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Chromosomal variation in dividing protoplasts derived from cell suspensions of barley (*Hordeum vulgare* L.)

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Summary. Numerical and structural chromosome variation was analysed in dividing protoplasts isolated from suspension cells of barley. Five cell lines exhibited distribution patterns in chromosome number with different peaks and ranges. Embryogenic/morphogenic cell lines showed a peak at 2n = 14 (ca. 50%) after 6–7 months in culture, while older non-embryogenic cell lines had peaks at aneuploid or polyploid chromosome numbers. Culture duration had a clear effect on numerical and structural chromosome variation in embryogenic cell lines. With ageing of the cultures chromosome variation accumulated and the proportion of 2n = 14 cells decreased. The effect of protoplast isolation and culture on chromosome variation was examined; more cells with normal chromosome sets (12%) were maintained in protoplast-derived colonies than in source suspension cells (4%) of the same culture age.

Key words: Chromosome variation – Embryogenic/regenerable suspension – *Hordeum vulgare* L. – Protoplasts

Abbreviations: *DC*, Dicentric; *F*, fragment; *T*, telocentric

Introduction

In cereal cell cultures, protoplasts are of particular interest because of their use in transformation via direct DNA uptake and in genetic manipulation via fusion. However, dividing protoplasts are routinely obtained only from long-term suspension cultures, and these cultures lose their morphogenic capacity with culture time. This loss is attributed to somaclonal variation (for review, see Karp 1991).

Because chromosomal variation occurs frequently in barley calluses but appears only at low frequencies in regenerated plants, it has been suggested that cells with normal chromosomal constitutions are selected for during the process of regeneration (Singh 1986; Gaponenko et al. 1988; Karp and Lazzeri 1991). Several researchers have reported that the regenerative capacity of barley callus cultures declines with time and that the increase in chromosome variation is responsible for this reduction (Jelaska et al. 1984; Goldstein and Kronstad 1986; Gaponenko et al. 1988). For long-term cultured suspension cells and protoplasts, there is a special need to know how much chromosome variation has occurred in an established cell line in order to ensure the use of the cell cultures during the suitable period and to control or modify culture conditions. In the paper presented here we report for the first time the chromosomal variability in dividing barley protoplasts isolated from morphogenic, non-morphogenic cell suspensions and protoplast-derived colonies.

Materials and methods

Dividing protoplasts were isolated from five established cell suspension lines of *Hordeum vulgare* L. (Lazzeri et al. 1991; Jähne et al. 1991 a, b). The origins, culture age and other features of these lines are presented in Table 1. All cell suspensions and protoplasts were cultured according to Lazzeri et al. (1991). Protoplast-derived suspension cells were obtained by transferring 1.5-month-old protoplast-derived microcalli to liquid L1 medium supplemented with 2 mg/l 2,4-D.

Preparation of metaphase chromosomes from protoplasts was performed using a modification of the method of Wang et al. (1989) as follows: suspension cells were subcultured at 3- to

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Table 1. Origins, culture age and embryogenic features of the cell lines used in the experiments

Cell line	Cultivar	Explant source	Culture age	Embryogenic
1	Igri	Anther	7 months	Yes and Regenerable
2	Dissa	Zygotic embryo	6 months	Yes
3	Igri	Anther	1.5 years	No
4	Dissa	Zygotic embryo	3 years	No
5	Dissa	Zygotic embryo	5 years	No

4-day intervals before preparation. For the last subculture 80% of the medium was replaced with fresh medium. On the 1st or 2nd day, the cells were pretreated with 0.15% colchicine for 3 h and then digested in enzyme solution for 2–4 h in order to isolate protoplasts. The enzyme components and washing solution were as in Lazzeri et al. (1991). The protoplasts were rinsed through sieves with a mesh size of 100 and 50 μm diameter, collected by centrifugation at 50 *g* for 5 min and fixed in fresh 3:1 methyl alcohol:acetic acid fixitive for 30 min. The fixed protoplasts were washed once with distilled water, then immersed in distilled water for 30 min, which induced hypo-osmosis to expand the cells. They were subsequently resuspended twice in fresh fixative for 15 min. For spreading of the chromosomes, the protoplast suspension was dropped onto cold wet slides from a low height (1–2 cm). The slides were immediately dried over the flame of an alkohol burner and then air dried to completion. Ten percent Giemsa R66 (Gurr) diluted with 1/15 *M* Sörensen phosphate buffer at pH 6.8 was used to stain the slides for 30 min. Fifty metaphases were analysed per sample according to Karp et al. (1987).

Mitotic chromosome behaviour at anaphase was observed by a squashing technique. Fixed suspension cells were macerated in 1 *N* HCl at 60 °C for 4 min, stained with modified carbol fuchsin stain (Kao 1975) for 3 min and subsequently squashed with a coverslip.

Results

Distribution patterns of chromosome number and structural variation in embryogenic and nonembryogenic cell lines

Variation in both chromosome number and structure was found in protoplasts of all of the cell lines assayed (Figs. 1 and 2). Different cell lines had various patterns of chromosome number distribution. The regenerable cell line 1 (7 months old) and embryogenic cell line 2 (6 months old) had distinct peaks in chromosome number at the diploid chromosome number (both 48%: 2n = 14) (Fig. 2a I, II). Also in line 1 10% of the cells were found to be tetraploid (4n = 28). In two non-embryogenic cell lines, peaks in chromosome number were located at 2n = 18 (36%) and 2n = 19 (38%) in the 1.5 year-old cell line 3 and at 2n = 16 (72%) in the 3-year-old cell line 4;

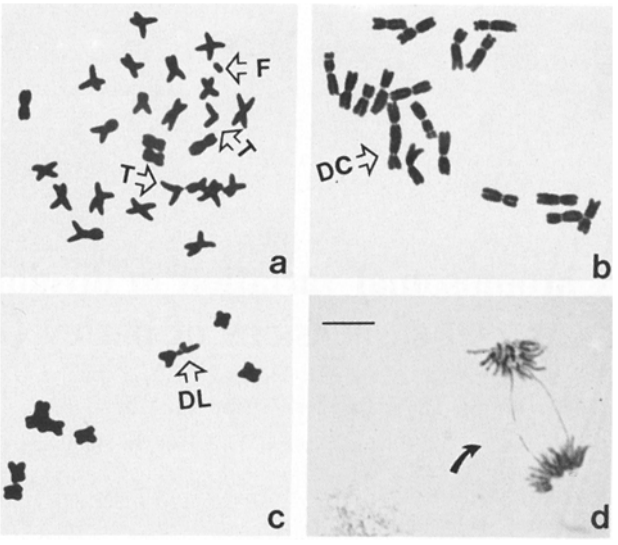

Fig. 1a–d. Chromosome variation in protoplasts and cells of barley. **a** Telocentric (*T*) and fragment (*F*), **b** dicentric (*DC*), **c** chromatid deletion (*DL*), **d** two chromatid bridges at anaphase. *Bar:* 10 μm

only 6% and 4%, respectively, of the cells maintained in the two lines were diploid (Fig. 2a III–IV). In the 5-year-old non-embryogenic transgenic cell line 5, the distribution of chromosome number showed specific peaks, with 32% of the cells being tetraploid (4n = 28) and 30% hypo-tetraploid (2n = 27); 2n = 2x = 14 diploid cells were never detected (Fig. 2a V).

Variations in chromosome structure included telocentrics, dicentrics, fragments and a very small number of chromatid deletions (Figs. 1, 2b). Mitotic aberrations comprised lagging chromosomes, micronuclei and one or two chromatid bridges (Fig. 1). Telocentrics were present at the highest frequency in metaphase cells in the 1.5-year-old line 3 (60%), in the 3-year-old line 4 (82%) and (at lower frequencies) in both regenerable cell line 1 (12%) and embryogenic cell line 2 (14%) (Fig. 2b I–IV). In the oldest (transgenic) cell line, line 5, telocentrics (T), dicentrics (DC) and fragments (F) were all present at high frequencies: 28% T, 34% DC and 24% F (Fig. 2b V).

Among the five cell lines examined, the embryogenic cell lines contained more cells with normal chromosome numbers and less structural variation than non-embryogenic cell lines.

Effect of culture duration on numerical and structural chromosome variation in an embryogenic cell line

The effect of culture duration on chromosomal stability and variability was investigated in an embryogenic cell line (line 2) by carrying out monthly examinations of protoplasts isolated from this same suspension cell line

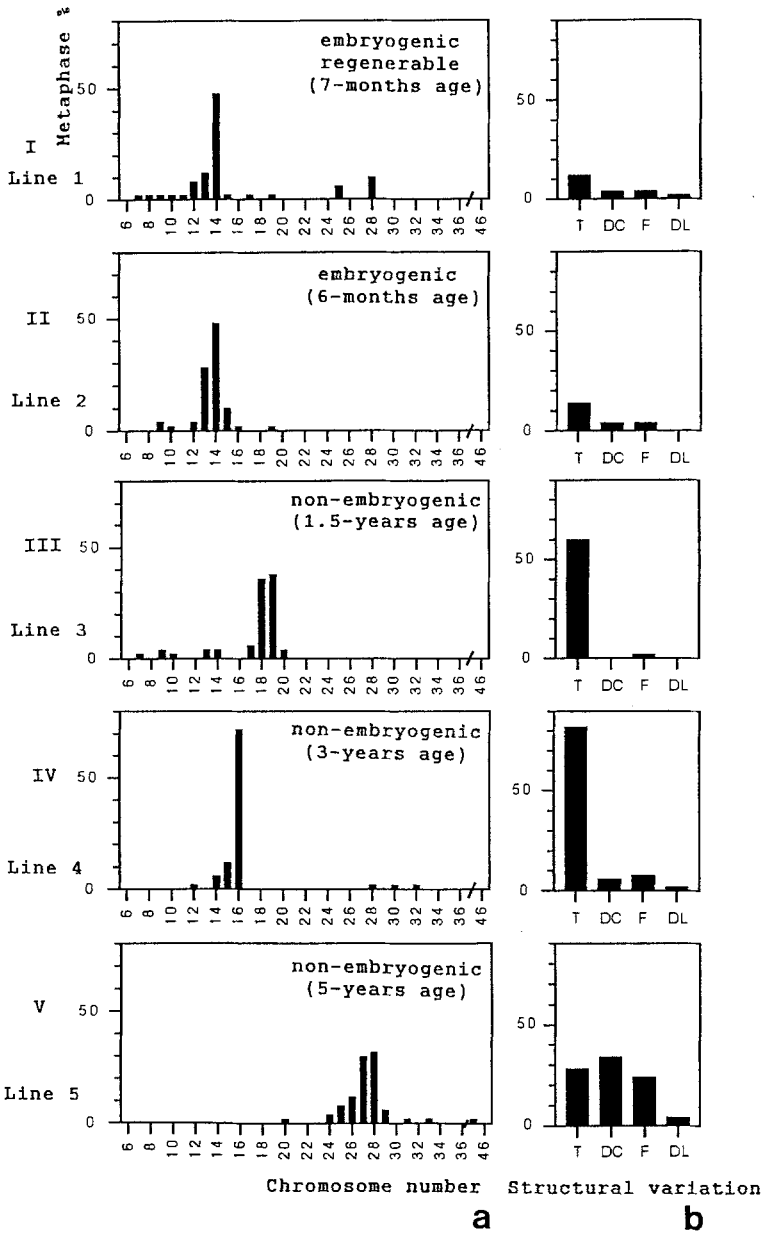

Fig. 2 a, b. Frequency distribution of chromosome number (**a** *I–V*) and of chromosome structural variation (**b** *I–V*) in dividing protoplasts derived from five suspension cell lines. *T* Telocentric, *DC* dicentric, *F* fragment, *DL* chromatid deletion. $N=50$ per sample

during the 6th to 9th month of culture. After 6 months of culture (the first 2 months on solid medium) the protoplasts exhibited a chromosome number distribution pattern with a clear $2n=14$ peak (48%) and with a narrow range of chromosome number, 9–18 (Fig. 3a I). After 7 months, the main chromosome peak had shifted to an aneuploid number ($2n=13$, 32%); the $2n=2x=14$ frequency had decreased from 48% to 16%, while the frequencies of hypodiploid, hyperdiploid and polyploid cells had increased (Fig. 3a II). After 8 months, the main peak was still situated at $2n=13$ (34%) and the frequency of $2n=14$ (14%) cells had changed little, but there was a general shift in chromosome numbers from diploid to polyploid cells (Fig. 3a III). At 9 months of culture no clear chromosome number peaks could be seen. Most cells were polyploid and aneupolyploid (between $3n=21$ and $4n=28$), and only 4% of the cells in the population were diploid (Fig. 3a IV).

The analysis of structural chromosome variation indicated that dicentrics were present at similar frequencies (4%, 4% and 6%) at 6, 7 and 8 months, but at 9 months the proportion had rapidly increased to 36%. Alterations in the frequency of telocentrics and fragments during the same period (6, 7, 8 and 9 months, respectively) were T: 14%–10%–18%–12% and F: 4%–6%–2%–8% (Fig. 3b).

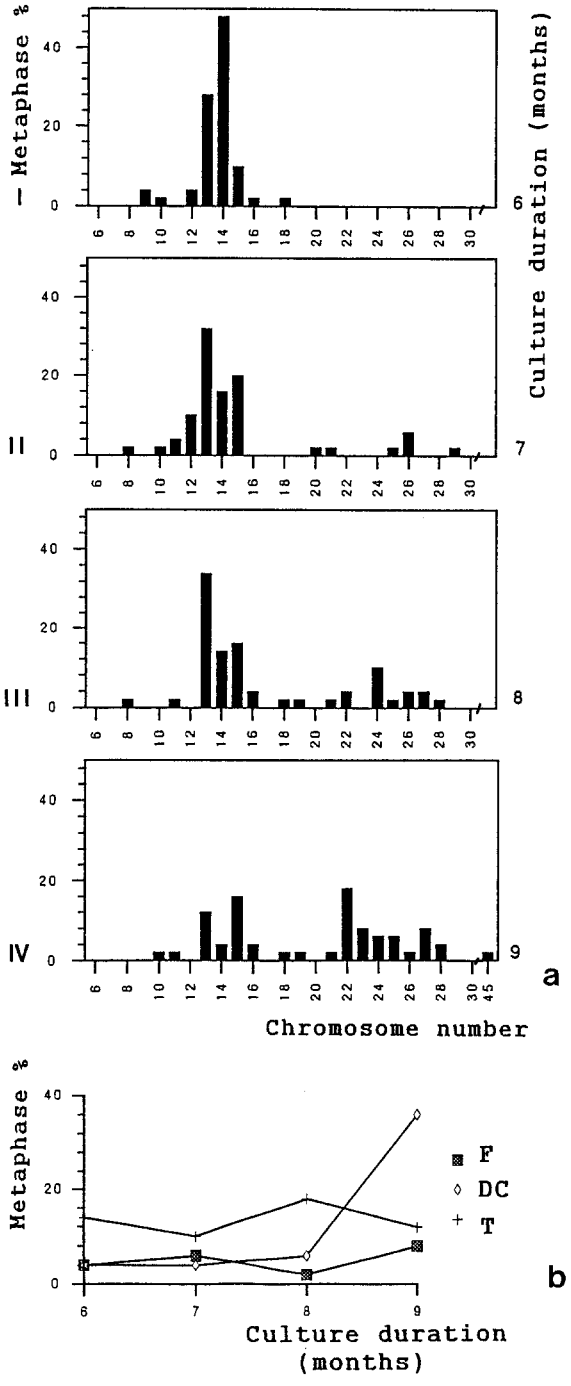

Fig. 3 a, b. Effect of culture duration on the frequency distribution of chromosome number (**a**) and on chromosome structural variation (**b**) in dividing protoplasts of embryogenic cell line 2. *T* Telocentric, *DC* dicentric, *F* fragment. $n=50$ per sample

Changes in chromosome number before and after protoplast culture and influence of culture conditions on variation

Protoplasts freshly isolated from 6-month-old embryogenic suspension cells (line 2) showed a peak in chromosome number at the normal $2n=14$ value (48%), and the chromosome numbers were dispersed in a narrow range (9–18, Fig. 4 I). After the protoplasts were cultured in agarose (1.5 months) and their microcalli used to initiate a suspension (a total of 9 months in culture), a large peak in chromosome number appeared at the hyperdiploid chromosome number $2n=15$ (54%), the frequency of $2n=14$ cells fell from the previous 48% to 12% and chromosome numbers ranged widely from 6 to 49 (Fig. 4 II).

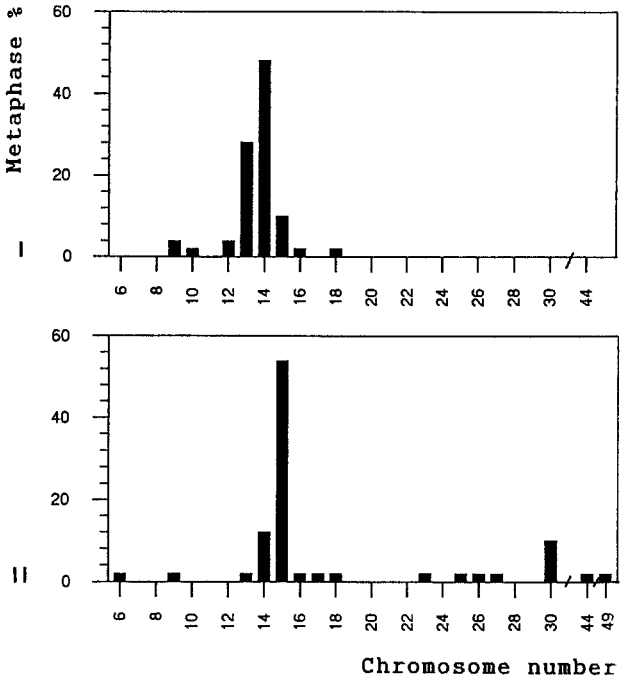

Fig. 4. Frequency distribution of chromosome number in freshly isolated protoplasts of line 2 (6 months old) (*I*) and in a protoplast-derived suspension 3 months after protoplast culture (*II*) $n=50$ per sample

In the comparison of two different culture types, protoplasts isolated directly from the source cell suspension, also after a total of 9 months in culture, maintained only 4% $2n=2x=14$ cells, which was lower than that found in protoplast-derived cultures (12%), and no clear chromosome number peak was observed (Fig. 3 a IV). These two different results (Fig. 3 a IV and Fig. 4 II) revealed that culture conditions had influenced chromosomal stability.

Discussion

It has been frequently suggested that the accumulation of chromosome variation in vitro is associated with the loss of regenerative potential (Muir 1965; Murashige and Nakano 1965; Torrey 1967; Singh 1986; Karp et al. 1987). In the study presented here the correlation between chromosome variation and culture duration confirms that

variation is accumulated with prolonged in vitro culture. Approximately 50% of the cells of younger suspension cell lines, about 6 months of age, contained normal chromosome complements, but after that the frequency of diploid cells declined rapidly. This observation possibly explains the finding that in diploid barley the regeneration capacity of cell suspensions or protoplasts is generally lost after about 6 months in culture (Lührs and Lörz 1988, Lazzeri and Lörz 1990, Jähne et al. 1991 a, b).

The frequency of variations in chromosome number that we observed in barley protoplasts is higher than that previously reported for callus (Orton 1980; Singh 1986; Gaponenko et al. 1988). These increases could be attributed to factors before protoplast culture such as frequent subculture and prolonged culture. In addition, more chromosome variation occurred in protoplast-derived colonies than in the protoplasts before they were cultured. The phenomenon of a larger number of chromosomal aberrations in protoplasts than in cell suspensions has been recorded in wheat (Karp et al. 1987). These results suggest that novel genetic variation is created during the process of protoplast culture. Further, protoplast colonies maintained more normal chromosome complements (12%) than continuously suspended cultures (4%). This means cell suspension is a very unstable culture type with respect to maintaining genetic constitutions.

The distribution peak of chromosome numbers in barley protoplasts isolated from cell suspensions showed a shift from 2n, through hyperdiploid, to polyploid cells during 6–9 months of observation. Nevertheless, the process of polyploidization did not proceed uncontrolled. In older cell lines some stabilization process appeared to take place: chromosomal numerical distributions tended to cluster around some particular genomic complement between 2n and 3n. This suggests that after extended culture the chromosome number shifts back to a low ploidy level under some selection for particular chromosome complements that are "fit" in prolonged in vitro culture. This is indirectly supported by Orton's observation that in calli of *Hordeum vulgare* × *H. jubatum*, the mean chromosome number increased drastically to about 54 during 6–10 months in culture, then decreased and stabilized at 35–36 during 10–15 months (Orton 1980).

In view of the negative effect of culture duration on barley genome stability in vitro, younger cell suspensions, up to about 7 months in culture, should be used whenever possible for protoplast isolation, and particularly when a normal chromosome complement and regeneration capacity are important.

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