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On the occurrence of somatic meiosis in embryogenic carrot cell cultures

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Abstract During the establishment of an embryogenic cell line from a carrot hypocotyl explant, processes closely resembling meiotic divisions are seen. A microdensitometric analysis revealed that the amount of cellular DNA diminished in the majority of cells to the haploid level. However, the diploid level was re-established in a matter of a few days. The genetic consequences of this segregation were studied by analyzing restriction fragment length polymorphisms (RFLP) and randomly amplified polymorphic DNAs (RAPD). The results showed that the great majority of embryos regenerated from segregants and that different segregants had different genetic constitutions.

Key words *Daucus carota* L. · Somatic segregation
Somatic embryogenesis · Somaclonal variation

Introduction

It is an established fact that somatic cell cultures show an excess of recessive genetic variants (Siminovitch 1976; Terzi and Sung 1986; Terzi 1974). This is true both in animal and plant somatic cells in vitro, where recessive mutants appear with frequencies far in excess of those expected on the basis of the square of the mutation frequency observed in haploid conditions. Abundant data exist to show that in somaclonal regenerants expression of recessive mutations is extraordinarily frequent (Evans 1989): in a recent study of somaclonal variation, 18 out of 19 mutated regenerants bred true for at least three generations (Gavazzi et al. 1987).

Various proposals have been put forward to explain this excess of recessives: suggested mechanisms range from small deletions or gene inactivation (Terzi 1974) to mitotic crossing over (Evans 1989), but the direct experimental evidence, obtained in attempts to quantify the importance of these various mechanisms, seems to favour a mechanism involving chromosome loss and reduplication (Campbell and Worton 1981).

To our knowledge these facts have never been related to meiosis, the occurrence of which in somatic cells was described long ago: chromosome segregation and reduction in somatic tissues – usually followed at once by a restoration of the diploid number – have been described in a number of cases (Nuti Ronchi et al. 1992a,b; see Huskins 1948 for older work). The phenomenon, however, as pointed out by Huskins (1948) is "very likely to be missed by the cytologist unless the organism has grossly heteromorphic pairs of chromosomes".

Segregation events leading to haploidy were recently shown to occur in embryogenic carrot cell lines (Nuti Ronchi et al. 1992a,b). In the present work we describe meiotic figures in somatic cells and compare them with those that appear during microsporogenesis. In addition randomly amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) patterns of pure lines, their sexual hybrid, and regenerants obtained from the latter via somatic embryogenesis, show that, in regenerants, segregation is indeed the rule rather than the exception. Hence somatic meiosis seems to be associated with embryogenicity or, more precisely, with its pre-requisite, viz. developmental totipotency.

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Materials and methods

Initiation of embryogenic cell lines

Embryogenic cell lines were initiated from hypocotyl explants according to de Vries et al. (1988), starting from carrot (cv. St.

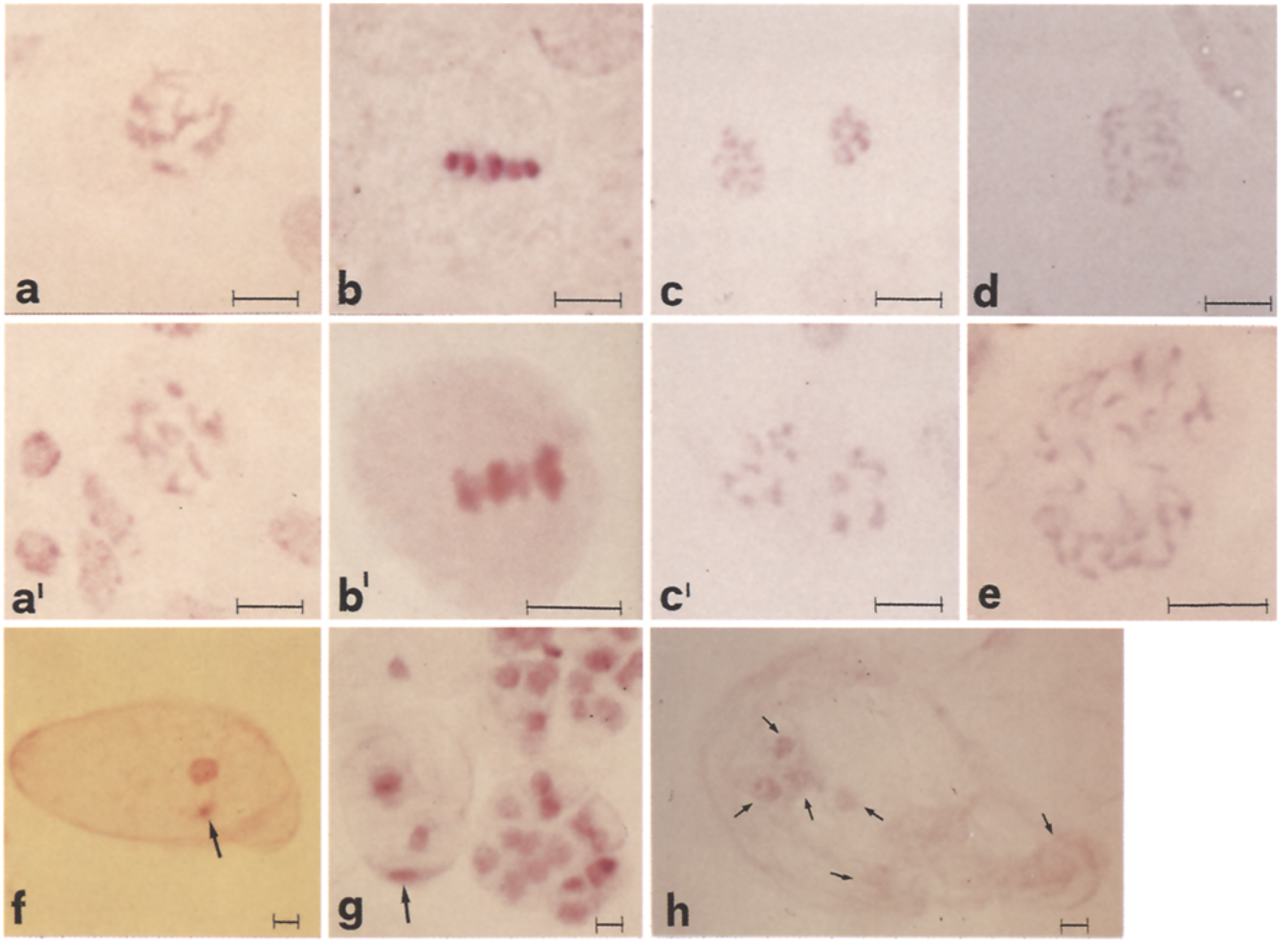

Fig. 1a–h Differentiation of gamete-like cells by means of reducing mechanisms. Different phases (**a**, **b**, **c**) of somatic meiosis in cultured carrot hypocotyls compared with the same phases (**a'**, **b'**, **c'**) observed during microsporogenesis in carrot anthers. **a**,**a'** Diakinesis, **b**,**b'** metaphase I, **c**,**c'** anaphase I. **d**,**e** Segregation of two haploid parental sets by a prophase chromosome reduction mechanism (Nuti Ronchi et al. 1992a,b); the precocious separation of the bipartite sister chromosomes restores diploidy (2C level DNA). The bottom row shows: **f** elongate pollen-like committed cell of the carrot C90 cell line with two nuclei of different sizes resembling the vegetative and generative (*arrow*) nuclei in an immature pollen cell. **g** Initial proembryogenic mass (PEM) formation: *left* further division of the larger nucleus in a pollen-like cell; the smaller nucleus is still evident (*arrow*); *right* a large PEM cluster with more cells of smaller size; **h** embryo sac-like cell with six nuclei (*arrows*); two others are out of focus. The cytological analysis was carried out on Feulgen-stained nuclei (Nuti Ronchi et al. 1984), counterstained, when necessary, with haematoxylin to reveal the cell wall. Bar represents 5 μm

Valery) hypocotyl explants kept for 20 days in B5 liquid medium (Flow laboratories). After that time the resulting cell suspension (C90) was subcultured every 14 days. Embryogenesis was induced by transferring cells at their 7th day, to hormone-free medium (for more details see Nuti Ronchi et al. 1984).

DNA cytophotometry

DNA cytophotometry was carried out on tissues or cells squashed on slides after fixation with 4% formaldehyde and 15 min digestion with 0.5% pectinase (Sigma) and then submitted to 5 N HCl hydrolysis for 1 h at room temperature. After Feulgen staining, slides were washed three times in SO_2 for 10 min and mounted in Eukitt. Feulgen/DNA absorption in individual nuclei (minimum 250 nuclei for each time point) was measured with the two-wavelength method (Patau 1952) using an MPV Compact Leitz microscope photometer equipped with an HP97S computer.

RFLP analysis

RFLP analysis was carried out using two pure lines, 218A and 218C, and their sexual hybrid, F1. From these lines, kindly provided by Zaadunie (Enkhuizen, Netherlands), plantlets were collected 1 week after germination and DNA extracted according to Murray and Thompson (1980). F1 plantlets were dedifferentiated and a cell line obtained. From this F1 cell line somatic embryos were regenerated: ten such embryos were dedifferentiated and DNA was extracted from the resulting lines. Five microgram samples of DNA from the above sources were loaded, after digestion with the appropriate endonuclease (*Dra*I, *Taq*I or *Cla*I) on an agarose gels, electrophoresed and blotted according to Sambrook et al. (1989). The probes used were three genomic clones, randomly selected from our carrot genomic library (Vergara et al. 1990) and labelled using a Boehringer Random Priming kit (250 ng of DNA, 40 μCi of ^{32}[P]dCTP, 3000 Ci/mmol); hybridisations were carried out at 42°C, 50% formamide, according to Sambrook et al. (1989).

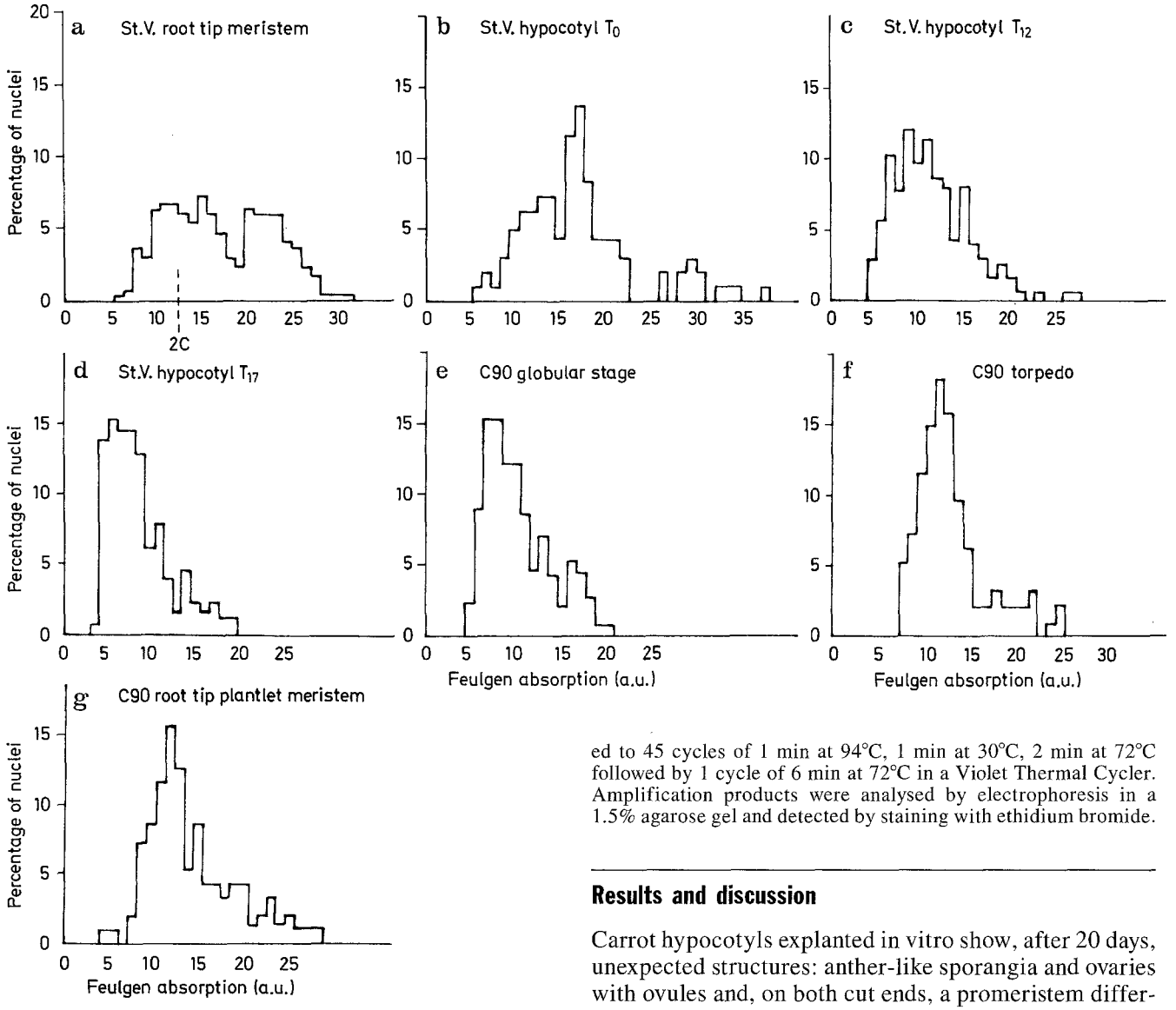

Fig. 2a–g Feulgen DNA absorption levels measured in individual carrot nuclei. Values are expressed in arbitrary units (A.U.). **a** Control carrot root tip meristems of cv. St. Valery (St. V.); **b** hypocotyls at the time of excision (T0); **c** same at 12 days; **d** same at 17 days. **e** Globular embryo stages differentiated from the cell line C90 originated from **d**; **f** meristems of regenerated torpedoes and **g** at plantlet stage.To provide an internal standard and convert Feulgen absorption units into picograms of DNA, diploid human lymphocytes (2C=6 pg) were analyzed on some slides in each experiment. The DNA content of meristematic root tip nuclei in the first peak of the distribution (13 A.U.) corresponds to 2 pg, which is the DNA content reported for 2C diploid carrot cells (Bennet and Smith 1976)

RAPD analysis

The same DNA samples as used for RFLP were used as templates for polymerase chain reaction (PCR) amplification with four different random primers (Oligo 1, 5′-CACGTGGACG-3′; Oligo 2, 5′-CGGCCCCTGT-3′; Oligo 3, 5′-TCGTCACTGA-3′; Oligo 4, 5′-GGTAGCAGTC-3′). Amplification reactions were performed in 25 μl volumes containing 0.2 μM primer, 0.1 mM dNTP, 45 ng genomic DNA, 0.5 units Taq polymerase and 1 × reaction buffer (enzyme and buffer supplied by Epicentre). Samples were subjected to 45 cycles of 1 min at 94°C, 1 min at 30°C, 2 min at 72°C followed by 1 cycle of 6 min at 72°C in a Violet Thermal Cycler. Amplification products were analysed by electrophoresis in a 1.5% agarose gel and detected by staining with ethidium bromide.

Results and discussion

Carrot hypocotyls explanted in vitro show, after 20 days, unexpected structures: anther-like sporangia and ovaries with ovules and, on both cut ends, a promeristem differentiating into floral primordia (a complete study will be presented elsewhere). Cells were extruded into the medium from the above structures: they showed features characteristic of chromosome reduction (somatic meiosis and prophase reduction), behaving like immature mononucleate pollen (Fig. 1f) or embryo sac cells (Fig. 1h).

Dividing cells showed homologous chromosome pairing and chiasma-like figures (Fig. 1a,b), very similar to those observed during microsporogenesis in vivo (presented for comparison: Fig. 1a′,b′). Anaphase I (Fig. 1c) with nine sister chromatids, was followed only rarely by the second equational division. At telophase I, in fact, the diploid chromosome number may be restored by precocious separation of sister chromatids, which leads to two 2C nuclei, which are distributed into two reduced cells by cytokinesis. The second division leading to haploid 1C cells is somewhat delayed, and it is normally achieved by means of a process of prophase reduction, which also occurs in cultured cells when unpaired chromosomes are segregated into two haploid sets (Fig. 1d,e) (see Nuti Ronchi et al. 1992a). These two reducing mechanisms

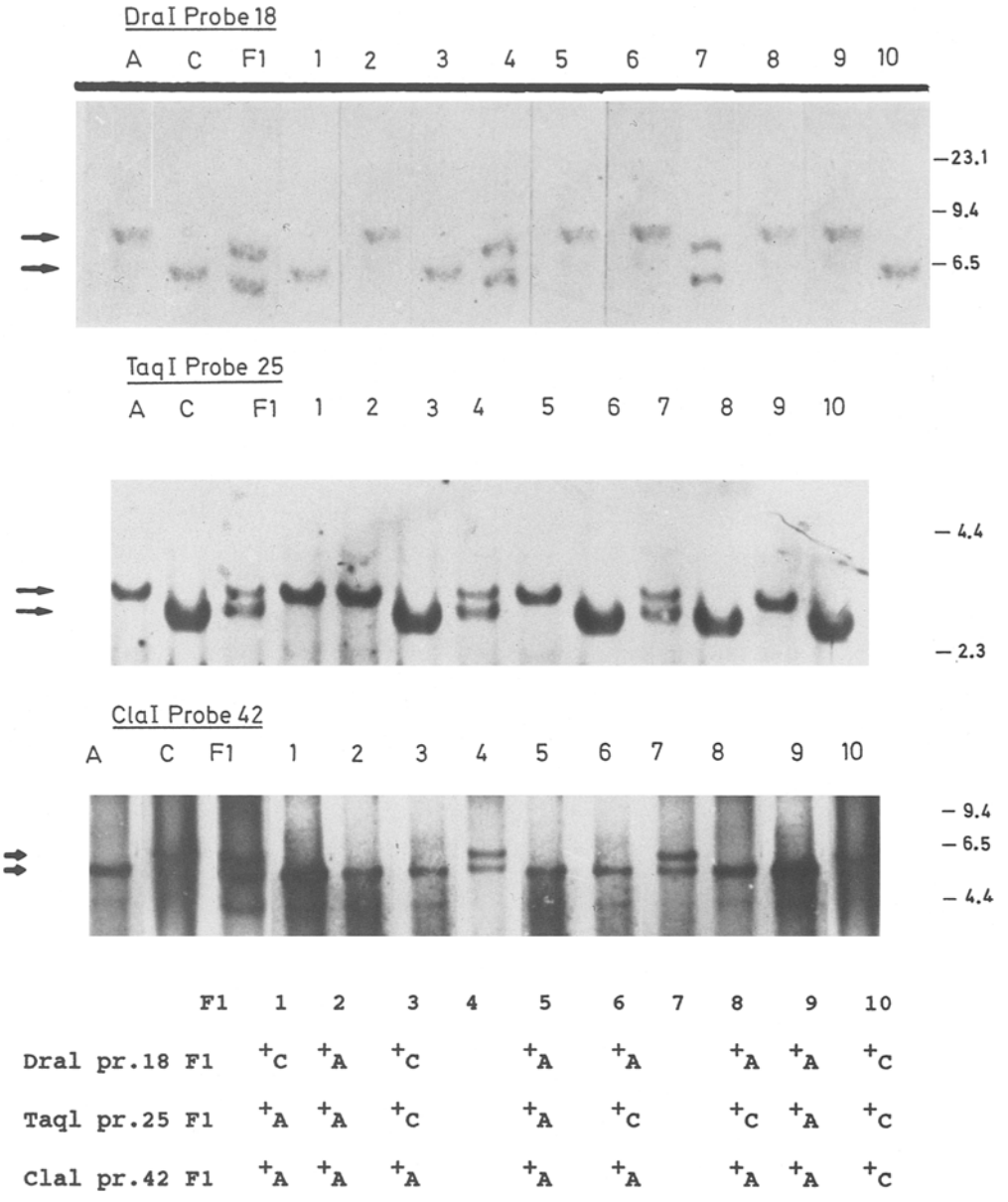

Fig. 3 Restriction fragment length polymorphism (RFLP) patterns shown by two parental lines (A and C), their sexual hybrid (F1) and ten cell lines established by dedifferentiating ten somatic embryos obtained from F1 hypocotyls. The different DNA sources were digested with restriction endonucleases and hybridized as indicated. The patterns obtained are summarized at the bottom: segregation of the parental types A and C is marked by +

have been discovered and studied previously in some stabilized carrot cell lines (Nuti Ronchi et al. 1992a,b) where they represent the only mode of cell division. However, genetic proof of segregation and of a possible relationship to the acquisition of embryogenic capacity was not obtained. In hypocotyls, the meiosis-like divisions have been observed to occur at a constant rate (1%–3%) in the presence of auxin; the committed embryogenic cells thus generated, elongate and undergo an asymmetrical division. The pollen-like cells divide, giving two nuclei of different sizes that resemble vegetative and generative nuclei (Fig. 1f). Apparently only the largest cell is able to divide further, thus originating a proembryogenic mass (PEM) (Fig. 1g). The other mode of formation of PEMs follows closely the first stages of development of carrot zygotic embryos (Borthwick 1931) and frequently occurs inside the embryo sac-like cell.

Cytophotometric DNA absorption analysis (Fig. 2) of nuclei of the hypocotyl-derived suspension culture and of the different developmental stages of regenerated embryos confirms the gradual appearance of a haploid cell population, presumably derived from the chromosome-reducing divisions (this was in agreement with chromosome counts). The same sort of cycling between a 1C- and 2C-type constitution has been described in detail elsewhere (Nuti Ronchi et al. 1992a). Figure 2b–d shows that the hypocotyls undergo a progressive reduction in the amount of DNA from the time of explant (t_0) to the moment when an embryogenic cell culture is generated (t_{17}). The trend is reversed in the progression from the cell line to a plantlet root meristem through the globular to torpedo-shaped embryonal stages (Fig. 2e–g).

The genetic result of somatic segregation followed by endoreduplication is generalized homozygosity. We have

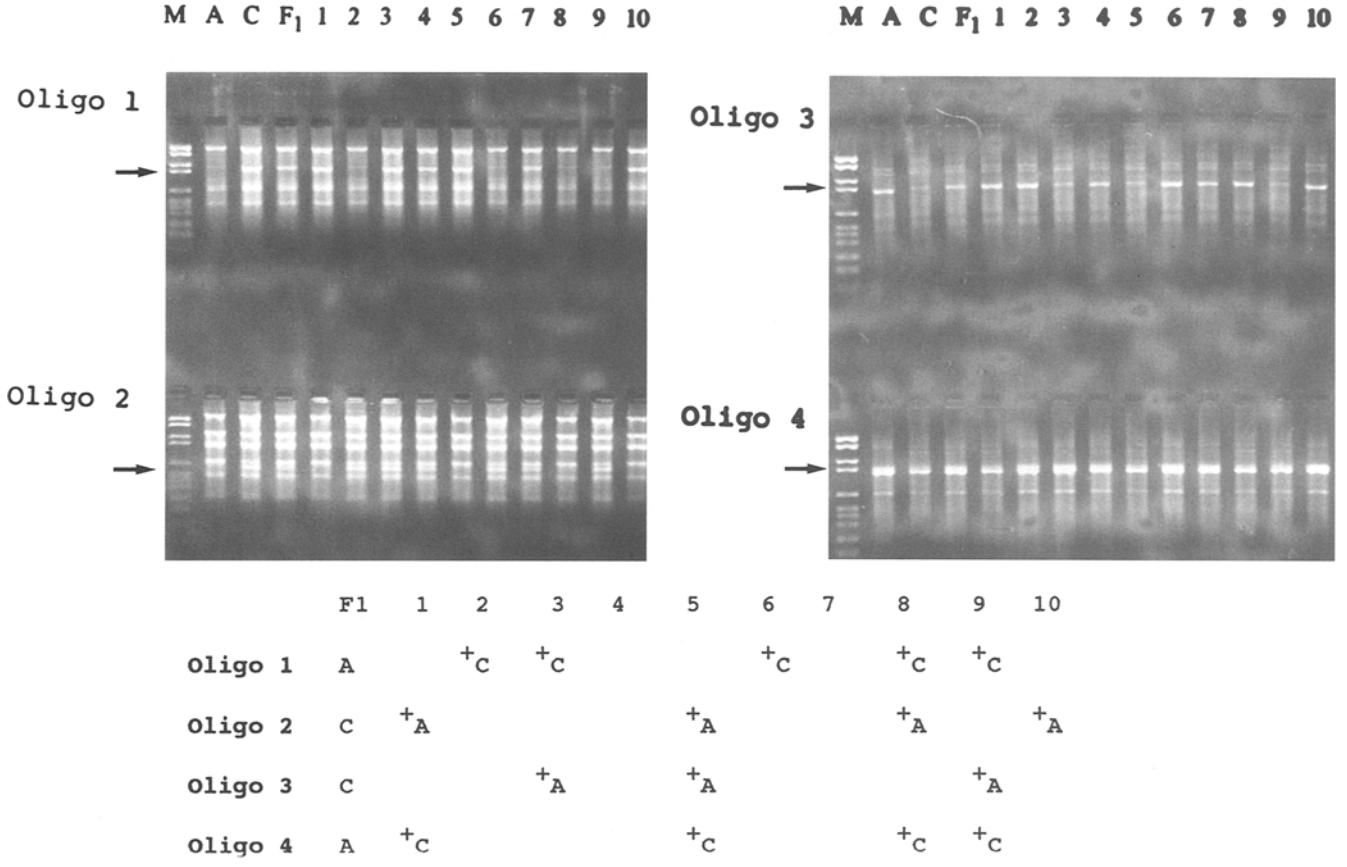

Fig. 4 DNA samples, as in Fig. 3, were amplified using the arbitrary primers, described in Material and methods, designated oligos 1 to 4. Polymorphisms are indicated by *arrows*. On the left of each pattern is shown the pBR328 *Bgl*I-*Hin*fI size marker. The patterns obtained were of parental types A and C (dominant/null expression); segregation is marked by + in the scheme at the bottom.

already said that this is a common observation at the phenotypic level but, in order to exclude complications caused by mechanisms preventing gene expression, segregation has to be demonstrated at the DNA level. This was done using two approaches: RAPD analysis, which is easier and faster, and RFLP analysis whose main advantage in this case is co-dominant expression of markers (Waugh and Powel 1992). For both approaches we used two carrot pure lines and their sexual hybrid, and considered the RAPD and RFLP patterns of the pure lines, of the hybrid and of cell lines obtained by dedifferentiating ten embryos regenerated from the sexual hybrid (somatic embryos derive from single cells; Backs-Husemann and Reinert 1970). Figure 3 presents the results of the RFLP analysis of the sexual hybrid and of the ten lines obtained from somatic embryos derived from it. As can be seen, lines 4 and 7 did not show segregation with any of the probes. All the other eight embryo lines, however, showed, in a random way, only the pattern characteristic of either of the parental pure lines.

The results of the RAPD analysis using four different primers are presented in Fig. 4, again comparing the sexual hybrid and ten lines obtained from somatic embryos derived from it. The expression of the RAPD pattern is not co-dominant as for RFLP, but of the dominant/null type. From Fig. 4 we see that segregation was apparent in 16 out of 40 patterns analysed. With oligo 1, which, in the hybrid, shows the parental A pattern, segregation is demonstrated in embryo lines 2,3,6,8,9; with oligo 2, showing the parental C pattern in the hybrid, segregation is demonstrated in 1,5,8,10; with oligo 3, showing the parental C pattern in the hybrid, segregation is apparent in 3,5,9; with oligo 4, showing the parental A pattern in the hybrid, segregation is apparent in 1,5,8,9. If the frequency of segregation (16/40) demonstrated with RAPD, is multiplied by two, to take into account the lack of co-dominant expression, we have with RAPD a frequency of segregation of 32/40, not much different from the 24/30 frequency obtained by RFLP analysis.

Two embryo lines (4 and 7) show no signs of segregation for any marker, including those of the RAPD analysis and may have derived from cells that had not undergone somatic meiosis: altered patterns of the meiotic process, which may be very frequent in plants, such as nuclear restitution due to failure of karyokinesis, failure of cytokinesis and nuclear fusion etc. (Miller 1963; Golubovskaya 1989), or a multicellular origin of those particular embryos may account for the result.

Co-segregation was not observed in our sample for any pair of markers. Therefore, the markers all seem to be non-syntenic: in other words the majority of the

genome is involved in these segregations and this practically excludes alternative explanations of our results based on selective DNA losses. The occurrence in culture of somatic segregation can explain at least in part the genetic variation known as somaclonal (Larkin and Scowcroft 1993) and may be of great interest to breeders.

Segregational events have already been confirmed to occur during hypocotyl or other organ culture of different species (tomato, sunflower, apple) that are however unable to perform somatic embryogenesis because of precocious degeneration of the gamete-like cells thus formed (Nuti Ronchi et al. 1992c). The data suggest that floral induction, inclusive of a meiotic process, can be expressed, in a plant explant, as a consequence of growth factor action and wounding, and that totipotency is expressed exclusively, or at least preferentially, by cells that have undergone a transition to the gametic condition in vitro.

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