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Cytogenetics of in vitro cultured somatic cells and regenerated plants of barley (*Hordeum vulgare* L.)

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Summary. Cytogenetic analysis of immature embryo-derived calli and regenerated plants of barley has demonstrated high heterogeneity of callus cultures and significant differences in cytogenetic processes between different callus lines. Regenerated plants usually have a normal chromosome complement ($2n = 14$). Tetraploid plaints occur with a frequency of 1%. No chromosome aberrations have been detected by Feulgen staining. The phenomenon of chromosome stickiness recorded from the 2nd day of culture was discovered in a majority of callus lines as well as the phenomena of chromatin hypercondensation and chromosome supercoiling. A possible contribution of cytogenetic and molecular processes to somaclonal variation is discussed.

Key words: Somaclonal variation – Chromosome behaviour – Chromosome stickiness – Chromatin condensation – *Hordeum vulgare*

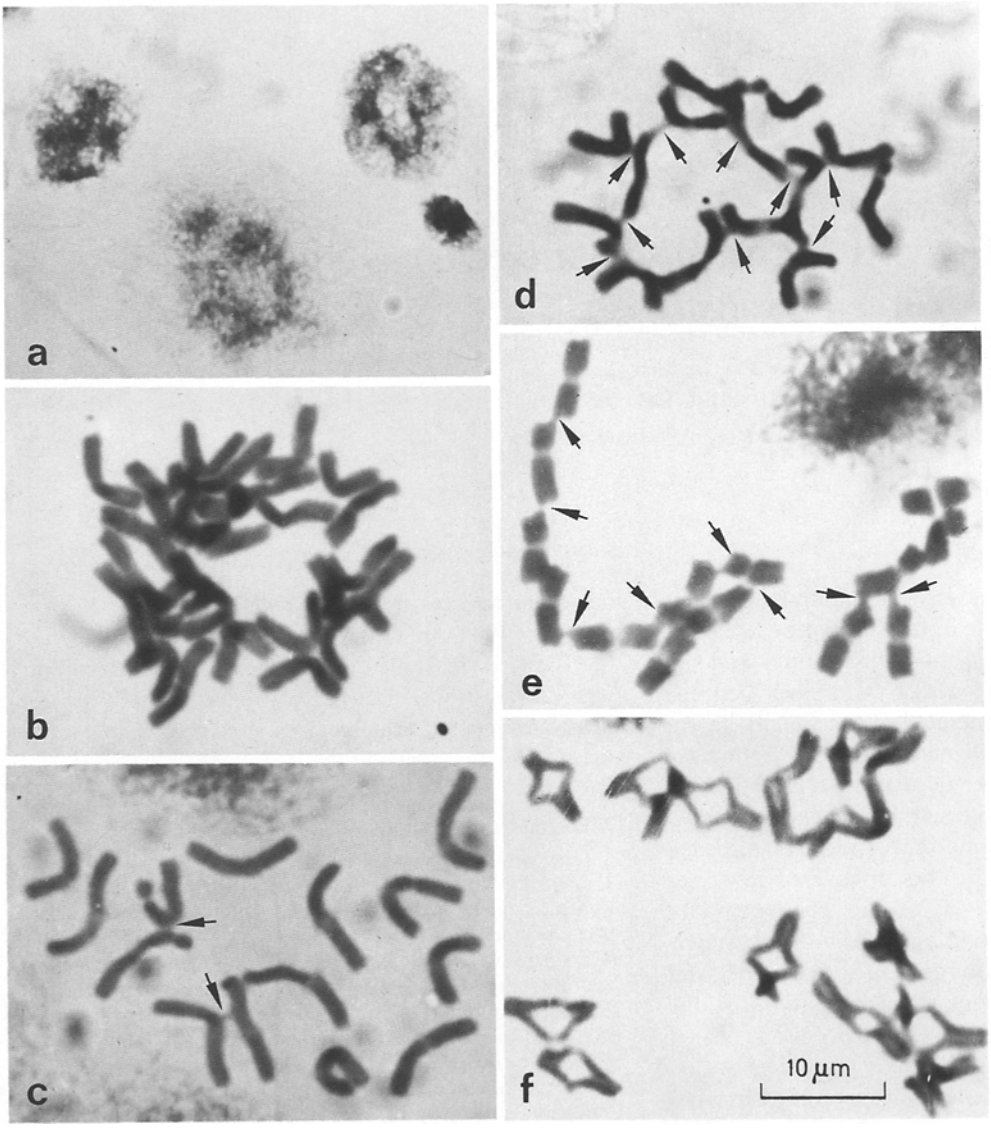
Introduction

The question of using cell cultures for development of principally new, nontraditional methods of selection has become a controversial topic in recent years. The study of plant genome stability in in vitro cultured cells is of great interest. A voluminous amount of experimental material is presently available on genetic instability of plant cells in vitro and on genetic heterogeneity of regenerated plants: this phenomenon is known as somaclonal variation (Larkin and Scowcroft 1981; Shepard 1981). To date, cytogenetic studies permitting the establishment of causal relationships of this variation to genome and chromosome mutations in barley are scant and contradictory (Maddock 1985; Vazquez and Ruiz 1986).

The present study was undertaken to determine a possible contribution of karyotype mutations and structural chromosomal changes to somaclonal variation.

Materials and methods

Two spring barley varieties,Moskovsky-121 and Moskovsky-3, were used in the study. Callus induction, cell cultivation and plant regeneration were performed as described previously (Gaponenko et al. 1985). Cytogenetic studies were carried out with morphogenic calli for 1 year of cultivation beginning from the day of immature embryo inoculation and with roots of regenerated plants. Thus, the study falls into the following divisions: 1) immature embryos 10–14 days old with a scutellum on the day of inoculations; 2) calli on the 2, 5, 8, 12, 20, 30, 120, 240 and 360th days of cultivations; 3) roots of regenerants upon their transfer from test tubes into perlite in passages II and III, and roots of seedlings from seeds of SC_1 regenerants. Embryos and calli were not subjected to pre-fixation treatment to avoid artifacts. Roots were treated before fixation with 0.2% colchicine for 2 h and with water for 2 h at 2 °C. The objects under study were fixed for 24 h in acet-alcohol (3:1). The fixed material was stained in Schiff's reagent according to Feulgen. Meristematic growth zones were isolated from stained calli and apical meristems were cut off from roots. Calli and stained root meristems were macerated for 10 min at 37 °C in a mixture of 1% macerase and cellulase "Onozuka", pH 4.8. Chromosome counts in roots were accompanied by staining meristems in aceto-carmine with preliminary maceration in 0.2% pectinase for 30 min. The necessity of using two procedures was dictated by the fact that both of them affect cell integrity and may lead to a possible loss of chromosomes from the metaphase plate. At the same time, the exclusion of such a procedure as maceration entails great efforts in squashing the preparation and may consequently cause breakage of chromosomes. In all these cases the analysis of cytogenetic processes in calli and regenerated plants is strongly influenced by the subjective factor, therefore the employment of different approaches may be highly desirable for control purposes. Permanent callus pre-



parations were used for statistical analysis of the karyotype structure and the frequency of karyotypic abnormalities. In root meristems chromosome counts of 5–10 metaphases each were conducted on temporary preparations.

Results

Cell division in scutellum and calli was equal even. In scutellum, on the day of inoculation, mitoses were concentrated in epidermal cells directly adjoining the endosperm as was demonstrated for wheat by Gaponenko et al. (1988). The mitotic index or percentage of dividing cells in meristems varied during the first month of culture and reached a level of 3%–4% in a 30-day callus. On leaving the meristem the cell either represented the non-morphogenic parenchymal part of the callus or participated in the formation of morphogenic structures. Morphogenic calli formed meristems on their surface and non-morphogenic calli had internal meristems.

We investigated dividing cells – metaphases and anaphases – in the meristematic zones to determine stability and variability of the karyotype during 1 year of callus cultivation (Table 1).

At the moment of embryo inoculation all mitoses in scutellum were diploid ($2n = 14$) without any changes as compared to root meristem mitoses. The same was also observed on the 2nd day of culture. On the 5th day, tetraploid metaphases with 28 chromosomes appeared (Fig. 1 b). On the 8th day, polyploids with 56 chromosomes were also recorded. It should be emphasized that after equal periods in culture, cell lines differed in their degree of expression of cytogenetic processes. On the

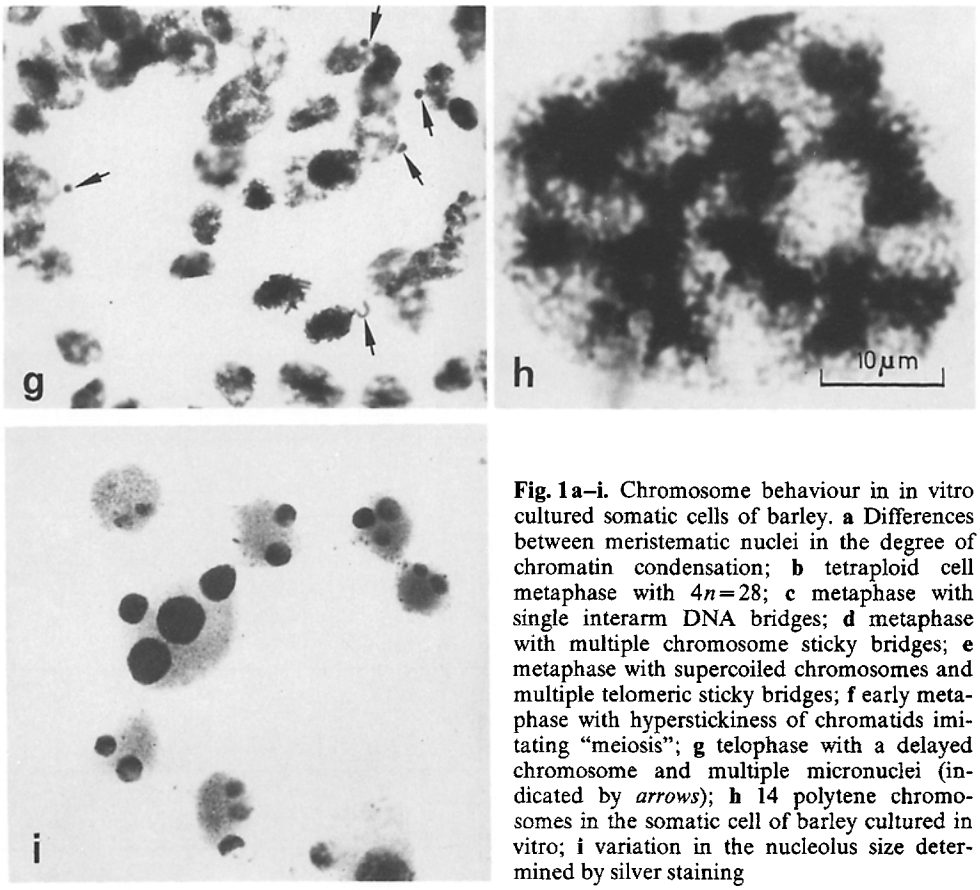


Fig. 1a–i. Chromosome behaviour in in vitro cultured somatic cells of barley. **a** Differences between meristematic nuclei in the degree of chromatin condensation; **b** tetraploid cell metaphase with $4n=28$; **c** metaphase with single interarm DNA bridges; **d** metaphase with multiple chromosome sticky bridges; **e** metaphase with supercoiled chromosomes and multiple telomeric sticky bridges; **f** early metaphase with hyperstickiness of chromatids imitating "meiosis"; **g** telophase with a delayed chromosome and multiple micronuclei (indicated by *arrows*); **h** 14 polytene chromosomes in the somatic cell of barley cultured in vitro; **i** variation in the nucleolus size determined by silver staining

8th day of culture 5 callus lines were analysed. In one of them, line 8, the frequency of polyploid cells was 4.8%, aneuploid cells were absent, and chromosome stickiness and polyteny were not observed. However, lines 10 and 11 had cells with polytene chromosomes, and the frequency of chromosome stickiness in them was 16.6% and 12.5%, respectively. Line 7 had 29.6% of aneuploid cells and the frequency of chromosome stickiness was very high –37.0%; polyploids were absent.

From the 12th–30th day of culture the callus lines displayed mainly diploid cells with the exception of line 12, in which the frequency of polyploid cells was 4.7%.

By the 4th month the cultured calli lost, to a considerable extent, the capacity to regenerate plants. By this time differences between the callus lines with respect to cytogenetic processes became much more pronounced. In addition to calli with 100% of diploid metaphases, there were calli in which polyploid metaphases constituted 39.1% and 82%. After 8 months in culture calli had the non-morphogenic structure and meristems were deep inside the callus. In addition to diploid metaphases representing 77%, polyploid and aneuploid ones were also found. In a 1-year-old callus the number of diploid metaphases decreased to 26% and the number of polyploid and aneuploid metaphases increased. Thus, the data presented in Table 1 show that the greater part of meristematic cells in a morphogenic regenerable callus are diploids. The percentage of polyploid and aneuploid metaphases increases with the age of the callus. Polyploids, who appear on the 5th day, always have even chromosome numbers – 28 and less frequently 56 chromosomes. There are prophases with a larger but uncountable number of chromosomes.

The appearance of aneuploids was also recorded. This was due to loss of chromosomes in mitosis and was always accompanied by the presence of micronuclei near surrounding interphase nuclei. At the early culture stages this process is rare, while in a 1-year-old callus aneuploid cells account for about 30%. In a population of interphase nuclei of this age there is a large number of micronuclei of different sizes. As the mitotic analysis shows, these micronuclei are not composed of acentric fragments, but of whole chromosomes lying separately from the mitotic figure (Fig. 1 g).

Analysis of normal diploid metaphases demonstrates the existence of DNA connections between chromosomes in the form of strands or closer contacts between homologous as well as nonhomologous chromosomes. This phenomenon was first recorded in a 2-day-old callus. The number of metaphases in which chromo-

Table 1. Variation of the karyotype structure in somatic cells of barley cv Moskovsky 2 depending on the time of cultivation

Callus nos.	Time of cultivation (days)	Metaphase no.	$2n = 14$ metaphase no.	$2n = 14$ metaphase frequency (%)	$4n = 28$ metaphase no.	$4n = 28$ metaphase frequency (%)	$4n = 56$ metaphase no.	$8n = 56$ metaphase frequency (%)	Aneuploid metaphase no.	Aneuploid metaphase frequency (%)	Frequency of metaphases with chromosome adhesions (%)	Other phenomena
1	0	40	40	100	–	–	–	–	–	–	–	–
2		25	25	100	–	–	–	–	–	–	–	–
3	2	82	92	100	–	–	–	–	–	–	2.4	–
4		115	115	100	–	–	–	–	–	–	4.5	–
5	5	53	50	94.3	3	5.7	–	–	–	–	–	–
6		64	62	96.9	2	3.1	–	–	–	–	–	–
7	8	27	19	70.4	–	–	–	–	8	29.6	37.0	
8		381	363	95.2	17	4.5	1	0.3	–	–	–	
9		53	52	98.1	1	1.9	–	–	–	–	30.2	
10		64	64	100	–	–	–	–	–	–	16.6	polyteny
11		72	72	100	–	–	–	–	–	–	12.5	polyteny
12	12	43	41	95.3	2	4.7	–	–	–	–	30.2	
13		47	47	100	–	–	–	–	–	–	14.3	polyteny
14		60	59	98.3	1	1.7	–	–	–	–	26.6	
15	20	22	22	100	–	–	–	–	–	–	9.1	
16		67	66	98.5	1	1.5	–	–	–	–	44.8	small chromosomes
17	30	26	24	92.3	–	–	–	–	2	7.7	34.6	
18		53	53	100	–	–	–	–	–	–	60.4	small chromosomes
19		39	39	100	–	–	–	–	–	–	35.9	
20	120	150	149	99.3	1	0.7	–	–	–	–	30.7	small chromosomes
21		110	110	100	–	–	–	–	–	–	100.0	
22		23	14	60.9	9	39.1	–	–	–	–	100.0	
23		44	8	18	36	82.0	–	–	–	–	100.0	
24	240	26	20	76.9	2	7.7	–	–	4	15.4	100.0	polyteny
25 b	360	27	7	26	12	44.4	–	–	8	29.6	100.0	micronuclei

somes are connected by DNA strands increases with aging up to 100% in 4-month-old and older calli. Chromosome contacts observed were single (Fig. 1 c) and multiple (Fig. 1 d). They were nonspecific and involve telomeric ends of chromosomes (Fig. 1 e) and intercalary regions of chromosome arms. In metaphase, one may observe pictures resembling "meiotic metaphase" (Fig. 1 f) due to hyperstickiness of chromatids that impedes their normal anaphase separation. Interarm bridges do not usually prevent anaphase separation. In case of a high level of stickiness stable bridges may be formed. It should be noted that the length of callus metaphase chromosomes not subjected to artificial condensation can reach 8–10 μm. At the same time, in individual calli metaphases with considerably condensed chromosomes (4–5 μm) were found on the 12th and 30th days and after 4 months. When analysing the causes of natural strong condensation of chromosomes we discovered that such metaphases were characteristic of meristematic regions with nuclei in which chromatin was strongly condensed at interphase. On the whole, the meristematic cell population of calli, both morphogenic and non-morphogenic, appears to be highly heterogenous with respect to the structural morphology and size of interphase nuclei.

We have detected four classes of nuclei differing in size and in the degree of chromatin condensation at interphase (Fig. 1 a): 1) rough reticulate structure of chromatin (structural norm); 2) rough reticulate structure with one or several regions of strongly condensed

chromatin; 3) homogeneous structure with totally condensed chromatin; and 4) nuclei of larger size and with different structures. The structure of barley interphase nucleus chromatin in callus and root meristems may be defined as chromonematic or rough reticulate without chromocenters. Such a structure is determined by the mass and distribution of structural heterochromatin along the length of barley chromosomes. The lack of large heterochromatin blocks determines the absence of chromocenters, and the centromeric location of heterochromatin determines the presence of a "condensed" ring near the nucleus pole (Fig. 1 a). There is a small number of nuclei with a tight condensed structure in scutellum by the moment of inoculation. Their number increases to such an extent that this cannot be accounted for by divisions of such cells only, but is associated with the transition of normal nuclei into the completely or partially condensed state in the course of differentiation. The phenomenon of chromatin hypercondensation is characteristic mainly of non-morphogenic regions of calli and has a gradient pattern with condensation increasing from meristematic centers towards periphery. Simultaneously, there occurs in these cells the reduction of rRNA synthesis, which is expressed in diminution of the size of nucleoli (Fig. 1 i) up to their total disappearance. The occurrence in calli of such events as chromatin condensation, on the one hand, and stickiness and supercoiling of chromosomes, on the other hand, suggests them to be related phenomena.

Some large interphase nuclei frequent in almost all callus cultures undergo mitosis and display a great number of chromosomes, indicating the polyploidization process. Other large nuclei preserve the normal diploid structure, but at the same time DNA strands assembled in a compact ring in the nucleus center and with their ends stretching against the membrane can be observed. These nuclei have a typical structure with polytene chromosomes at interphase which were described previously for barley antipodes (Petrova et al. 1985). As a result of chromatin condensation in interphase nuclei in the course of callus aging, 14 large (12 × 7 μm) chromosomes are seen in these nuclei, which are connected by numerous DNA strands (Fig. 1 h).

Along with an investigation of karyotype structure in the callus culture we determined the chromosome number in regenerated plants upon their transfer from test tubes into perlite (Table 2).

To determine the chromosome number in seeds of regenerated plants after meiosis, a cytological analysis of seedlings from seeds of the first reproduction of mature plants (SC_1) was carried out. Regenerated plants of five barley varieties obtained in different passages were analysed. The results are presented in Table 3. Among 124 regenerated plants there was one tetraploid (cv Odessky 100) with 29 chromosomes. Plants from subsequent reproductions of this plant had 28 chromosomes in SC_2; in SC_3 some plants had 27 chromosomes and the other part had 29 chromosomes. Instability of the chromosome number observed in the tetraploid regenerant in a series of generations points to the disturbance of meiosis, which is characteristic of autopolyploids. The third passage in which the tetraploid was obtained corresponds approximately to a 4-month-old callus that may have up to 82% of polyploid metaphases (Table 1).

Table 2. Chromosome count in seedlings of barley cv Moskovsky 121 transferred from the callus culture into perlite

Passage	Plant no.	Chromosome no.	Diploid (%)
II	27	14	100
III	13	14	100

Table 3. Chromosome count in seeds of regenerated plants of different barley varieties in SC_1 obtained in different passages

Variety	$2n=14$ plant no. Passage						$2n=29$ plant no.
	I	II	III	IV	V		
Moskovsky 3	7	4	23	19	2	55	
Moskovsky 121	–	5	12	–	–	17	
Odessky 100	9	4	21	–	–	34	1
Nosovsky 9	–	–	6	–	–	6	
Nutants 518	–	–	11	–	–	11	
	16	13	73	19	2	124	
$2n=29$			1				

It should be stressed that our analysis of metaphases and anaphases has revealed no bridges, fragments, dicentrics or other chromosome aberrations, which suggests that the absence of mutation process affects the integrity of chromosomes. The frequency of anaphase and metaphase disorders is lower than that in the roots of control seeds in experiments on radiation and chemical mutagenesis (Gilyarovskaya 1979).

Discussion

Among all crop cereals, it is probably most difficult to obtain somatic cells in culture from barley. Stable regeneration of barley plants in vitro also presents great difficulties. The morphogenic capacity of barley calli, mostly determined by the donor plant genotype, quickly decreases in time. We have shown that this process is correlated with an increase of chromatin condensation in meristematic nuclei when a meristem turns into a non-morphogenic callus. Hypercondensation of

chromatin blocks the action of genes, which is expressed by the discontinuation of RNA synthesis and morphogenetic processes (Nagl 1982); this was also confirmed by our observation. Difficulty in obtaining regenerated barley plants explains the small amount of literature on genetics of barley calli and regenerated plants. Only a few papers are devoted to stability and variability of callus cultures and regenerated plants of barley. The detailed work of Orton (1980) deals mainly with a hybrid between *H. vulgare* and *H. jubatum*. Therefore the variability of karyotype, individual chromosomes, morphological and biochemical characters of hybrid plants is apparently due to the interaction of two different genotypes and cannot be attributed to somaclonal variation. Deambrogio and Dale (1980) reported the appearance among barley diploid regenerated plants of albinos, plants with poor fertility, different growth rates and morphological changes. Our study of 299 regenerated plants of different varieties in SC_1, SC_2 and SC_3 has additionally revealed hereditary changes in the composition of esterases and hordeins as well as in a number of morphological traits (Gaponenko et al. 1986).

As was demonstrated in early studies by Cheng and Smith (1975), regenerated plants of barley have the diploid chromosome complement ($2n = 14$). Ruiz and Vazquez (1981) observed a high frequency of diploid cells in immature embryo-derived calli after 4 months in culture, and this predominance of diploid cells was maintained for over 3 years. However, in the early culture the authors noted some instability of the karyotype. Singh (1986) attempted to investigate the chromosome constitution of morphogenic and non-morphogenic immature embryo-derived calli. Morphogenic calli carried the normal chromosome complement ($2n = 14$) in a majority of cells. In contrast, non-morphogenic calli had a large number of cells carrying numerical and structural chromosomal changes. The author concluded that plant regeneration is possible only from calli without chromosomal abnormalities and that karyotypic and chromosomal changes in non-morphogenic calli are induced during the culture.

Our studies support the findings of the abovementioned authors since the analysis of barley callus culture during 1 year and for 124 regenerated plants has shown that the majority of regenerated plants are represented by diploids (99.2%). In other words, plants regenerate only from cells with the normal karyotype.

Aneuploid cells characterized by the loss of chromosomes result from disorders in the spindle mechanism. They are subjected to somatic selection and do not occur in regenerated plants.

We recorded a high percentage of polyploid metaphases in some callus cultures and studies the occurrence of polyploids as a result of the formation of early anaphase restitution nuclei, which is due to a disturbance in spindle functioning and consequently nondisjunction of chromosomes to the cell poles. As a rule, polyploid nuclei form an individual population in meristems that may prevail, and, as experience has shown, which may sometimes regenerate polyploid plants.

Chromosome aberrations, anaphase bridges in particular, were recorded in appreciably lower frequencies than is accepted to be the norm for root meristems (2.1 ± 0.2). In our opinion, this proves that our methods of cultivation (particularly the growth of calli in the dark to regeneration), do not possess a strong mutation effect leading to chromosome breaks. Since chromosome aberrations occurring in non-morphogenetic calli and causing their non-regenerability do not occur in the genotypes of regenerated plants (Singh 1986), we concentrated on cytogenetic events in chromosomes of morphogenic regenerable calli.

We discovered a process that began during the first days of culture and involved at late stages up to 100% of all metaphases: stickiness of chromosomes described for plants in works on radiation biology (Ivens 1963) beginning in 1920. This process represents nonspecific sticky adhesions between chromatids at metaphase and anaphase. Three types of adhesion are distinguished: 1) induced by various physical (radiation in the first place) and chemical agents (Ivens 1963); 2) spontaneous adhesions resulting from disturbances in the genetic balance (Beadle 1932; Sokolov 1959); and 3) functional adhesions occurring during normal differentiation of certain tissues (Romanov 1965; Petrova 1969). All three types of adhesions may be of different intensity – from single interarm bridges at metaphase to total agglutination of chromatin. Another frequent phenomenon observed from the 8th day of culture is the appearance of metaphases with hypercondensed chromosomes (4–5 μm). This phenomenon is recorded in nuclei with hypercondensation of chromatin at interphase. The study of wheat callus sections (Gaponenko 1987) showed that the distribution of nuclei with hypercondensed chromatin in meristems has a gradient pattern with an increase toward periphery. The process of chromatin condensation during tissue differentiation inplants is described in detail by Nagl 1982).

Analysis of the fate of nuclei with hypercondensation of chromatin permits this process to be regarded as the phenomenon of callus differentiation which does not give rise to plant regeneration. At the same time, chromosome stickiness associated with hypercondensation of chromatin and entailing a high percentage of metaphases with interarm and telomeric DNA bridges may lead to point or gene mutations.

The existence of somaclonal variation in regenerated plants with the normal karyotype ($2n = 14$) and no

large chromosome aberrations detected by Feulgen staining suggests that, in barley, somaclonal variation is caused by genetic events on the molecular level. This suggestion is indirectly supported by our work on rDNA demethylation in cultured calli of barley (Khvyrleva et al. 1986) and by the works of Breiman and Rotem (1986, 1987) who reported a high molecular variation in the spacer region of ribosomal genes in regenerated plants.

References

Beadle GW (1932) A gene for sticky chromosome in *Zea mays*. Z Indukt Abstamm Vererbungsl 63:195

Breiman A, Rotem D (1986) Somaclonal variation at the biochemical and molecular level in barley (*H. spontaneum*). Abstracts IVth Int Congr Tissue Cell Cult. Minneapolis, p 218

Breiman A, Rotem-Abarbanett D, Karp A, Shakin H (1987) Heritable somaclonal variation in wild barley (*Hordeum spontaneum*). Theor Appl Genet 74:104–112

Cheng TY, Smith HH (1975) Organogenesis from callus culture of *Hordeum vulgare*. Planta 123:307–310

Deambrogio E, Dale PJ (1980) Effect of 2.4 D on the frequency of regenerated plants in barley and on genetic variability between them. Cereal Res Commun 8:417–423

Gaponenko AK, Malikova NI, Okhrimenko GN, Sozinov AA (1985) Obtaining of somaclonal lines in cereals (*Triticum aestivum* L. and *Hordeum vulgare* L.). Dokl Akad Nauk SSSR 6:1471–1475

Gaponenko AK, Okhrimenko GN, Iskakov AR, Sozinov AA (1986) Genetic analysis of somaclonal variation in cereals. Abstracts IVth Int Congr Tissue Cell Cult. Minneapolis, p 217

Gaponenko AK, Golubeva ON, Petrova TF, Sozinov AA (1988) Morphogenesis in wheat somatic cell culture. Ontogenez (in press)

Gilyarovskaya GT (1979) On the influence of different periods of barley storage on the variation in the chromosome aberration spectrum induced by gamma-rays (metaphase method). Genetika 7:12–25

Ivens Kh (1963) Chromosome damage by ionizing radiation. Atomizdat, Moscow, pp 1–96

Khvyrleva TsD, Ryzhik MV, Ananyev EV, Gaponenko AK, Iskakov AR, Sozinov AA (1986) rDNA demethylation in barley callus tissue cultured in vitro. Dokl Akad Nauk SSSR 5:1249–1252

Larkin PJ, Scowcroft WR (1981) Somaclonal variation – a novel source of variability from cell culture for plant improvement. Theor Appl Genet 60:197–214

Maddock SE (1985) Cell culture, somatic embryogenesis and plant regeneration in wheat, barley, oats, rye and *Triticale*. In: Bright SWJ, Jones MGK (eds) Cereal tissue and cell culture. Dordrecht Boston Lancaster, Nijhoff/Dr. W. Junk Publ, pp 131–174

Nagl W (1982) Condensed chromatin: species-specificity, tissue-specificity, and cell cycle specificity, as monitored by scanning cytometry. In: Nicolini C (ed) Cell growth. Plenum Press, New York, London, pp 171–218

Orton TJ (1980) Chromosomal variability in tissue culture and regenerated plants in *hordeum*. Theor Appl Genet 56:101–112

Petrova TF (1969) L'agglutination de la chromatine an cours des mitoses dan's l'albumen chez *Ezythronium sibiricum*. Rev Cytol Biol Veg 32:391–395

Petrova TF, Solovyanova OB, Chentsov YuS (1985) Ultrastructure of giant chromosomes in barley antipodes. Cytologia 5:499–503

Romanov ID (1965) Abnormal mitoses and cytoplasmatic gradient in embryo sacs of several flower plants. Cytologia 7:1–13

Ruiz ML, Vazquez AM (1981) Cell population evolution in tissue culture from embryo of barley (*Hordeum vulgare*L.) after caffein treatment. Protoplasma 107:13–20

Shepard JF (1981) Protoplasts as sources of disease resistance in plants. Annu Rev Phytopathol 19:145–166

Singh RJ (1986) Chromosomal variation in immature embryo derived calluses of barley (*Hordeum vulgare* L.). Theor Appl Genet 72:710–716

Sokolov NI (1959) Nucleus-cytoplasm interaction in remote hybridization of animals. Nauka, Moscow, pp 1–117

Vazquez AM, Ruiz ML (1986) Barley: Induction of genetic variability through callus cultures. In: Bajaj IPS (ed) Biotechnology in agriculture and forestry, vol 2, Crops I. Springer, Berlin Heidelberg, pp 204–219