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Chromosome variation in dividing protoplasts and cell suspensions of wheat

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Summary. The cytology of bread wheat (*Triticum aestivum*) suspension lines, recycled lines (selected for high division frequency) and their dividing protoplasts, have been examined. Extensive numerical and structural chromosome variation was present in all the lines. The most frequently observed chromosome numbers were around 2n = 32, indicating that considerable chromosome loss from the normal wheat complement (2n = 6x = 42) had occurred during selection of the lines. Chromosome aberrations also indicated loss of chromosome arms and chromosome segments. The implications of this variation for studies on transformation and for the potential regeneration of whole plants from protoplasts of bread wheat are discussed.

Key words: Wheat – Protoplasts – Cell suspensions – Aneuploidy – Structural chromosome variation

Introduction

With present techniques of recombinant DNA technology the potential exists for direct manipulation of plant genomes in novel ways.

In the *Gramineae*, transformation has now been demonstrated in a number of species (eg. Lörz et al. 1985; Potrykus et al. 1985; Jones 1985a; Fromm et al. 1986), but for most cereals the major limitation is the lack of a reliable system for regeneration from the transformed cells (Jones 1985a, b). Recent success in rice, where plants have been regenerated from protoplasts of morphogenetic cell lines derived from immature tissues (eg. Fujimura et al. 1985), has heightened optimism for cereals such as bread wheat, where considerable effort is being placed to obtain similar morphogenetic lines.

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Successful division of protoplasts of bread wheat ha recently been achieved in cv. 'Copain' by Maddock (1987 after testing a large number of cell suspension cultures. Th division frequency was, however, relatively low (< 1% c plated protoplasts divided). In an accompanying paper, w describe procedures which have led to a significant increase i the division frequency (Wu et al. 1987) but it has not yet bee possible to achieve regeneration of plants from the dividin protoplasts.

Numerous reports in the literature indicate that ce suspensions of a wide range of plant species show variation i chromosome number and structure (Heinz et al. 1969; Kao e al. 1970; Bayliss et al. 1977; Singh et al. 1981; Browers an Orton 1982; Murata et al. 1983). Furthermore, loss of morphc genetic capacity has been related to a high degree of chrc mosome instability (Murashige and Nakano 1967; Sagorska e al. 1974).

In this report, we described the results of a cytologi cal study of dividing wheat protoplasts and the cel suspensions from which they were isolated. The impli cations of the variation observed are discussed i relation to the morphogenetic capacity of this tissu culture system and its potential use for experiments i which DNA constructs can be introduced into brea wheat protoplasts to examine both transient expressio and stable integration of the introduced genes.

Materials and methods

Cell suspension and protoplasts

Cell suspension cultures derived initially from two brea wheat lines have been studied in detail. The culture used mos extensively was from *Triticum aestivum cv.* 'Copain', name C82d, isolated by Maddock (1987), and the second line fror cv. 'Maris Butler', named MBE6. Both cell suspensions origi nated from embryogenic callus obtained from immatur embryos (approximately 12 days post anthesis) cultured o solid medium containing 1 mgl^{-1} 2,4-D (Maddock 1987 Protoplasts can be isolated from both cell cultures, but onl line C82d yields protoplasts that divide. In order to improv

the division frequency of protoplasts from line C82d, a strategy, described elsewhere by Wu et al. (1987), that involved recycling and selection of the largest protoplast-derived colonies, was followed. The selected colonies were used to produce new cell suspension cultures, designated C82d^2, C82d^3 and C82d^4 obtained after, one, two and three rounds of selection respectively. Protoplast isolation, and subsequent division and growth parameters, are described by Wu et al. (1987), the salient point being that the division frequency of protoplasts from these selected lines was increased up to 7 fold (7%) over that of the original line C82d (1%).

Chromosomes have been examined in both the original cell suspension cultures (C82d, MBE6), and in the recycled line C82d^2. Chromosomes have also been examined in dividing protoplast-derived cells from the original and recycled cell suspension lines, (designated P-C82d, P-C82d^2, P-C82d^3 and P-C82d^4 respectively).

Cytological procedures

Methods for cytological examination of suspensions and protoplasts were modified after Kao et al. 1975.

Pretreatment and fixation of suspension cultures

Fifty ml of each suspension culture were taken 4 days after subculturing, an equal volume of 0.2% colchicine added (to give a final concentration of 0.1%) and the mixture shaken in a conical flask for 2h at 25 °C on an orbital shaker (100 rpm). The suspension was then transferred to tubes which were centrifuged at 1100 rpm for 10 min. The supernatant containing colchicine was removed and the pellet resuspended in 20 ml of fixative (3 parts ethanol:1 part glacial acetic acid) and kept overnight at 4 °C. The fixed cells were centrifuged at 1100 rpm for 10 min, the fixative removed, the pellet resuspended in 0.1 M sodium acetate buffer (pH 4.5) and centrifuged at 1100 rpm for 10 min. The buffer was discarded and the pellet transferred to a conical flask. Enzyme solution (20 ml, containing 0.25 g Onozuka cellulase R10, 0.25 g Macerozyme R10 (Yakult Pharmaceutical Industry Co. Ltd., Japan) and 49.5 ml 0.1 M sodium acetate buffer pH 4.5) was added and the cells incubated at 25 °C for 2 h. The cells were washed as before with 0.1 M sodium acetate buffer pH 4.5 and the pellet resuspended in 10 ml 45% acetic acid.

To examine chromosomes, 20 µl of the suspension was pipetted onto a clean slide and the fixative allowed to evaporate. A solution of 1% macerozyme R10 was added for 15 min in order to soften the cell walls and aid squashing. A few drops of modified carbol fuchsin (Carr and Walker 1961) were then pipetted onto the slide and the mixture allowed to stain for 4 min. A coverslip (22×50 mm) was then applied and the preparation squashed flat. Chromosomes were counted in the first 50 well-spread cells (approximately 20 slides).

Pretreatment and fixation of protoplasts

Protoplasts (5 ml) in culture medium containing mannitol (Wu et al. 1987) were taken, and 5 ml of 0.2% colchicine (made up in the same medium) added. The protoplasts were incubated in a conical flask for 6 h at 25 °C on an orbital shaker, then transferred to tubes and centrifuged at 1,000 rpm for 10 min. The colchicine supernatant was removed and the pellet resuspended in 1 ml of a fixative (75 ml 3 ethanol:1 acetic acid, 25 ml distilled water and 3.6 g sorbitol) and stored at 4 °C overnight. The fixed protoplasts were then centrifuged at 1100 rpm for 20 min and the fixative supernatant removed. The pellet was resuspended in 2 drops of 45% acetic acid.

In order to examine chromosomes in the protoplast culture approximately 20 µl was pipetted onto a clean slide and the fixative allowed to evaporate. A few drops of modified carbol fuchsin were then added and the preparation allowed to stain for two min. A coverslip (22×50 mm) was then applied and the preparation was squashed gently. Fifty cells were examined per culture.

Results

Considerable variation in both chromosome number and structure was observed amongst all wheat cell suspension and dividing protoplast lines. Out of a total of 350 cells, none were found to contain the normal wheat chromosome complement (2n=6x=42). The cultures were so heterogenous that no attempt has been made to compare them statistically but characteristics of individual cell suspension and dividing protoplast lines have been summarised in Table 1 and can be described as follows.

Table 1. Cytological characteristics of the cell suspensions and their dividing protoplast lines

	Cell line	Range in chromosome nos.	Mean no.	Modal no.	% cells with one or more			
					T[a]	DL[a]	DC[a]	F[a]
Suspension	MBE 6	22–62	33	33	4	0	26	6
	C82d	29–75	36	36	34	2	12	8
	C82d^2	28–130	39	31	80	2	2	48
Protoplast	P-C82d	29–163	53	30, 32	80	4	16	32
	P-C82d^2	29–127	51	32	98	6	4	20
	P-C82d^3	29–269	61	31	100	16	24	22
	P-C82d^4	29–209	53	32	100	6	16	26

[a] T=telocentric chromosome; DL=deleted chromosome; DC=dicentric chromosome; F=chromosome fragment

Cell suspensions

MBE6. Cell suspension MBE6 contained cells with the lowest chromosome number observed amongst all the lines investigated. The frequency of cells with different chromosome numbers is shown in Fig. 1a. The majority of cells had between 30–39 chromosomes, but 10% had less than 30 chromosomes, and the mean and modal chromosome numbers, were only 2n = 33 (Table 1). A high frequency of cells (26%) had one or more dicentric chromosomes, and fragments and telocentric chromosomes were also observed (Fig. 2a). In addition, 4% of the cells contained megachromosomes.

C82d. Cell suspension C82d had, on average, higher chromosome numbers than MBE6 (Table 1). The distribution of cells with different chromosome numbers depicted in Fig. 1b shows that the majority of cells contained 30–39 chromosomes, but the mean and modal chromosome numbers were 2n = 36 (Table 1).

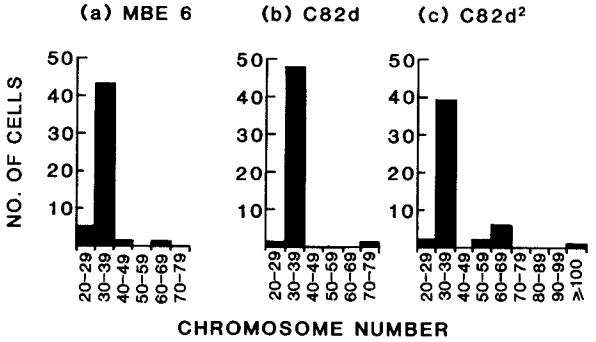

Fig. 1a–c. Frequency distribution of cells with different chromosome numbers for cell suspensions (**a**) MBE 6, (**b**) C82d and (**c**) C82d²

Fewer dicentric chromosomes were observed than in MBE6, but over 30% of the cells were found to contain from one to three telocentric chromosomes (Fig. 2b). Chromosome deletions and fragments were also seen (Table 1).

C82d². Cell suspension C82d² was derived from C82d by protoplast recycling. The distribution of cells with different chromosome numbers (Fig. 1c) differed from C82d and MBE6, in that a higher proportion of cells were observed with high chromosome numbers. The majority of cells still contained between 30–39 chromosomes, and the modal chromosome number was 2n = 31, but the mean chromosome number was higher than for C82d (Table 1). The second major grouping in the frequency distribution was around chromosome number 2n = 50–2n = 69, and a single cell was observed with 2n = 130 chromosomes (Fig. 1c).

Telocentric chromosomes, ranging from 1–6 in number, were observed in a very high frequency of cells (Table 1). Chromosome fragments, deletions and dicentrics were also seen.

Dividing protoplast lines

P-C82d. An examination of chromosomes in the dividing protoplasts derived from cell suspension C82d (P-C82d) revealed a different distribution of chromosome numbers from the original cell supension (Fig. 3a). Most cells had chromosome numbers within three ranges; 30–39, 50–69 and 80–100. The major class was still in the 30–39 chromosome range and the most frequently observed chromosome numbers were 2n = 36 and 2n = 32 but the mean chromosome number was 2n = 53 because of the increased proportion of cells with high chromosome counts (Table 1).

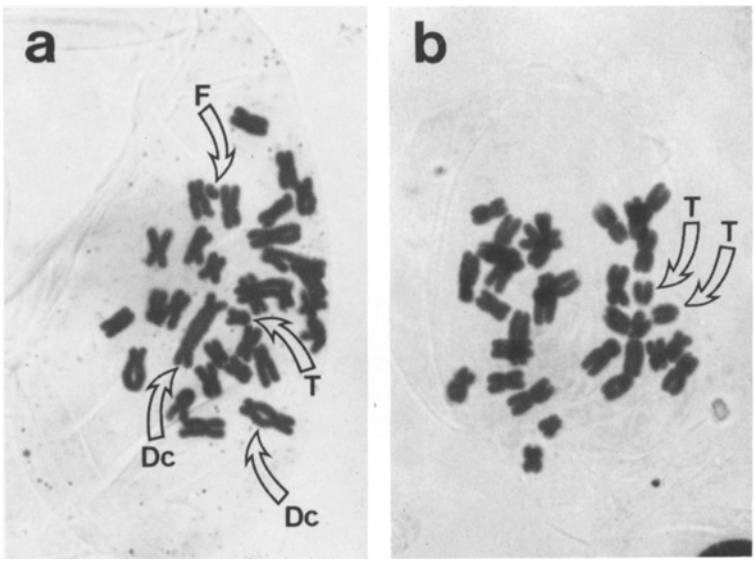

Fig. 2a, b. Chromosome variation in cell suspensions of (**a**) MBE6 (2n = 35) and (**b**) C82d (2n = 32). (Dc = dicentric, F = fragment, T = telocentric)

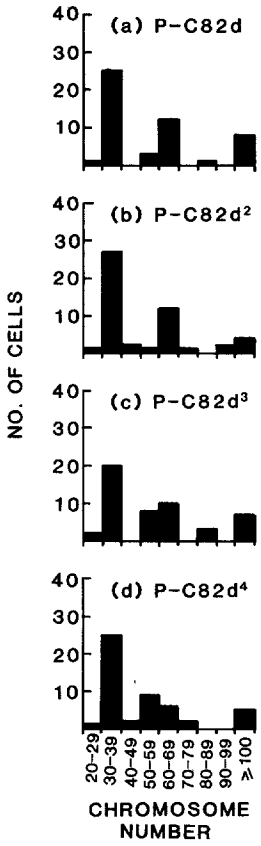

Fig. 3a–d. Frequency distribution of cells with different chromosome numbers for dividing protoplast lines (**a**) P-C82d, (**b**) P-C82d², (**c**) P-C82d³ and (**d**) P-C82d⁴

A closer examination of the dividing protoplasts revealed major karyotypic alterations (Fig. 4). Deletions were sometimes observed (Fig. 4a) whilst a high frequency of cells (Table 1) contained telocentric chromosomes (Fig. 4b, c). Dicentric chromosomes (Fig. 4a, b) and chromosome fragments (Fig. 4a, b, c) were also found at high frequency (Table 1). In addition, diplochromosomes (Fig. 4c) were present in 4% of the cells scored and were seen in many highly polyploid cells.

P-C82d². Dividing protoplast cultures derived from cell suspension C82d² had similar characteristics to P-C82d, but a higher proportion of cells contained telocentric chromosomes (Table 1) and chromosome counts were distributed over a broader spectrum of numbers (Fig. 3b).

P-C82d³. Dividing protoplast cultures of C82d³ were similar in cytological characteristics to P-C82d and P-C82d². However, telocentric chromosomes were observed in all the cells and dicentrics, deletions and chromosome fragments were present at high frequencies (Fig. 5) (Table 1). Some cells were found with very high chromosome counts (2n=269), raising the mean chromosome number to 2n=61, and proportionally fewer cells contained 30–39 chromosomes (Fig. 3c) although the modal value was 2n=31.

P-C82d⁴. The distribution of cells with different chromosome numbers was spread most extensively in dividing protoplasts isolated from C82d⁴ (Fig. 3d). The modal chromosome number was 2n=32, and the most frequent class of cells contained 30–39 chromosomes, but more cells were observed with 40–59 chromosomes compared with P-C82d, P-C82d² and P-C82d³. As for P-C82d³, the telocentric chromosomes were present in all the cells screened (Fig. 5) and often in high numbers. Dicentrics, chromosome deletions and fragments were also observed.

Discussion

Extensive chromosome variation was observed in cell suspensions and dividing protoplasts of wheat cv. 'Copain'. This is in accordance with numerous earlier reports describing cytological instability in cell suspensions of a number of plant species (see Wersuhn and Dathe 1983), but to date, it is the first study of this kind in dividing bread wheat protoplasts.

Cell suspension MBE6 (cv. 'Maris Butler') had cells with very few chromosomes (e.g. 2n=22) and a mean chromosome number that was lower than for C82d, although both suspensions were isolated at the same time (Maddock 1987). The high degree of chromosome loss in MBE6 may be reflected in the failure to obtain division in MBE6 protoplasts.

The modal chromosome number (between 2n=30 and 2n=36) was similar for all the cell suspensions and dividing protoplast cultures of C82d. This suggests that during the cyclic selection for fast dividing cells (Wu et al. 1987) considerable chromosome loss has occurred from the normal bread wheat complement (2n=6x=42), and that this loss has stabilized at about 30–33 chromosomes. Stabilising of particular chromosome numbers has also been observed in other cell suspension systems (Heinz et al. 1969; Pijnacker et al. 1986a). The appearance of a modal chromosome number of about 32 in *all* the selected lines suggests that some factors may have to be lost from the wheat genome before sustained division of protoplasts can be attained. This aspect will be studied further.

Since chromosome variation was already present in the wheat cell suspensions it was expected that protoplasts isolated from the suspensions would show similar variability. Dividing protoplasts of C82d had different cytological characteristics from the C82d cell suspension from which they were isolated, but similar characteristics to the C82d² cell suspension to which they eventually gave rise (see Methods for re-cycling sys-

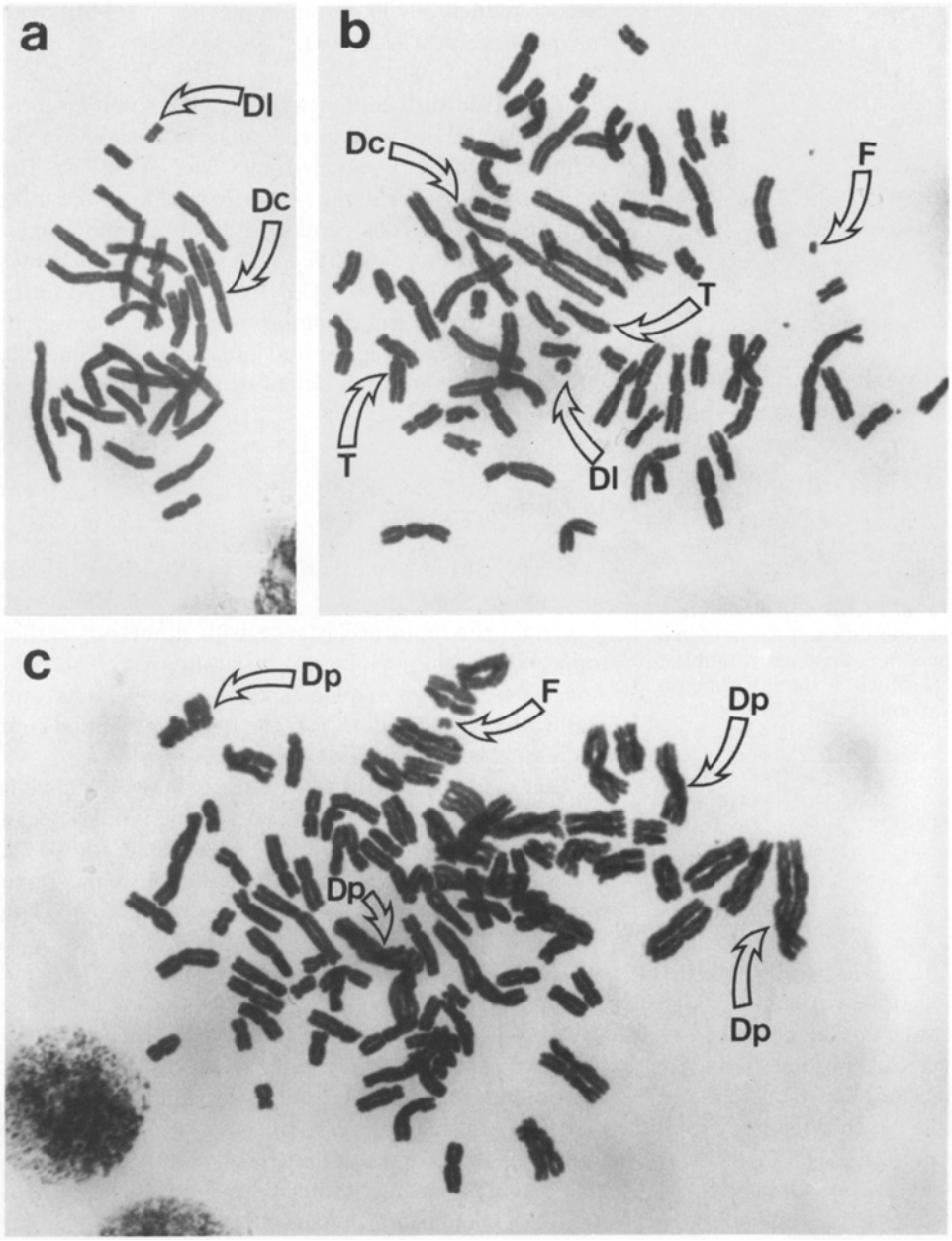

Fig. 4a–c. Chromosome variation in dividing protoplast line P-C82d. (**a**) 2n = 31, (**b**) 2n = 65 and (**c**) polyploid cell with diplochromosomes (ca. 2n = 120). There are numerous structural changes and only a few are arrowed as examples (Dp = diplochromosome, Dc = dicentric, F = fragment, T = telocentric, Dl = deletion)

tem). Comparison of chromosome numbers between P-C82d and C82d indicated that the dividing protoplasts (P-C82d) showed an increased proportion of cells with around 60 chromosomes (i.e. twice the modal value of C82d) and an increased proportion with around 100 chromosomes (i.e. four times the modal value of C82d). During routine isolation of protoplasts, including those of the *Gramineae* from cell suspension cultures, bi- and multi-nucleate protoplasts are observed (Jones and Dale 1982).

It is usually thought that multinucleate protoplasts do not normally grow on to form colonies. However, the cytological data suggest that a small proportion of multinucleate and a larger proportion of binucleate cells do grow, and that this may result in some of observed chromosome doubling. Thus, the mean chromosome number increased with successive protoplast re-cycling. Accurate highest chromosome counts were difficult to obtain for the later cycles because of the presence of diplochromosomes in some of the cells.

The difference between dividing protoplasts and parental cell suspension was greatest after the first re-cycling (i.e. between C82d and P-C82d) suggesting, once again, that

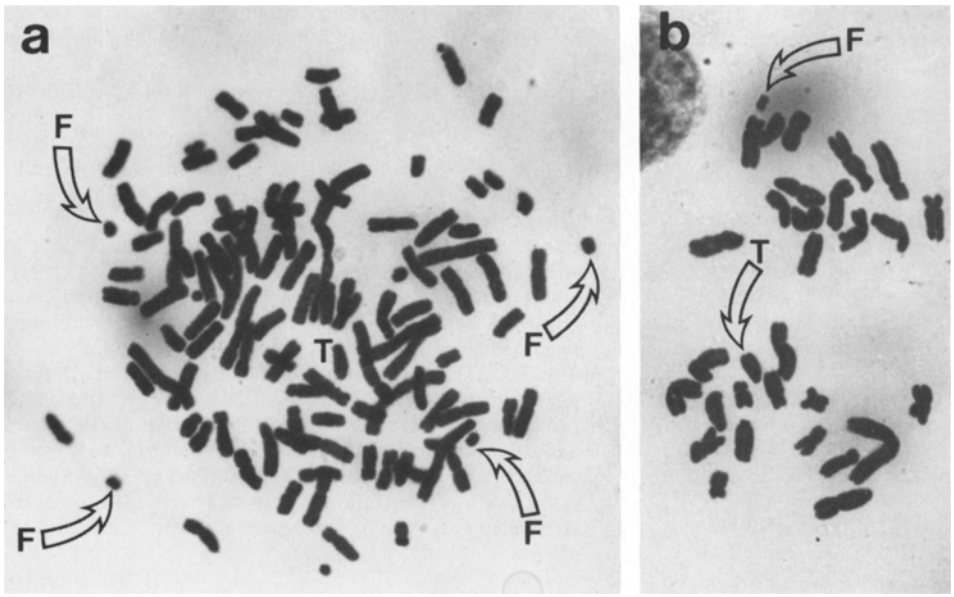

Fig. 5a, b. Chromosome variation in dividing protoplast lines (**a**) P-C82d^3 (2n = 111) and (**b**) P-C82d^4 showing numerous fragments (F) and telocentrics (T) (not all arrowed)

chromosome numbers were stabilising in the cell lines. Similarly, the basal chromosome number (2n = 29) was almost identical for all of the lines. Interestingly this correlates with the observations on division frequency where the largest increase was also achieved in the first cycle (Wu et al. 1987).

Chromosome structural changes were observed in all of the cell lines examined indicating that karyotypic restructuring occurs in cell suspensions, as has been described earlier (Browers and Orton 1982; Murata and Orton 1983). In this study it is not possible to draw too many conclusions about the frequency of different structural changes, because of the difficulty of accurate scoring, particularly in cells with high chromosome numbers. However, it appeared that the numbers of telocentric chromosomes increased with successive protoplast recycling and that more chromosome aberrations were observed in dividing protoplasts than in the cell suspensions. Similar chromosome aberrations (dicentrics, fragments, deletions and megachromosomes) have been reported earlier in cell suspensions of the diploid *Triticum monococcum* and *T. aestivum* (Kao et al. 1970) indicating that MBE6 and C82d are not peculiar in this respect.

The origin of the diplochromosomes is unclear. It is possible that they are an effect of the colchicine pretreatment, but this seems unlikely as they have been reported in cell suspensions earlier (Ono and Harashima 1983; Pijnacker et al. 1986b) and in C82d dividing protoplast lines they were often only present in half of the cell. It is most likely that they result from endoreduplication, as suggested earlier (Ono and Harashima 1983; Pijnacker et al. 1986b) although it is also possible that they arise from the induction of S-phase in a G2 cell (Pijnacker et al. 1986). It is curious that diplochromosomes were present often in only part of the wheat cells (Fig. 4c) suggesting some out-of-phase replications were occurring in the dividing protoplasts. Such repeated replications may also contribute to the polyploidization that occurs during protoplast re-cycling.

The chromosome variation described here is of relevance not only from a morphogenetic point of view, but also for the studies we are carrying out on the transformation of bread wheat (Jones et al. 1986). Furthermore, the results have wider relevance than just to bread wheat. Protoplasts from similar cell suspension lines of other *Gramineae* including *Oryza sativa, Sorghum bicolor, Saccharum officinarum, Pennisetum* spp, *Panicum maximum, Lolium multiflorum, Triticum monococcum* and *Zea mays* (Potrykus et al. 1985; Ou-Lee et al. 1986; Lörz et al. 1986; Hauptmann et al. 1986; Fromm et al. 1986) are all being used to study both stable integration and transient expression of introduced gene constructs in graminaceous cells. Despite the cytological abnormalities reported here, the cells should retain characteristics typical of *Graminea.* Nevertheless, the chances of regenerating transformed plants from cell suspensions of bread wheat, or their isolated protoplasts, appear rather low when the cytological data are considered. Polyploid species can tolerate cytological changes more readily than diploid species (Karp and Maddock 1984), but cell suspensions have long been recognised as sources of cytological instability, and more effort should be placed into studying protoplasts and cells derived directly from the plant tissues. If methods of culture could be devised in which cytological stability is maintained, the probability and ease of regeneration of cereal plants from protoplasts would be greatly improved.

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