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Ploidy reduction and genome segregation in cultured carrot cell lines. I. Prophase chromosome reduction

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Abstract

Cytological analysis of different carrot cell lines in culture has shown various cytogenetic anomalies generating new levels of ploidy and novel chromosome numbers. Polyploidy may be considered a reservoir of variability that can be released in the form of distinct new segregants of different ploidy. Mechanisms alternative to mitosis (reductional grouping, prophase chromosome reduction) operate from a polyploid state (possibly reached by means of endopolyploidy, endomitosis, nuclear fusion, or restitution nuclei) to generate new levels of ploidy and novel chromosome numbers necessary for selection to operate *in vitro*. The segregational phenomena require chromosome recognition in haploid set complements and abnormal behaviour of mitoses; the resulting chromosome variability suggests that chromosomes are arranged, in the resting nuclei, in an orderly and predictable manner.

The knowledge of the molecular events governing these mechanisms, and how to control them, would be of great help for future applications of plant cell culture.

Introduction

Numerical variations and rearrangements of chromosomes in plant cells in culture and their role in somaclonal variability of regenerated plants have been widely discussed and interpreted in the last few years, but very little is known of the mechanisms underlying the phenomenon. Genome change definitely plays a role in response to the *in vitro* conditions of growth, which may be considered as conditions of stress, but the kinetics and functions of polyploidization and depolyploidization events and their fate during growth and regeneration of plant cells *in vitro* are unknown.

Knowledge of cytological mechanisms adopted by cells in order to survive under conditions of stress and their molecular regulation may make the process of somaclonal variation and chromosome variability amenable to manipulation.

In this, and in the accompanying paper (Nuti Ronchi et al. 1992) we describe the results of a cytological analysis of some carrot cell lines, several years old or recently constituted, obtained from different laboratories. Although the small size of the carrot chromosomes does not offer easy material, we have been able to detect some cytological chromosome-reducing mechanisms, not described before in cell tissue culture.

Prophase chromosome reduction, reductional grouping and somatic meiosis, described in this and in the accompanying paper, also frequently occur in fast growing cells of other species cultured *in vitro* (Nuti Ronchi 1990). Similar phenomena have been reported in the past to occur in plants after chemical treatments (Huskins

1948; Mehra 1986) or under natural conditions, but often have been dismissed as due to bad fixations. The high frequency of their occurrence under tissue culture conditions has allowed their identification as an alternative to normal mitosis.

Materials and methods

Cell lines and growth conditions

Three different carrot (*Daucus carota* L.) cell lines were used:

- $E_2A_1C_6$: 6-year-old methotrexate (10^{-4} M)-resistant line (Cella et al. 1984) derived from Cv. Lunga of Amsterdam, non-embryogenic.
- A+: 4-year-old highly enbryogenic line, initiated from seedling hypocotyls of *D. carota*, cv. St. Valery.
- T_2: 2-year-old, highly embryogenic line obtained from a somatic embryo of A+ line.

Line $E_2A_1C_6$ was grown either in MS medium (Murashige & Skoog 1962) supplemented with 2.2 μM 2,4-dichlorophenoxyacetic acid (2, 4-D), or 1.1 μM kinetin or in MS growth regulator-free medium. Lines A+ and T_2 were grown in B5 medium (Gamborg et al. 1968) supplemented with 2.2 μM 2,4-D and 1.1 μM 6-benzyladenine (BA).

All the cultures were kept in flasks at 24°C on rotary shakers (80 rpm) under continuous light (6-12 $\mu mol\ m^{-2}\ s^{-1}$), and subcultured by transferring, in 50 ml of fresh medium every 14 days, 2 ml of settled cell volume after centrifugation (5 min at 1200 rpm).

Cell growth of the three lines was determined by measuring the settled cell volume in graduated sidearm flasks.

Induction of embryogenesis

To induce embryogenesis, subcultures of the different lines were filtered through two nylon sieves of 120 μm and 50 μm pore size 7 days after subculturing; the cell clumps (referred to as cellular units or C.U.) retained by the second filter were washed several times with a growth regulator-free medium and resuspended in flasks containing 50 ml of the same medium at a density of 3000 $C.U.ml^{-1}$.

Cytological analysis

At different times of culture, the cells and the embryos were fixed with Carnoy's fluid (ethanol:acetic acid 3:1; v/v), washed in distilled water, hydrolyzed in 1N HCl at 60°C for 7-9 min, stained with Feulgen's reagent and squashed on slides. The mitotic index was determined by counting 3000 nuclei per sample.

Chromosome numbers were determined in all lines at the 3rd day after subculturing: colchicine treatment was not necessary, since metaphases presented well-spread chromosomes. Determination of the presence of chromosome groups in the same cell was made by counterstaining, after Feulgen, with light-green or haematoxylin (Shandon haematoxylin Activity 2).

DNA cytophotometry

For DNA cytophotometry, cells or embryos were fixed in 4% formaldehyde, treated with a 5% aqueous solution of pectinase (Sigma) and squashed under a cover slip in a drop of 45% acetic acid. After hydrolysis in 5.0 M HCl for 60 min at room temperature, preparations were washed three times for 10 min each in SO_2 water prior to dehydration and mounting. DNA content was measured with the two-wavelength method (Patau 1952) with an MPV compact Leitz microdensitometer equipped with an HP97S computer.

As a standard to compare results and convert Feulgen arbitrary units into picograms of diploid DNA, human lymphocytes (2C = 6 pg) smeared in a corner of some slides in each experiment were measured.

Meristematic root-tip nuclei with the value 13 in arbitrary units (mean of the first peak of the curve of Fig. 2a) was close to the DNA content of 2 pg reported for diploid carrot cells (Bennett & Smith 1976).

Cloning experiments

Suspension cultured cells of line $E_2A_1C_6$ were filtered through two nylon sieves of 120 μm and 80 μm pore size 4 days after subculturing; the cells retained by the second filter were plated at a final concentration of 5000 cells ml^{-1} in 0.7%

agar (Oxoid n°3) solidified MS medium supplemented with 2.2 μM 2,4-D and 1.1 μM kinetin.

Three weeks later, 57 colonies were isolated, grown separately as calluses and then transferred to liquid medium. Cytological analysis was carried out on 50 of these $E_2A_1C_6$ clones.

Results

Prophase chromosome reduction mechanism

The proposed prophase chromosome reduction mechanism separates chromosomes into two or more groups, according to ploidy, during different prophase stages. The mechanism gives rise to two prophase-like nuclei situated close to each other in the same cell, often appearing still partly intermingled or partly divided (fig. 1a-c), with chromosomes being directly unthreaded into two or more prophase configurations, depending on the ploidy of the nucleus. The separation of the chromosome complements into two or more groups may lead later to their inclusion, as reduced nuclei, in separate new cells. Alternatively, they progress to metaphase and then directly to an interphase stage; anaphase figures are normally absent whereas a new cell wall is formed in between the two reduced nuclei.

Carrot lines T2 and A+ show these cytological features not only in the suspension cultures but also in regenerated globular and heart stage embryos. In cell line T2 the proposed mechanism appears to work in the haploid-diploid range separating two groups of 9 metaphase bipartite chromosomes; rarely two of 18 or two groups of 9 and 18(=27, the triploid number) are found. Line A+ presented a higher frequency of polyploid cells that could also separate more nuclei in prophase as shown in Fig. 1e-f.

Table 1 shows the chromosome number distribution of the cell lines T2 and A+. Both appear to have haploid cells, presumably arising by means of this separation of chromosome complements. Fig. 1 shows the separation of two haploid prophases (Fig. 1a), which may in most cases proceed to metaphases, i.e. two haploid metaphases from a dipoid nucleus (Fig. 1b-c).

It is worth noting that by this mechanism a haploid set of bipartite chromosomes segregating in prophase may restore the diploid number if the chromosomes separate into their constituent chromatids. Alternatively, an endoreduplication process may re-establish diploidy. The frequency of segregational events in lines T2 and A+ was (on 200 total analyzed mitoses per cell line) 45% and 30% of reduced prophases and 40% and 34% of reduced metaphases respectively, whereas total normal anaphases were only 0.4% for line T2 and 9% for A+.

In order to confirm the presence of a haploid cell population, a cytophotometric Feulgen/DNA absorption analysis in individual nuclei was performed (Fig. 2) in T2 and A+ cultures and in T2 globular and heart-stage embryos.

A peak of 1C-2C values confirms that the cell population of lines T2 and A+ is mostly cycling in the haploid-diploid range (an event that also has positive evidence from chromosome counts) (Table 1). Besides the presence of haploid nuclei, Fig. 2 shows that, compared to the control carrot root-tip meristem nuclei, the DNA content of individual nuclei of T2 (Fig. 2b) and A+ (Fig. 2d) cultured cells and regenerated T2 embryos (Fig. 2c) is shifted to lower values. In fact the mitoses, indicated in dark in the histograms, were either haploid (n = 9) or diploid (2n = 18). In all cases their DNA content was much lower than expected when compared to the values of carrot root-tip mitoses.

Table 1. Chromosome number distribution and frequencies in T_2 and A+ suspension cell cultures and regenerated embryos.

Cell lines	Cell number	Chromosome numbers				
		n = 9	2n = 18	3n = 27	4n = 36	5n – 6n = 45 – 54
		Frequency (%)				
T_2 cells	308	24.0	68.8	1.9	4.2	1.6
T_2 embryos	103	38.8	60.1	0.9	0.9	0.0
A+ cells	142	9.8	69.0	1.8	14.7	3.5
A+ embryos	116	9.4	73.2	1.7	12.9	2.5

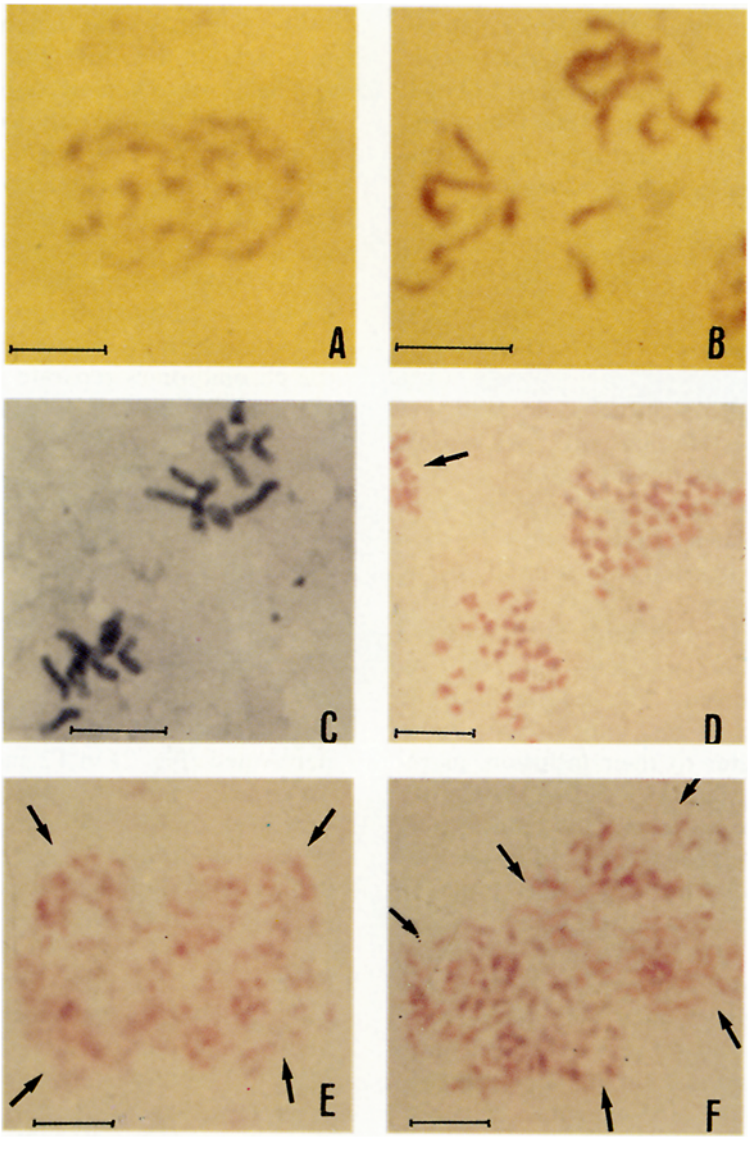
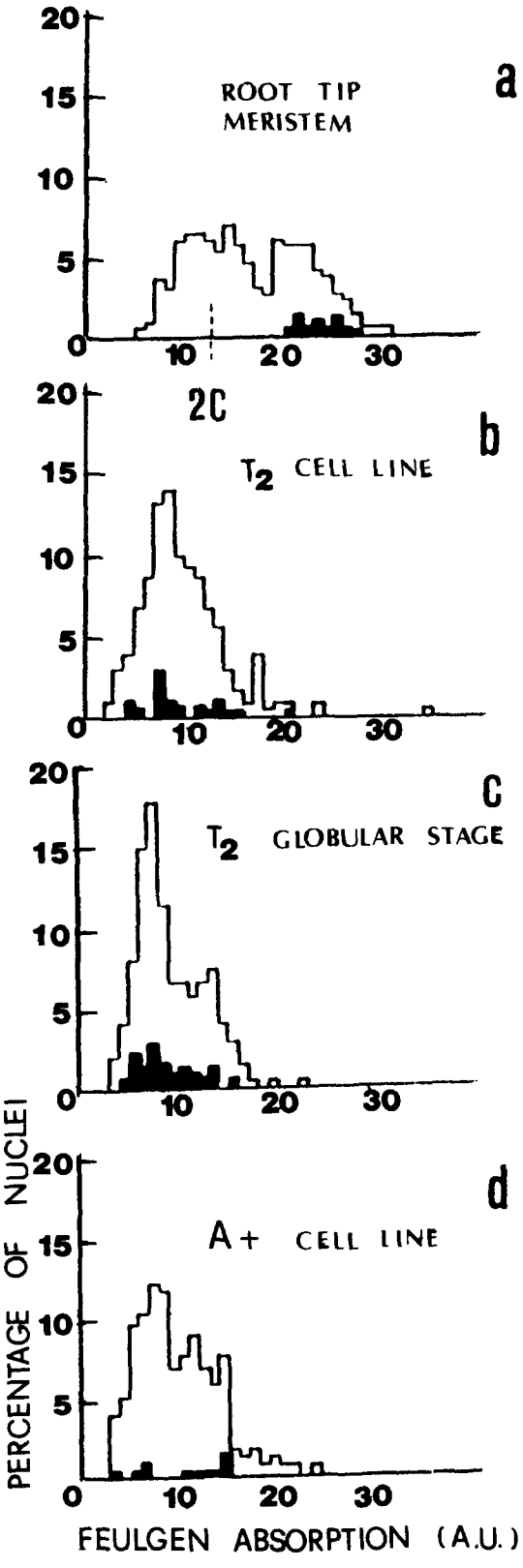


Fig. 1. Prophase-chromosome reduction and reductional grouping in carrot cell lines. (*A*) segregation of two haploid chromosome sets during prophase in T2 line; (*B* – *C*) two subsequent phases of the prophase-reduction nuclei as in (*A*), resulting in (*B*) two separate prometaphase and (*C*) two metaphase haploid groups in line T2; (*D*) reductional grouping in line $E_2A_1C_6$ resulting in three metaphase groups of n = 9 (arrow), 3n = 27 and 4n = 36 chromosomes in the same cell; (*E* – *F*) prophase-like chromosomes, still partly intermingled separating more reduced chromosomes complements from highly polyploid nuclei. Arrows point to the different groups. Bars = 5 μm.

Reductional grouping mechanism

A variant of the previously described prophase chromosome reduction mechanism was studied in the 6-year-old nonembryogenic carrot cell line $E_2A_1C_6$. In this line highly polyploid nuclei are split, after prophase, directly in numerous arrested metaphase chromosomes (Fig. 1d) arranged in groups in the same large cell. The arrested metaphases represented 92% of the mitotic figures analyzed (no pretreatment with colchicine was necessary), normal anaphases being almost absent (7.5%). The consequent chromosome variability of the cell line is shown in Fig. 3,



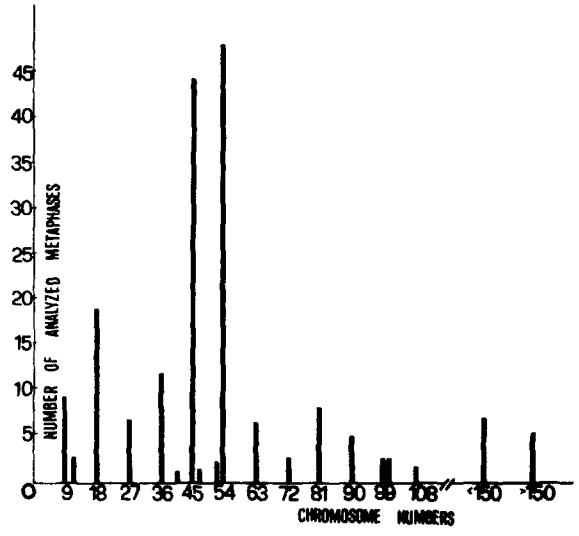


Fig. 3. Distribution of chromosome numbers of cell line $E_2A_1C_6$. Total analyzed metaphases: 182. The carrot haploid number 9 is represented in 5% of the metaphases. Different chromosome numbers in separate groups could be found in the same cell.

chromosome numbers falling in the range of multiples of the haploid number n = 9, this last number being present in 5% of the metaphases. Table 2 shows the distribution of metaphases with groups of chromosome of different ploidy. About 10% of the metaphases had groups of different chromosome sets, multiples of the haploid number 9. Chromosome groups of different ploidy could vary from a minimum of 2 to 6 or more. As shown in Fig. 3, aneuploidy could occur particularly in the case of higher polyploidy.

Fig. 1d shows three metaphases (haploid, triploid and tetraploid) in the same cell. It is worth noting that this last figure shows that all chromosomes are endoreduplicated, therefore indicating that in line $E_2A_1C_6$ DNA endoreduplication is a

Fig. 2. Nuclear DNA content of line T2 cells, its regenerated embryos and line A+ cells compared to carrot root tip meristem. (Clear bars) Feulgen/DNA absorption in arbitrary units (a.u.) measured in individual nuclei of (*a*) root-tip of cv. St. Valery seedling meristems, (*b*) T2 cell line, (*c*) globular regenerated embryos of T2, and (*d*) cell line A+ (minimum 250 nuclei at each time). (Solid bars) Mitoses could be either haploid (n = 9, 2C), or diploid (2n = 18, 4C), or segregated followed by centromere disjunction (2n = 18, 2C) metaphases or prophases. Beside the accumulation of cells at haploid values, an overall shift of DNA contents to lower values is evident.

Table 2. Line $E_2A_1C_6$: percentage of metaphases with groups of different sets of chromosomes: numbers are indicated in brackets.

Chromosome groups in the same cell	Number of analyzed metaphases	%	Chromosome numbers analyzed in well spread metaphases
1	313	90.4	
2	13	3.8	(18 – 27)(45 – 45)(27 – 18) (45 – 24)(54 – 45)
3	13	3.8	(9 – 18 – 54)(18 – 18 – 18) (45 – 9 – 9)(2 – 9 – 12)(42 – 9 – 18) (9 – 20 – 36)(19 – 45 – 9)
4	2	0.6	(10 – 12 – 27 – 27)(9 – 2 – 15 – 25)
5	3	0.9	
6	2	0.6	
	Total 346		

common source of the polyploidy besides the one derived from restitution nuclei.

Line $E_2A_1C_6$ presents also other growth anomalies, possibly related to the cytological mitotic aberrations and to a tumorous transformation. As shown by Fig. 4, a high mitotic index (indicating a capacity of indefinite growth) was detected at all times of the growth curve, whereas line A+ showed the usual mitotic peak at the 3rd day after subculturing. Moreover, the cytological characteristics of all the 50 colonies derived from line $E_2A_1C_6$ cloned single cells were completely comparable with the original cell line (data not shown) suggesting the presence of a self-perpetuating mechanism of variability.

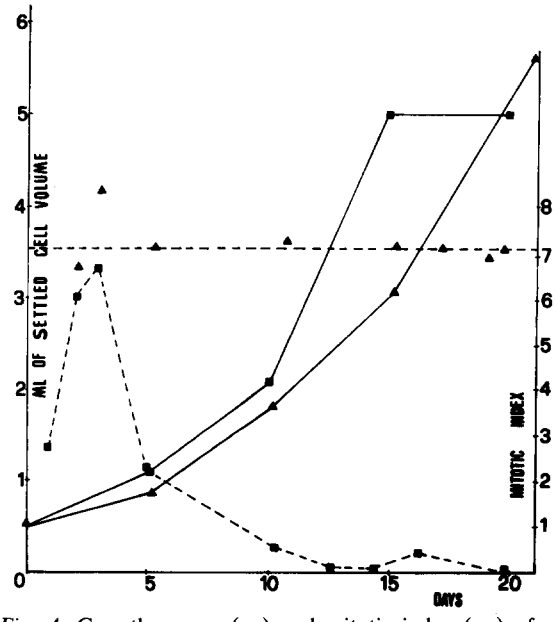


Fig. 4. Growth curves (—) and mitotic index (---) of cell lines A+ (■) and $E_2A_1C_6$ (▲). Non-embryogenic 6-year-old line $E_2A_1C_6$ growth curve and mitotic index indicate a tumorous transformed phenotype.

The selective pressure used to isolate this MTX resistant line has no relationship to the reductional grouping phenotype, since the cell line E_2I, from which $E_2A_1C_6$ derived, presented the same anomaly.

Discussion

The most interesting information emerging from our data is that most A+ and T2 regenerated embryos are haploid, haploidy possibly being established by means of chromosome reducing-segregating events.

Moreover, our data confirm that polyploidy is a reversible process, segregational division being a method to control the level of ploidy that has *in vivo* and *in vitro* an evolutionary function both in animals and in plants (Dobzhansky 1964; Afon'kin 1989; Huskins 1948).

Our data provide evidence that different mechanisms and strategies may work separately or in unison to obtain this purpose in carrot cells dividing in culture.

The prophase-like chromosome partition of A+ and T2 cells, resulting often in two haploid prophases and metaphases, appears as a segregational process, during which the chromosome number is halved from 2n (diploid) to n (haploid or gametic number). The cytophotomertic Feulgen/DNA measurements clearly confirm

that, in culture, a high percentage of cells are cycling between 1C-2C levels, and that the regenerated somatic embryos are haploid as well. Since aneuploidy was not detected, the reducing events appear fairly precise as far as chromosome separation is concerned, as also demonstrated by the high embryogenic capacity of the two cell lines. The phenomenon presumes that the parental chromosomes of each haploid set in a nucleus occupy distinct domains in interphase. This is what has been demonstrated to occur occasionally in barley root-tip cells and as a rule in premeiotic mitosis using an electron microscope-serial thin-section reconstruction technique (Bennett 1984). We propose that a similar premeiotic mean spatial order of heterologues in two separate haploid sets arrangement may be very frequent in carrot cultured cells, allowing the segregation of the single chromosome haploid sets from a diploid nucleus as alternative to mitosis (and meiosis) in culture. This chromosomal disposition would therefore also favour the meiosis-like mechanism described in the accompanying paper (Nuti Ronchi et al. 1992). Moreover, the segregational devices, which lead to the same result of a meiotic process, i.e. haploid nuclei, may be essential in the acquisition of totipotency and embryogenic capacity of cultured carrot cells (Nuti Ronchi et al. 1990). A similar mechanism of prophase reduction appears also to work in reducing polyploid nuclei allowing, as in line $E_2A_1C_6$, the separation of nuclei with a reduced number of chromosomes, mainly in the range of multiples of the *n* number.

It is worth noting that the variant phenotypes described in this and the accompanying paper have been detected also in cell cultures of several other species, as *Nicotiana glauca*, *Lycopersicon esculentum*, *Malus domestica*, etc. and therefore appear to be of frequent occurrence *in vitro*.

Unfortunately, due to the small size of the carrot chromosomes, we have not been able to ascertain the integrity of the haploid complement nor of the different chromosome sets. On the other hand, the regularity of the chromosome numbers, as multiples of *n* is very striking and suggests a non-random spatial disposition of chromosomes in the resting nuclei and a consequent mechanism for the partition of different chromosomal sets.

Reductional grouping has been reported by numerous authors to occur during meiosis of different plant species and described (Tai 1970) by such terms as incompact spindle (Darlington & Thomas 1937), double-plate metaphase (Upcott 1939; Vaarama 1949; Vasek 1962), multipolar spindle (Therman & Timonen 1950, 1954; Walters 1958; Knudson 1958) or complement fractionation (Thompson 1962). The reductional grouping denomination is here preferred to multipolar division since, in the scattered metaphase chromosomes, no spindle has been detected (Mehra 1986), at least with the light microscope; neither has microtubule presence nor orientation been investigated. Moreover, the metaphase chromosomes showed features similar to colchicine-induced arrested metaphase, where the spindle is not assembled. In fact, colchicine pretreatment of the cells did not change the general pattern of the metaphase configuration nor of the prophase reduction process.

The shift to lower values, compared with root-tip meristem, of DNA content of both the embryogenic lines T2 and A+, is particularly intriguing. It may be tentatively interpreted as a 'purge' of somatic DNA, possibly repeated regulatory DNA sequences that are either extruded in the cytoplasm (Nagl 1983) or underreplicated (Nagl et al. 1976; Stern & Hotta 1983). Deumling & Clermont (1989) have recently described a similar DNA reduction in *Scilla* tissue cultures, a reduction that the authors considered a prerequisite to render cells 'omnipotent'. In plants *in vivo*, variations in the frequency of repeated DNA sequences were shown to occur during different developmental events (Cavallini & Cionini 1986; Cavallini et al. 1986). It is possible that, in carrot, the chromosome reduction mechanisms and eliminated DNA sequences play a role in the acquisition of embryogenic capacity. Appropriate experiments are required to confirm this hypothesis (Nuti Ronchi et al. 1990).

The occurrence of the aberrant divisions, which may become constitutive of a cell line, suggests that the different mechanisms are part of a complex of genes participating in cell division: the deregulation in one of those genes results in the phenotypic expression of a defect in some function of the whole process.

In the near future, a great deal of effort should be devoted to discovering the molecular events governing these mechanisms and how to control

them for future applications in plant cell culture. The use of temperature-sensitive mitotic mutants, isolating cell lines with a normal phenotype only at the permissive temperature, should help to overcome the difficulty of instability of spontaneous mutant lines.

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