and photographed under 302 nm light using Polaroid type 55 film.

Estimation of DNA fragment lengths and genome sizes

The lengths of the fragments resulting from Sa*l*I digestion of mtDNA from the cell lines PR, PH4 and HCl-3 were determined by the method of Schaffer and Sederoff (1981), using HindIII fragments of lambda DNA as the standard molecular weights. Photographic negatives were scanned using a Gilford spectrophotometer (model no. 240) and the relative molar ratios of the fragments were determined by comparing the areas under the peaks.

Results

Selection of somatic hybrids

In the experiments described here the resistance carried by the cell strains (PR resistant to Glp, HCl-3 resistant to 5MT, C-1 resistant to SC and S232 resistant to SS) was tested with protoplast derived cells. As shown in Table 1 and Fig. 1, each strain is able to grow with the respective inhibitor in the medium while the other strains will not form any colonies. Growth of all cell strains is also prevented when two different inhibitors are present in the medium.

When protoplasts of the four cell strains are mixed in three combinations and are not treated with the

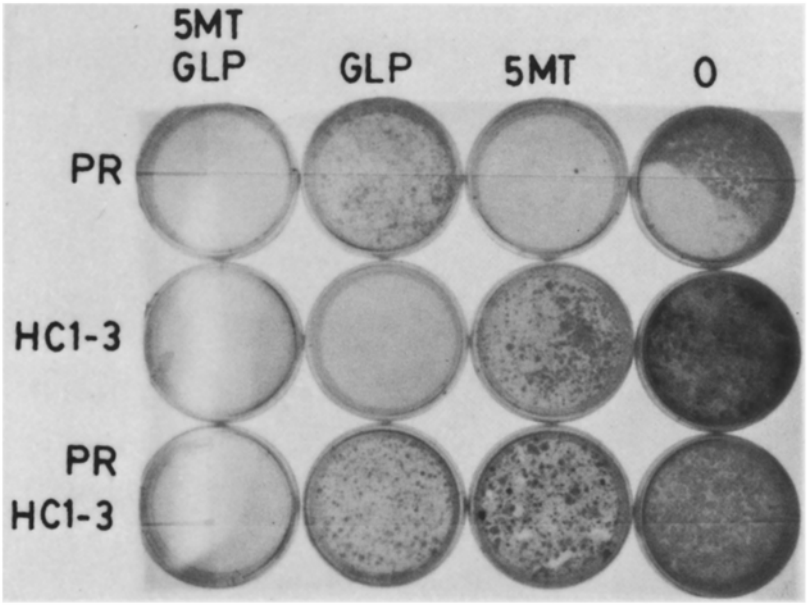

Fig. 1. Growth of PR and HCl-3 protoplasts under selective conditions. Parental protoplasts (PR and HCl-3) and their unfused mixture (PR + HCl-3) grown for 14 days in MXG medium without any inhibitors and then plated under double selective (0.46 mM 5 MT + 30 mM Glp), complementary selective (0.46 mM 5 MT or 30 mM Glp) and nonselective (MXG) conditions showing no growth under double selective and complementary selective conditions

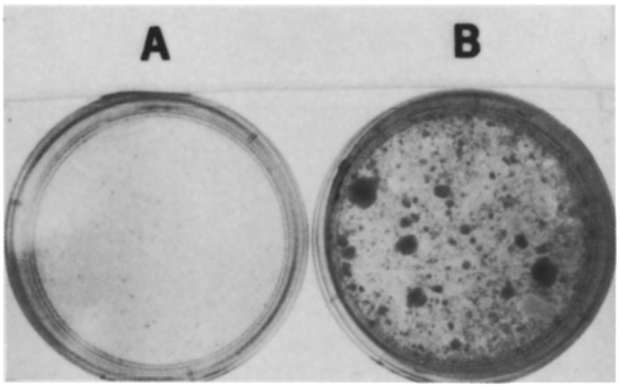

Fig. 2. Growth of unfused (*A*) and dextran fused (*B*) PR + HCl-3 protoplasts under selective conditions. Under double selective conditions. (0.46 mM 5 MT + 30 mM Glp) the plate with fused cells shows formation of colonies while control plate with a mixture of unfused PR + HCl-3 protoplasts shows no colonies

dextran fusigen (Table 2), again no colonies form when the cells are plated for 14 days in medium containing two of the inhibitors. However, following dextran fusion, a large number of colonies form in the presence of the two respective inhibitors of the parental strains (Fig. 2, Table 2). These results indicate that crossfeeding does not occur in mixed but unfused protoplasts and that following fusion many stable hybrids were formed which expressed the resistance trait present in the individual parental strains. Successful hybrids were formed in combinations between PR and HCl-3 (denoted PH1, PH2, etc.), C-1 and HCl-3 (denoted CH1, etc.) and PR and S232 (denoted PS1, etc.).

Evidence for the hybrid nature of these selected colonies include their appearance only after fusion induction and the high frequency of appearance in comparison to the controls (unfused or single strain alone) selected in the same way. Also, as shown in Table 3, the mean chromosome numbers found in two putative hybrids examined from each fusion combination are very close to that expected by adding the parental chromosome numbers together.

Besides showing inhibitor resistance at the callus level, the hybrids also exhibit single and double resistance as suspension cultures (data not shown). In all cases, growth of the parental lines was completely inhibited by the respective double inhibitor combinations used while the hybrids grew at a rate near that of controls grown without inhibitors.

Characterization of the mtDNA

Initial restriction endonuclease analysis of the mtDNA from the parental cell lines with the enzymes BamHI and HindIII showed that the mtDNA restriction fragment patterns of the carrot cell lines C-1 and S232 were identical. However, the patterns of C-1 and S232 were different from PR which in turn was different from HCl-3. These different restriction endonuclease patterns fit with the origin of the strains since C-1 and S232 were selected from the HA line (Furner and Sung 1982, 1983), PR was selected from a different C-1 line maintained in this laboratory (Nafziger et al. 1984) and HCl-3 was selected from a newly initiated carrot cell line HCl (unpublished). The mitochondrial genomes of the carrot cell lines appear to be stable since the restriction fragments are the same in parental and selected

Table 3. Chromosome numbers of parental and protoplast fusion hybrids

Chromosome no. ranges	Cell types									
	PR	HCl-3	C-1	S232	PH1	PH2	CH3	CH5	PS2	PS5
	No. of figures in each class									
1– 8			2	2						
n= 9			10	7						
10–17	1		2	5						
2n=18	6	4	23	12						
19–26	2		3							
3n=27	10		1							
28–35	3	3	4	2	1				1	
4n=36	6	11	7	4					3	2
37–44	2	6	2						1	2
5n=45										
46–53		1					2		1	2
6n=54						1	5	5	5	7
55–62						2	7	7	5	9
7n=63						10	3	1		6
64–71					1	6	2	2	1	2
8n=72		2			3		1	3	5	
73–80					10	1			5	
9n=81					1					
82–89					7	1				
Total no. of the metaphase figures counted	30	27	54	32	23	21	20	18	27	30
Average no. of chromosomes	27.6	36.7	20.5	18.3	73.3	65.0	54.8	61.2	52.3	55.1
Number expected in fusion hybrid					64.3	64.3	57.2	57.2	45.9	45.9

lines after many years (Matthews and Widholm 1985; Matthews and DeBonte 1985), so the differences seen in the mtDNA of the strains used here should be due to origin rather than to changes occurring during culture. It has also been demonstrated that different mtDNA restriction fragment patterns are found with different carrot cultivars (Matthews and DeBonte 1985) and *Daucus* species (DeBonte et al. 1984).

These differences in the mtDNAs restriction endonuclease fragment patterns noted with the parental carrot cells allowed the origin of the mtDNA to be investigated in the selected fusion hybrids. mtDNAs from the somatic fusion hybrids CH3, CH5, CH8 and CH20 (resulting from the fusion of C-1 and HCl-3), when digested with EcoRI, have fragment patterns identical to those of the parental cell line HCl-3 (Fig. 3). Similar results were obtained when the mtDNAs were digested with the enzymes XhoI and HindIII (data not shown). This indicates that the mitochondria in these four hybrids originated from the HCl-3 cells and not from C-1.

The mtDNAs from the somatic hybrids PS4 and PS5 (selected from the fusion between cell lines PR and S232) when digested with XhoI have fragment patterns identical to that of the PR cell line (Fig. 4). The same was observed for the hybrids PS1, PS2 and PS3 when digested with PstI (data not shown).

The mtDNAs of the somatic hybrids PH1, PH2, PH3, PH4, PH5, PH8, PH10 and PH20 (originating from the fusion between PR and HCl-3) were digested with the enzymes XhoI, BamHI, *Sal*I, and PstI. Although the mtDNAs of all the somatic hybrids are identical to each other, a novel mtDNA restriction endonuclease fragment pattern is observed (Fig. 5). The majority of the mtDNA fragments are of sizes similar to those of both parents, indicating the contribution of both parental genomes. There are however, differences between the hybrids and the parents which can not simply be explained on the basis of mixing of both mitochondria types. Some parental fragment bands were missing, and new fragments were present which were not found in either parental mtDNA.

Table 4 lists the DNA fragment sizes produced by *Sal*I digestion of mtDNA from PH4, PR and HCl-3 cells. The parental strains PR and HCl-3 have 29 and 30 fragments larger than 1 kb, respectively. Sixteen of these fragments are common to each strain while the rest are unique. The sum of the fragment sizes are ca. 250 kb for PR and 265 kb for HCl-3. When stoichiometries for each fragment were estimated by densito-

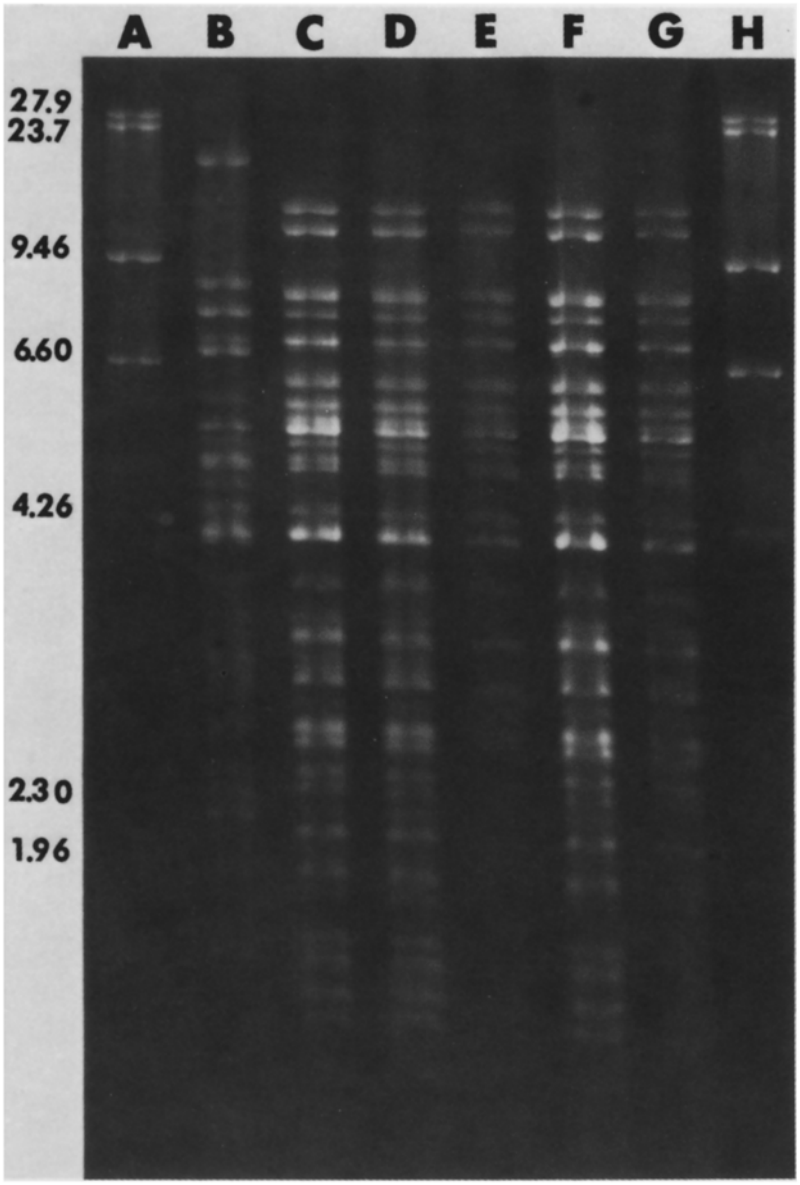

Fig. 3. Comparison of EcoRI restriction fragments of mtDNA from parental and somatic hybrids separated on 0.7% agarose gels. *Lane A* and *H:* HindIII digests of λ DNA; *lane B: Daucus carota* C-1; *lane C:* somatic hybrid CH3; *lane D:* somatic hybrid CH5; Lane E: somatic hybrid CH8; *lane F:* somatic hybrid CH20; *lane G: Daucus carota* HCl-3

metry and are used to calculate the net genome sizes, the values increased to 400 to 450 kb.

Discussion

The mtDNA of the parental strains of the PH4 somatic hybrid could be distinguished by the Sa*l*I restriction endonuclease. Sa*l*I digests of the mtDNA from the fusion hybrid PH4 produce 38 fragments larger than 1 kb (Table 4). Most of these fragments correspond to the sizes of those found in the parental mtDNA, but there are three new fragments (U in Table 4) not found in the parental mtDNA and four fragments for each parent are missing. Summation of the sizes of all the fragments found in the hybrid PH4 mtDNA gives a value approximately 100 kb larger than either parent. These observations suggest that the pattern of mtDNA fragments found in the PH4 fusion hybrid does not arise by simply adding the genomes together, but that recombination has taken place producing new fragments with the loss of some parental ones.

All eight of the PH fusion hybrid mtDNAs analyzed had identical restriction endonuclease patterns. These identical patterns could have arisen if the recombination were limited to specific sites on the mtDNA molecules such as repeats (Lonsdale et al. 1984; Palmer and Shields 1984). It is also possible that numerous new mtDNA arrangements are formed but that the one found in these hybrids allows maximal mitochondrial

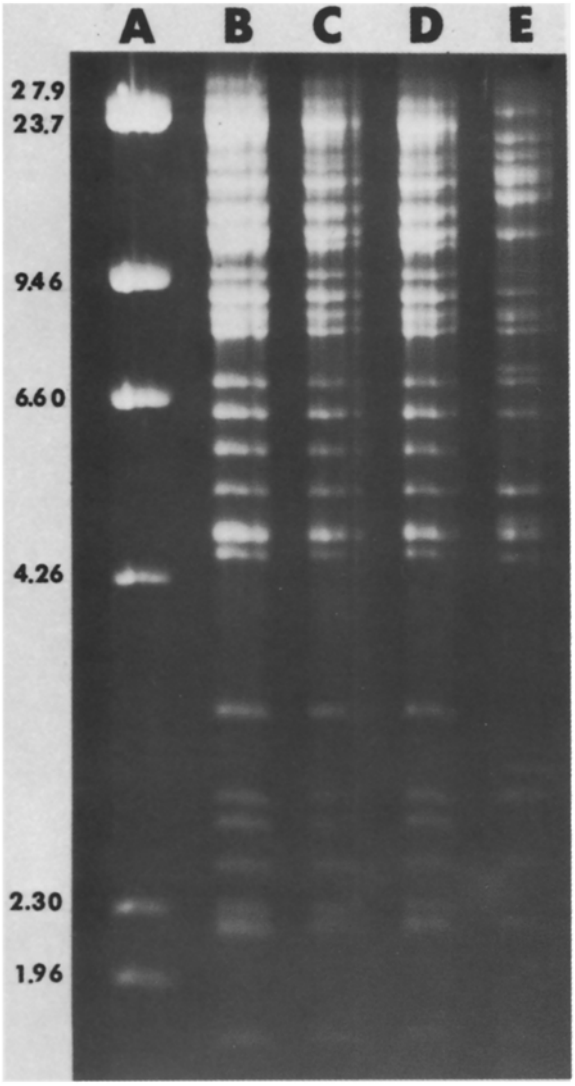

Fig. 4. Comparison of XhoI restriction fragments of mtDNA from parental and somatic hybrids separated on 0.7% agarose gels. *Lane A:* HindIII digest of λ DNA; *lane B: Daucus carota* PR; *lane C:* somatic hybrid PS4; *lane D:* somatic hybrid PS5; *lane E: Daucus carota* S232

Table 4. Fragment sizes (kb) produced by SalI digestion of mtDNAs from carrot cell strains PR, PH4 and HCl-3 determined from densitometer tracings of photographs of agarose gels following electrophoresis. HindIII fragments of lambda DNA were used as molecular weight standards * indicates that the fragment was shared by the three strains; a = shared by PR and PH4; b = shared by PH4 and HCl-3; U = unique to PH4

PR	PH4	HCl-3
	32.40 U	
	28.66 b	27.66 b
26.28 a	26.28 a	
26.57		
	24.25 b	24.25 b
	21.98 b	21.98 b
		20.53
19.26 *	19.26 *	19.26 *
17.78 a	17.78 a	
17.12		
	15.93 b	15.93 b
15.39		
	14.89 b	14.89 b
		14.42
13.55 *	13.55 *	13.55 *
13.00 a	13.00 a	
	12.26 U	
11.32 *	11.32 *	11.32 *
10.64 a	10.64 a	
10.15 *	10.15 *	10.15 *
8.62 a	8.62 a	
	8.37 b	8.37 b
	8.05 U	
6.64 *	6.64 *	6.64 *
5.95 *	5.95 *	5.95 *
		5.90
5.28 *	5.28 *	5.28 *
4.72 *	4.72 *	4.72 *
4.47 *	4.47 *	4.47 *
4.39 a	4.39 a	
		4.21
4.03 a	4.03 a	
3.90 a	3.90 a	
3.53 a	3.53 a	
3.42		
3.36 *	3.36 *	3.36 *
	3.09 b	3.09 b
	2.84 b	2.84 b
2.67 *	2.67 *	2.67 *
	2.58 b	2.58 b
	2.18 b	2.18 b
2.12 *	2.12 *	2.12 *
2.02 *	2.02 *	2.02 *
1.85 *	1.85 *	1.85 *
1.76 *	1.76 *	1.76 *
1.60 *	1.60 *	1.60 *

survival. The formation of identical hybrids should not have been due to formation from the same fusion event since the fused protoplasts were plated in agar-solidified medium after a 14 day incubation.

Our results differ from those of other workers since we found identical alterations in the eight somatic hybrids analyzed. In other studies with several species the mtDNA in the somatic hybrids was generally different from either parent and from each other (Belliard et al. 1979; Boeshore et al. 1983; Chetrit et al. 1985; Galun et al. 1982; Nagy et al. 1981, 1983). The reasons for the identical recombination in our studies with *Daucus* and the variable changes in the other cases listed is not known but one possibility is that different species may have different recombination mechanisms.

The other two somatic hybrid combinations where the parental mtDNA could be distinguished by restriction endonuclease analysis (CH and PS hybrids), did not show new mtDNA patterns, but the hybrids contained mtDNA identical to that of one of the parents.

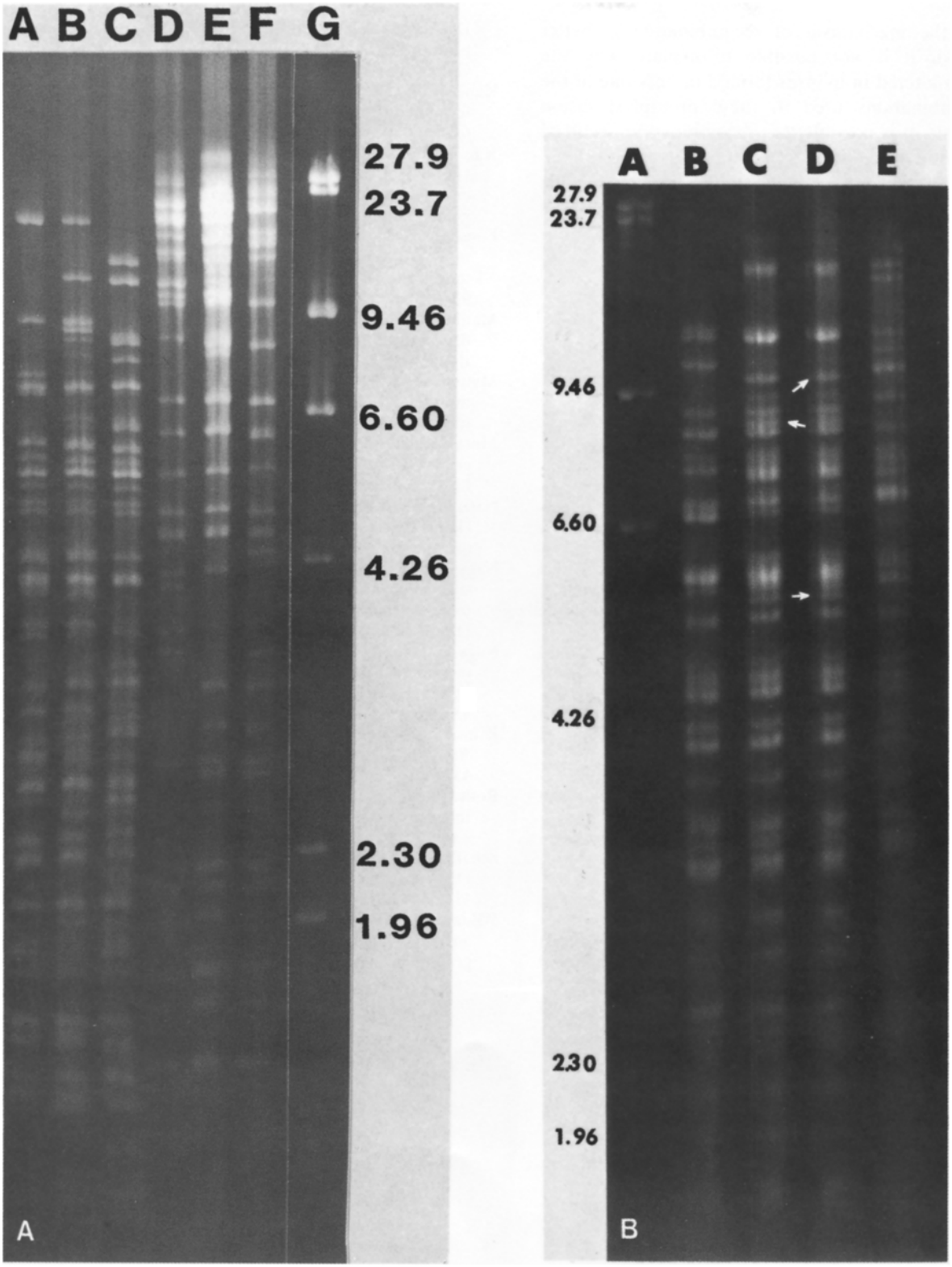

Fig. 5. **A** Comparison of restriction fragments of mtDNA from parental and somatic hybrids separated on 0.7% agarose gels. *Lane A: Daucus carota* PR, EcoRI; *lane B:* somatic hybrid PH4, EcoRI; *lane C: Daucus carota,* HCl-3, EcoRI; *lane D: Daucus carota* PR, SalI; *lane E:* somatic hybrid PH4, SalI; *lane F: Daucus carota* HCl-3, SalI; *lane G:* HindIII digest of λ DNA. **B** Comparison of BamHI restriction fragments of mtDNA from parental and somatic hybrids separated on 0.7% agarose gels. *Lane A:* HindIII digest of λ DNA; *lane B: Daucus carota* PR; *lane C:* somatic hybrid PH4; *lane D:* somatic hybrid PH5; *lane E: Daucus carota* HCl-3. *Arrows* indicate fragments unique to both hybrids

Until the mechanism of recombination is better understood, it is not possible to explain why the mtDNA is altered in hybrids formed in only one of the three combinations used in these protoplast fusion experiments. It is possible that recombination occurred but was not detected or was prevented for some reason. It is unlikely that the CH and PS lines are not hybrids since the selection system produced doubly resistant lines only from fused protoplasts and the chromosome numbers are the additive number.

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