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A specific rearrangement of mitochondrial DNA induced by tissue culture

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Summary. The induction, growth and regeneration of sugar beet callus to whole plants were all found to be highly genotype-specific. Regenerants of one line (of sterile cytoplasm) were obtained and a study of the chloroplast and mitochondrial DNA in these somaclones was undertaken by gel electrophoresis and cosmid hybridization. In one somaclone a rearrangement in the mitochondrial genome was observed; the novel arrangement of this part of the genome was identical to the corresponding area of the genome of the normal cytoplasm though it was otherwise of sterile type. This suggests that mitochondrial DNA may have a propensity to undergo certain types of rearrangement.

Key words: Mitochondrial DNA – Somaclonal variation

Introduction

Plant material regenerated from protoplasts and callus has been shown to sometimes undergo genetic changes ranging from variation in chromosome number and structure to single gene changes (Edallo et al. 1981; Prat 1983; Evans and Sharp 1983). The majority of these genetic mutations have been localized to the nucleus and reports of such 'somaclonal variation' being attributable to the chloroplast and mitochondrial DNAs are less frequent. Here we report experiments undertaken to regenerate somaclones of sugar beet from callus culture. We demonstrate that one such somaclone has undergone a rearrangement in its mtDNA.

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Materials and methods

Material used comprised partially inbred lines (listed in Table 1) from the Plant Breeding Institute sugar beet breeding programme. Standard culturing techniques were used. Short segments of influorescence carrying one or two closed flower buds were removed from flowering plants for culturing. Explants were surface-sterilized by treating for 20 min in 5% sodium hypochlorite followed by 15 s in 70% ethanol. After drying on autoclaved blotting paper and the removal of bleached tissue, they were transferred to medium. Syringe filters (0.2 μm) were from Nalgene. Expandable peat pellets were of the 'Jiffy-7' type.

Indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), α-naphthaleneacetic acid (NAA), kinetin, and all vitamins were from Sigma and gibberellin A_3 (GA_3) was from Calbiochem. Casein hydrolysate and agarose were from Sigma, bacto agar was from Difco.

Media preparation

All media used were based on that described by Kao and Michayluk (1974). The final compositions of media used are shown in Table 2. Hormones (with the exception of BAP, which was added beforehand) were sterilized by passing through 0.2 μm syringe filters and added after autoclaving.

Isolation of total plant DNA

Total DNA was isolated from fresh green leaf tissue; approximately 2 g was used for each isolation. Tissue was frozen in liquid nitrogen and ground to a uniform powder with a pestle and mortar and the help of a little acid-washed sand. Excess nitrogen was allowed to evaporate and the powder was transfered to a 50 ml polypropylene centrifuge tube containing 5 ml of buffer (0.1 *M* TRIS-HCl pH 8.0; 0.05 *M* EDTA, 0.1 *M* NaCl, 2% SDS, 0.1 mg/ml proteinase K). Tubes were incubated at 37 °C for 1 h with occasional swirling, and the contents were then extracted twice with phenol and once with a phenol/chloroform mixture before ethanol precipitation of the nucleic acids and recovery by centrifugation at 3600 × g. Nucleic acids

Table 1. Response of 23 genotypes to tissue culture

Line	A	B	C	D	E	F	G	H
83 RU 80	S							
83 RP 1-69	S							
82 RP 1-25	S							
83 SD 1-74 (monogerm)	N							
83 SD 1-74 (multigerm)	N	+	3	3	2	2		
83 RD 1-74	S	+	3	3	2	3	+	
81 RD 1-23	S							
82 RD 2-12	S	+	2	1	1	–		
82 RP 16	S	+	3	2	2	1	+	+
81 SW 10	N	+	1	4	1	2		
82 SU 82	N							
82 SP 117	N							
81 RA 7	S							
81 RA 674	S							
81 SA 9-9	N							
82 RF 11-4	S	+	2	3	3	–		
82 SP 112	N							
83 RW 3-31	S							
82 SP 41	N							
82 RP 41	S	+	3	3	2	3		
80 RB 585	S							
80 SB 585	N							
3 FA 80	N							

A, Cytoplasm; B, successful induction of callus; C, nature of callus; 1 – watery, 2 – medium structure, 3 – well structured; D, colour of callus; 1 – yellow, 2 – dark yellow, 3 – light brown, 4 – brown; E, speed of proliferation of callus; 1 – slow, 2 – medium, 3 – fast; F, growth rate in response to auxin; 1 – no response, 2 – medium response, 3 – considerable response; G, response to induction of organogenesis (see text); H, regeneration of somaclones

Table 2. Media preparation

Basic components (mg/l)

NH_4NO_3 250; KNO_3 2,500; $CaCl_2 \cdot 2H_2O$ 450; $MgSO_4 \cdot 7H_2O$ 250; $NaH_2PO_4 \cdot H_2O$ 150; $CaH_4(PO_4)_2 \cdot H_2O$ 100; $(NH_4)_2SO_4$ 134; Na_2EDTA 335; $FeSO_4 \cdot 7H_2O$ 276; H_3BO_3 10; $MnSO_4 \cdot H_2O$ 10; $ZnSO_4 \cdot 7H_2O$ 2; KI 0.75; $Na_2MoO_4 \cdot 2H_2O$ 0.25; $CuSO_4 \cdot 5H_2O$ 0.025; $CoCl_2 \cdot 6H_2O$ 0.025; Nicotinic acid 1; Thiamine HCl 10; Pyridoxine HCl 1; Casein hydrolysate 250; Sucrose 20,000

Additional components used in individual media (mg/l)

Medium	Z-I	Z-P0	Z-P1
Bacto agar	10,000	10,000	
2,4-D		0.3	0.3
BAP	0.25	0.1	0.1
Medium	**Z-R1**	**Z-R2**	**Z-R3**
Agarose (type I)	7,000	7,000	
IAA	0.1	0.1	
IBA			0.1
BAP	2	5	5
GA_3	0.2	0.2	0.2
Medium	**Z-K**	**Z-W1**	**Z-W2**
Bacto agar	10,000	10,000	10,000
IBA		3	
NAA			1
Kinetin	0.3		
Medium	**Z**		
Bacto agar	10,000		

All media were used at pH 5.8

were redissolved in 0.4 ml NTE (10 m*M* TRIS-HCl pH 8.0, 10 m*M* NaCl, 1 m*M* EDTA) and incubated with 100 µg/ml RNase A and 1000 units/ml RNase T_1 at 37 °C for 1.5–2 h to remove RNA. Subsequently, the DNA was extracted twice with a mixture of phenol/chloroform, ethanol precipitated in a 1.5 ml polypropylene Eppendorf tube and redissolved in 0.2 ml NTE.

Digestion of DNA with restriction endonucleases

Restriction endonuclease digestions were carried out under the conditions specified by the enzyme supplier. Typically up to 8 µg of total DNA were digested in a reaction volume of between 25 and 50 µl. A ten-fold excess of enzyme was usually used. Digestions were stopped by the addition of 10–20 µl gel loading buffer (0.1 *M* EDTA pH 8.0, 0.025% bromophenol blue, 50% glycerol).

Gel electrophoresis

DNA was fractionated in 0.8%–1% horizontal submarine agarose gels in 'TAE' buffer in the presence of 0.5 µg/ml ethidium bromide. After electrohoresis, gel were washed in 'TAE' buffer to remove excess ethidium bromide and the DNA was visualized on a 300 nm transilluminator. Blotting onto Gene Screen Plus was undertaken according to the manufacturer's recommendations.

Radioactive labelling of DNA and hybridizations

DNA was labelled by random priming using α-^{32}P-dATP (New England Nuclear, >800 Ci/mMol; Feinberg and Vogelstein 1983). Unincorporated nucleotides were separated from labelled DNA by chromatography on a 1.2 ml, 7 cm Sephadex G100 column.

Hybridizations with Gene Screen Plus were done according to the recommendations of the manufacturer. After hybridization, filters were washed twice in 3 × SSC, 0.5% SDS at 65 °C and once in 0.3 × SSC, 0.5% SDS at 65 °C and then finally rinsed in 0.3 × SSC at 65 °C to remove SDS. Each wash was for approximately 30 min. Filters were then air-dried and allowed to expose Kodak XAR-5 film at room temperature or at –80 °C using intensifying screens ('Cronex Lightening Plus', Dupont).

Hybridized DNA was removed from filters by 'stripping' in 0.4 *M* NaOH at 42 °C for 30 min, with subsequent neutralisation also at 42 °C in 0.2 *M* TRIS-HCl at pH 7.5, 0.1 × SSC, 0.1% SDS for 30 min.

Results

Callus induction on tissue cultures

Flower buds from a wide range of sugar beet genotypes were brought into in vitro culture with the aim of inducing callus and regenerating somaclones (Table 1). For each line, eight to ten cultures were established on medium Z-I; they were examined frequently over the first 4 months of growth, developing buds being removed to establish shoot cultures. Development of callus was sub-

sequently observed for seven lines, often not until at least 2 months after the establishment of the culture. The appearance and nature of these calluses are noted in Table 1.

Callus growth in the presence of auxin

Callus was cultured on plates using Z-P0 and in suspension using Z-P1 to observe its growth in the presence of auxin. Suspension cultures were agitated at approximately 100 rpm. Results for Z-P0 and Z-P1 were similar (Table 1).

Induction of organogenesis in callus

Attempts to induce organogenesis in callus for the regeneration of whole plants were made by plating callus from cultures on media Z-R1, Z-R2 and Z-R3. Only Z-R1 gave positive results. The lines which responded to this medium were:

(1) 83 RD 1-74. After 1 week on Z-R1, root structures bearing root hairs formed on several calluses of this genotype. These structures could not be maintained.

(2) 82 RP 16. After 3–4 weeks, green areas were observed on one callus on one plate. Shoot tips were visible 4 days later. These were subsequently regenerated to whole plants. The organization of callus into shoots was observed under the binocular dissecting microscope; they appeared to have multicellular origins. The regenerating callus was spread to several different media to observe regeneration.

Regeneration of callus of line 82 RP 16

Establishment of a regenerating cell line. Callus from line 82 RP 16, which was undergoing organogenesis and producing shoot structures, was transferred to fresh medium (Z-R1) and to two further media (Z, Z-I) to observe its continued regeneration. In each case organogenesis continued and new shoots were formed; in fact, it was not possible to distinguish between the behaviour of the regenerating callus on these three media. It seemed that once a specific hormonal regenerating stimulus (medium Z-R1) had been received, the callus continued undergoing organogenesis unaffected by its hormonal environment.

Subculture of shoot structures. Shoot structures which had developed on callus and which had grown to up to 1 cm in size were transferred to media Z, Z-I and Z-K in 60 ml specimen containers to encourage rapid shoot growth. Development was similar on each of these three media. After a period varying between 2 weeks and 2 months, plantlets were 4–6 cm in size. Some had visible root systems and were transferred directly to peat (see below). Others were transferred to Z-W1 or Z-W2 for rooting and subsequently to wetted expandable peat pellets and placed in large kilner jars with loose fitting tops and incubated at 22°–24 °C and 18 h light. There seemed to be little correlation between medium and rooting success. When plants had grown to approximately 12 cm, they were transferred (including peat pellet) to 13 cm pots with compost and placed in the glasshouse. Plants were grown on for several weeks before being vernalized for 16 weeks at 5°–8 °C and 16 h light in growth rooms, and were later returned to the glasshouse and brought to flower. A total of 30 plants or 'somaclones' reached this stage. All retained the sterile phenotype of the starting material.

Analysis of chloroplast and mitochondrial DNA of somaclones.

Total DNA was extracted from green leaf material of pre-vernalization somaclones. Digestion of total DNA with SmaI (palindromic recognition site: CCC'GGG) cut preferentially the chloroplast and mitochondrial DNA components but not methylated restriction sites in the nuclear DNA. Fractionation of SmaI-digested total DNA in agarose gels gave a predominantly undigested high molecular weight component corresponding to the mostly uncut nuclear DNA and also a series of bands representing a recognisable SmaI chloroplast profile, visible in preference to the mitochondrial profile only because of the larger quantity of chloroplast DNA present in the cells. From the chloroplast profile the degree of completion of the digest could be established: this was necessary information for the interpretation of subsequent hybridizations of mitochondrial cosmids to the filter-bound digests. Digested DNA was transferred to Gene Screen Plus and probed sequentially with cosmids 4D7, 6A8, 3H5, 6H9, 6H1, 1A8, 7B3, 6C11, 8A1, 5A3, 5E3 and 7C7. These cosmids span the entirety of the mitochondrial genome of the sterile cytoplasm, the complete physical map of which has already been determined (Brears and Lonsdale 1988). After each probing, the hybridized DNA was removed by stripping.

Comparison of the SmaI chloroplast profiles, visible in the ethidium bromide-stained gels, showed no variation from the expected profile (Brears et al. 1986), and clearly no major rearrangements of ctDNA had occurred affecting the SmaI restriction profile.

For all but one of the somaclones, the hybridization pattern produced by each of the 12 mitochondrial cosmids was identical, and in these cases no rearrangement in the mtDNA detectable by SmaI restriction endonuclease digestion and gel electrophoresis was observed. In the case of somaclone 2, however, a rearrangement was observed, and this was detected by hybridization with

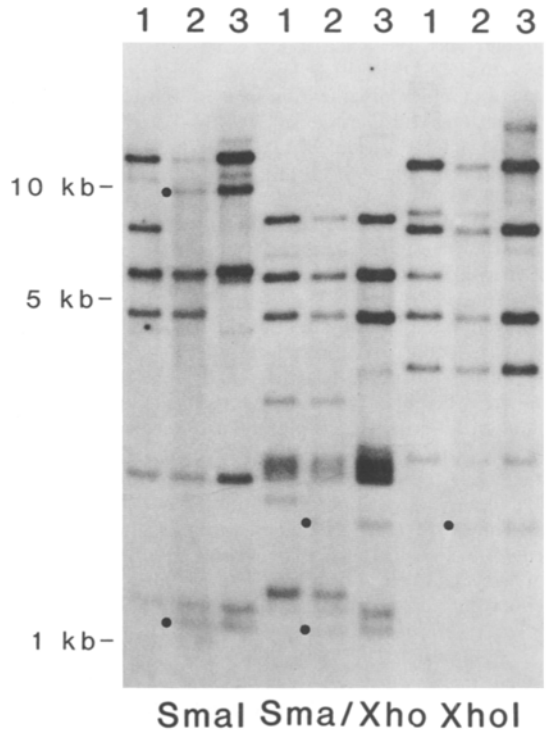

Fig. 1. Genomic rearrangement in a tissue culture regenerant. Total DNA from 82 RP 16 (S cytoplasm, 1) somaclone 2 (2) and 82 SU 82 (N cytoplasm, 3) was digested with SmaI, SmaI and XhoI together, and XhoI, and fractionated in 1% agarose. The DNA was transferred to Gene Screen Plus and the filter probed with radio-labelled cosmid 8G4. Somaclone 2 lacks a SmaI fragment of 7.35 kb and has instead homologous fragments of 9.5 and 1.1 kb. It also lacks a 5.62-kb XhoI fragment which is replaced by one of approximately 2 kb. The 2-kb XhoI fragment and the 1.1-kb SmaI fragment are also found on double digestion with SmaI and XhoI. All these fragments are found in the N cytoplasm digests (3) and presumably, therefore, somaclone 2 has undergone a genome rearrangement producing an organization similar to that found in the N cytoplasm at this locus

cosmids 1A8 and 7B3. Cosmid 1A8 did not hybridize to the predicted fragment of 7.35 kb (map coordinates 193.06–200.41; Brears and Lonsdale 1988), but hybridized instead to one of approximately 9.5 kb in somaclone 2. Cosmid 7B3 also failed to hybridize to the 7.35 kb fragment and hybridized instead to fragments of 9.5 kb and 1.1 kb.

To investigate the apparent rearrangement in greater detail, cosmid 8G4 (which spans the 7.35 kb SmaI fragment of the S cytoplasm more extensively on both sides than either c1A8 or c7B3) was used as a hybridization probe. Digests of total DNA with several restriction endonucleases allowed the comparison of plants carrying the sterile and the normal cytoplasm with somaclone 2 (Fig. 1). Each novel fragment identified in somaclone 2 was found to co-migrate with a corresponding cross-hybridizing fragment in the digest of N cytoplasmic DNA. From the previous analysis of the mitochondrial genome (Brears 1987), it was apparent that SmaI fragments of 9.5 and 1.1 kb were uniquely associated with this part of the genome in the normal cytoplasm and presumably the rearrangement in somaclone 2 had generated the normal cytoplasm arrangement at this locus. The rest of the genome of somaclone 2 was, however, found to be of sterile type, and the plant itself was of sterile phenotype.

Discussion

From the in vitro culture of a wide range of sugar beet lines, callus induction, growth and organogenesis were highly genotype-specific. Of the 23 lines brought into culture it was possible to induce callus on only seven. The calluses of five lines were tested for their response to auxin and found to vary considerably in their growth rate; this was presumably due to genotypic variation. Organogenesis was only observed in the callus of two genotypes. In one, root structures formed and in the other, shoot structures formed. The specificity of response of different genotypes to in vitro culture was observed at all stages. A similar specificity has been observed in a number of other plant species. For example, in bird's-foot trefoil (Glover and Tomes 1982) and lucerne (Bingham et al. 1975), the regeneration potential of callus has been shown to be heritable, and in lucerne, three cycles of selection improved the regeneration ability from 12% to 67% of genotypes in culture.

The organogenesis observed in sugar beet line 82 RP 16 was believed to be multicellular. In other studies, organogenesis in sugar beet callus has been found to be both by somatic embryogenesis and from multicellular origins (B.V. Ford-Lloyd, personal communication). The same has been found for other species and, at least in maize, has been linked to genotype. For example, using inbred lines of maize, Green and Phillips (1975) found three cultivars capable of multicellular organogenesis and only one to undergo somatic embryogenesis. If it is assumed that multicellular organogenesis of callus gives chimeric-regenerated plants, then presumably the different 'cell types' of an individual plant may be independently somaclonally variant and somaclones derived from a single cellular source will not exist until the subsequent generation.

Restriction endonuclease analysis of chloroplast DNA in the somaclones revealed no rearrangements, though this was based on a very limited analysis using a single restriction endonuclease. This lack of observable variability in chloroplast genomes is consistent with other studies. It reinforces a view that chloroplast genomes have a stable organisation with little tendency to undergo rearrangement: this may be due in part to the lethality of disrupting the closely packed transcriptional units of the chloroplast genome.

The observed alteration in the mitochondrial genome in one of the somaclones is of interest for two reasons. Firstly, tissue culture appears to stress mitochondrial genome organization. Variation in restriction fragment stoichiometries (McNay et al. 1984; Grayburn and Bendich 1987; Rode et al. 1987), appearance of novel restriction fragments (Kemble and Shepard 1984) and the disappearance of previously existing fragments (Rode et al. 1987) have all been reported. Secondly, in maize where genome structure and organization is well-understood, alterations in genome organization associated with different cytoplasms (Small et al. 1987) or those occurring as a direct result of the fertility phenomenon associated with the S and T cytoplasm appear to be limited to specific types (Schardl et al. 1985; Umbeck and Gengenbach 1983). In the T cytoplasm of maize, e.g., fertility reversion involves the deletion of a gene, T-*urf13* (Rottmann et al. 1987). This deletion reconstitutes a restriction fragment which is normally only found in the normal (N) fertile cytoplasm (Fauron et al. 1987). Similarly, in sugar beet, the observed alteration in the mitochondrial profile reflects the organization seen in the mitochondrial genome of the fertile cytoplasm though, unlike in maize, no phenotypic alteration is observed.

Clearly the rearrangement which has occurred at this locus in cell culture is similar to one of the several rearrangements which has distinguished the N cytoplasm from S. Presumably, therefore, mitochondrial DNA may have a propensity to undergo specific types of rearrangement. Such events undoubtedly lead to the rapid evolution of mitochondrial genome organization (Palmer and Herbon 1988; Small et al. 1987) and probably are promoted by recombination events occurring between short repeated DNA elements. The formation of chimeric genes such as T-*urf13* in maize (Rottmann et al. 1987) and *Pcf* in *Petunia* (Young and Hanson 1987) are the likely result of recombination events. By destabilizing mitochondrial genome organization, for example in cell culture, it may be possible to induce the formation of novel chimaeric genes, which may in turn affect plant phenotype.

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