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A. Fluminhan · T. Kameya

Behaviour of chromosomes in anaphase cells in embryogenic callus cultures of maize (*Zea mays* L.)

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Abstract Mitotic anaphase cells of highly friable and embryogenic calluses which had been induced from immature embryos of two inbred lines of maize that have contrasting levels of heterochromatic knobs were analysed for the presence of abnormalities 3, 6, 9 and 12 months after the initiation of culture. A total of 500 typical anaphases was scored at each time point, and various aberrations, such as delay in the separation of sister chromatides, chromosome bridges (single, double and multiple) and chromosome fragments, were revealed to occur extensively in the cultures of both genotypes. Preparations after C-banding revealed that primary breakages often occurred inside knobs or at junction regions between the euchromatin and the heterochromatin of the knobs. Figures characterized by the delayed separation of sister chromatids, which originated preferentially at the knob level and was considered to be an initial event in the development of breakages, were observed at constant frequencies throughout the experiment. Increasing numbers of aberrant cells were detected with time, mainly due to the accumulation of cells with chromosome bridges and fragments. Several mitotic figures suggested the occurrence of breakage-fusion-bridge cycles that were initiated by broken chromosomes. The overall frequencies of aberrant cells were similar for both genotypes, despite the differences in knob composition. However, callus cultures induced from the genotype having the higher level of knobs had more aberrant cells with abnormalities that involved several chromosomes, such as multiple bridges and multiple fragments.

Key words *Zea mays* L. · Tissue culture · Aberrations in chromosome structure · Heterochromatic knobs · Chromosome behaviour

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A. Fluminhan · T. Kameya (✉)
Institute of Genetic Ecology, Tohoku University, Katahira 2-1-1, Aoba-ku, Sendai 980-77, Japan

Introduction

Cultures of plant cells and tissues have a mutagenic potential that has been documented by numerous authors and extensively reviewed (for example, Larkin 1987; Karp 1991; Peschke and Phillips 1992; Phillips et al. 1994). In diploid species, the predominant type of chromosome variation that is detected in regenerated plants and morphogenic cultures is often a change in chromosome structure, as opposed to a change in chromosome number (for review, see Singh 1993). In analyses of meioses in regenerated maize plants, the largest category of detectable aberrations involves the breakage of knobbed chromosomes (Lee and Phillips 1987; Benzion and Phillips 1988). The relationship between late-replicating DNA in heterochromatin and the vulnerability of such regions to breakage has been described in several species (Sacristán 1971; Döbel et al. 1978; McCoy et al. 1982; Murata and Orton 1984; Johnson et al. 1987). Since knob heterochromatin is the latest-replicating chromosomal DNA (Pryor et al. 1980), its improper or delayed replication has been considered to be a possible underlying cause of both cytogenetic and genetic variations induced in cultures of maize cells (Lee and Phillips 1988; Phillips et al. 1988). According to one hypothetical mechanism (Phillips et al. 1990; Kaeppler and Phillips 1993a,b), changes in DNA methylation could affect the structure of the chromatin, leading to the even later or further delayed replication of heterochromatin. These alterations could be responsible for the formation and breakage of chromosome bridges at mitosis. Such breakage could release newly activated or newly generated transposable elements which have the potential of inducing the single-gene mutations that have been detected in the progeny of regenerated plants (Peschke et al. 1987, 1991; Peschke and Phillips 1991).

The increasing frequency of regenerants with structurally altered chromosomes in older cultures is another commonly observed phenomenon (for review, see Phil-

lips et al. 1988; Singh 1993). A pedigree analysis of cell lineage by Benzion and Phillips (1988) indicated that the frequency of regenerated plants that were cytogenetically abnormal increased with the age of the culture. They verified that many of the aberrations detected in meiotic cells originated early in the culture period, and they postulated that the effects of age were due to mutational events that occurred throughout the culture and not neccessarily at an increasing rate. Thus, aberrant cells would tend to accumulate over time.

In maize, a large amount of experimental data is available from analysis of numerical chromosome changes in cultured cells (Balzan 1978; Edallo et al. 1981; McCoy and Phillips 1982; Mohanty et al. 1986) and the analysis of meioses in regenerated plants (Rhodes et al. 1986; Lee and Phillips 1987; Armstrong and Phillips 1988; Benzion and Phillips 1988). However, according to the observations summarized above, the initial event responsible for the occurrence of structural changes in chromosomes should be one that results in chromosome breakage in somatic cells in callus cultures early in the period of development *in vitro*. Such configurations have been identified (Aguiar-Perecin and Fluminhan 1992; Fluminhan 1992), and they are characterized by the delayed segregation of sister chromatids in mitotic anaphases, originating preferentially at heterochromatic knobs. It has been proposed that this delay might prevent the transmission of two chromatids to opposite poles, thus causing the formation of a chromosome bridge and its subsequent breakage (Fluminhan 1992; Fluminhan et al. in press).

The purpose of the study presented here was to investigate the occurrence, nature, and frequency of chromosomal aberrations in mitotic anaphases of 3-, 6-, 9- and 12-month-old embryogenic callus cultures that were induced from two inbred lines of maize having contrasting levels of heterochromatic knobs. This report also provides further cytological documentation and a discussion of the events that lead to the occurrence of variations in chromosome structure in cultured maize cells.

Materials and methods

Plant materials and tissue culture

Two inbred lines of maize, derived from cvs 'Dangjin' and 'Mexico Amber Kernel', respectively, that have different levels of heterochromatic knobs were used in this study: the S10 progeny from 'Dangjin' has medium- sized knobs at 2L, $6L_2$ and 7L, and the S5 progeny from 'Mexico Amber Kernel' has large- and medium-sized knobs at 1S, 2L, 3L, 5L, $6L_2$, $6L_3$, 7L, $8L_1$, $8L_2$ and 9L. Tissue cultures were initiated and maintained as described by Green and Phillips (1975). A total of 150 immature embryos (1.5–2.0 mm in length) were removed from selfed ears and cultured on modified MS medium (Murashige and Skoog 1962) that contained 2 mg/l 2,4-dichlorophenoxyacetic-acid (2,4-D), 1.38 g/l L-proline, 100 mg/l casein hydrolysate 8 g/l bacto-agar and adjusted to pH 5.8. Callus cultures were incubated at 25°C in darkness. Subculturing was performed at 2-week intervals, and the most friable and embryogenic regions of actively growing tissues were selected for subculture.

Observations of chromosomes

Samples of five highly friable and embryogenic (type II) callus cultures that had been induced from each genotype were taken for cytogenetic analysis. Observations on the chromosomes were made with the same calluses 3, 6, 9, and 12 months after culture initiation, as described by Aguiar-Perecin and Fluminhan (1992) and Fluminhan (1992) Structures resembling somatic embryos were collected 5 days after subculturing. They were kept overnight in Carnoy's fixative (absolute ethanol and glacial acetic acid, 3:1, v/v) and stored in 70% ethanol at 4°C. Squash preparations were made in 45% acetic acid after incubation in a solution of 5% (w/v) pectinase and 1% (w/v) bacto-peptone (pH 6.0) for 30 min at 37°C. Mitotic anaphases were stained by Feulgen's method and scored in order to identify abnormalities during the segregation of sister chromatids. Anaphase cells were classified as being at one of three different stages, namely, early, typical and late. Attempts were made to analyse at least 100 typical anaphases per callus at each different time point. Some configurations were analysed by C-banding (performed as described by Aguiar-Perecin 1985) in order to verify the involvement of knobs in the formation of chromosome bridges and to monitor the occurrence of breakage-fusion-bridge cycles after breakage events.

Results

Chromosome aberrations at anaphase in embryogenic cultures

Embryogenic cultures were analysed individually for numbers of aberrant and non-aberrant typical anaphases. Differences in terms of frequencies of aberrations among calluses of the same genotype were not statistically significant at a probability level of 0.05 (Turkey test; data not shown). Thus, all of the mitotic figures scored for a given genotype at each of the time points are presented together. The number of typical anaphases with chromosome aberrations, their classification and the variations in 3-, 6-, 9- and 12-month-old cultures of both genotypes are summarized in Table 1. Mitotic abnormalities were mostly characterized by the occurrence of chromosome bridges, fragments or a combination of bridges and fragments. Among the aberrant cells with chromosome bridges, we could distiguish five classes of cells: those with a delayed separation of sister chromatids (Fig. la, b); those with single bridges, (Fig. 2a); those with double bridges (Fig. 2b); those with multiple bridges (Fig. 2c); and those with both bridges and fragments (Fig. 2e). Lagging chromosomes (Fig. 2f) and telophase cells displaying micronuclei were also recorded at low frequencies. Except in the case of configurations that revealed the delayed separation of sister chromatids and lagging chromosomes, all of the cells that we scored as aberrant were interpreted as being the result of one or more breakage event.

We focused our attention on the frequency of abnormal cells in which we observed the delayed separation of sister chromatids, thereby adhering to the previously proposed interpretation that this delayed separation could be the initial event responsible for the variations in chromosome structure in cultured maize cells (Fluminhan 1992; Fluminhan et al. in press). According to the proposed mechanism, perturbations affecting the normal

Table 1 Chromosome aberrations observed at different time points in mitotic anaphases of embryogenic callus cultures induced from maize inbred lines with contrasting levels of heterochromatic knobs (see text for a complete description of the genotypes)

Inbred line	Months in culture	Analysed cells [a]	Number of cells with aberrations [b]						Total abnormal	%
			D.S.C.	S.B.	D.B.	M.B.	Fragments	Others		
Dangjin										
	3	507	6	4	2	0	3	6	21	4.1
	6	510	5	5	4	0	5	6	25	4.9
	9	514	5	7	6	0	9	9	36	7.0*
	12	522	6	12	9	0	12	14	53	10.1**
Mexico Amber Kernel										
	3	502	3	3	2	4	4	5	21	4.1
	6	512	3	3	3	5	6	8	28	5.4
	9	530	3	5	6	6	11	13	44	8.3*
	12	536	4	10	9	5	14	15	57	10.6**

*,** Statistically significant at the 0.05 and 0.01 level, respectively, based on a Tukey test, relative to the frequencies of aberrations observed at the third month of culture

[a] Total number of cells analysed from five different callus cultures of each genotype

[b] D.S.C., Delayed separation of sister chromatids; S.B., single bridge; D.B., double bridges; M.B., multiple bridges, including also the delayed separation of chromatids; fragments, chromosome fragments; others, different combinations of chromosome bridges and fragments, lagging chromosomes and micronuclei

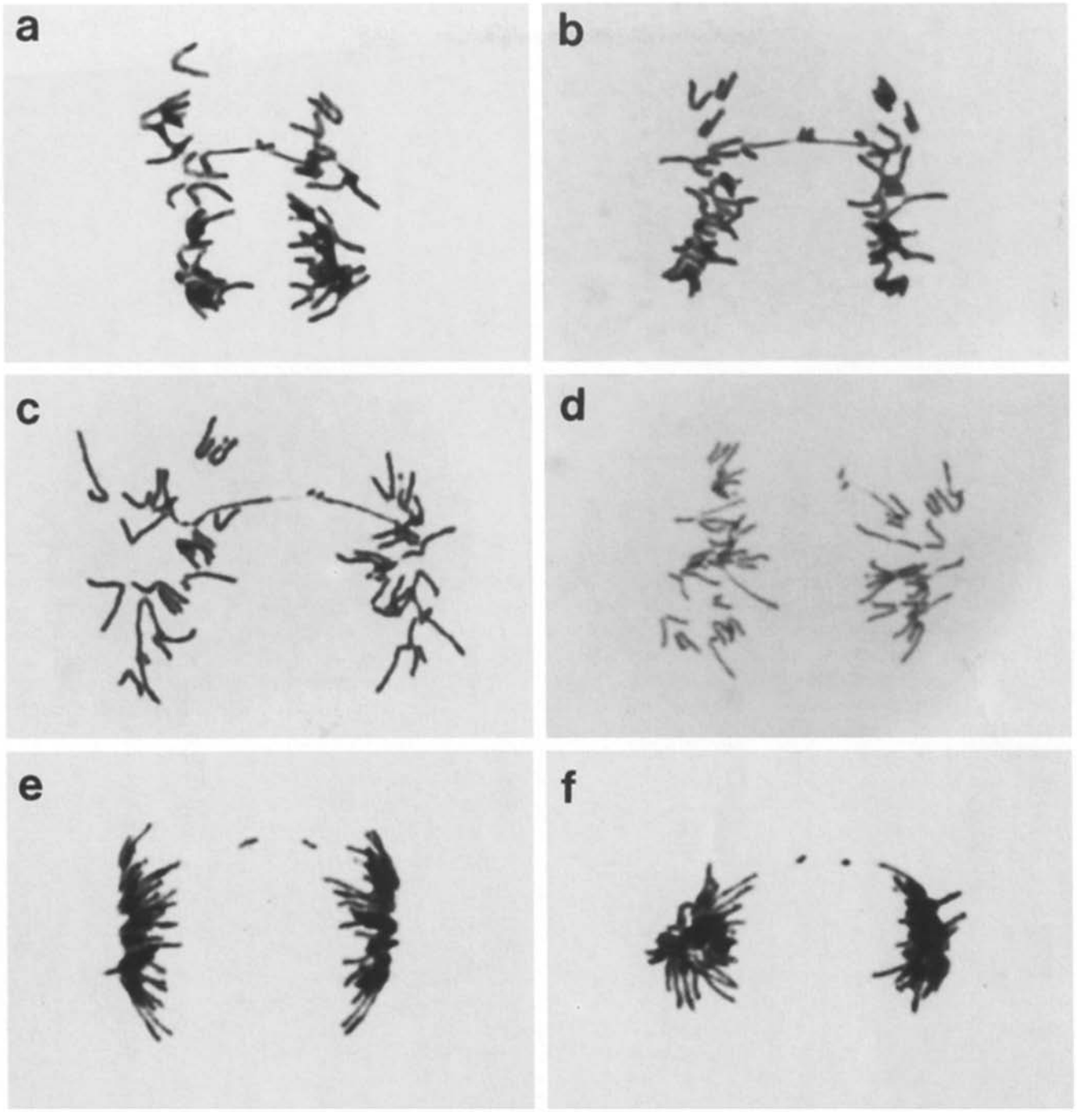
separation of chromatids at the level of the knob would lead to the formation of a chromosome bridge (Fig. 1a, b). Subsequently, the streching that occurs at anaphase would cause the bridge to break (Fig. 1c, d), with the resultant formation of chromosome fragments (Fig. 1e, f). Breakages were seen to occur frequently inside or adjacent to a heterochromatic knob (Fig. 3d–f), but breakages at other locations in a bridge were also detected (Fig. 2c, e). Upon the initial breakage of a bridge, one of the two sister cells received an intact chromosome and the other received a deficient chromosome with a freshly broken end (Fig. 1c). Fusion of the replicated broken ends occurred during the subsequent mitosis. A dicentric chromosome was formed that will undergo the chromatid type of breakage-fusion-bridge cycle during each successive division, thereby conforming to the behaviour described by McClintock (1939, 1941) during the development of gametophyte tissues. Breakage at different locations will give rise to diverse deficient-duplicated cell types. The frequent occurrence of breakage inside or adjacent to heterochromatic knobs was revealed in C-banded preparations (Fig. 3a–f). Chromatids with apparently heteromorphic broken ends (Fig. 3f) could be interpreted to be the results of successive breakage-fusion-bridge cycles.

The effects of genotype and of levels of heterochromatic knobs

Cultures of calluses of both genotypes yielded similar results in terms of the level of cytogenetic stability at different times after the initiation of cultures (Table 1). However, one particularly interesting result was observed. Embryogenic cultures induced from explants of 'Dangjin' (with a lower level of knobs) contained aberrant cells that displayed evidence of having only single and double rounds of delayed segregation of sister chromatids or chromosome bridges. In cultures of the genotype with a higher level of knobs, a greater number of abnormal cells contained multiple bridges (Fig. 2c) and multiple fragments (Fig. 2d). An aberrant anaphase cell observed in a culture of 'Mexico Amber Kernel' is shown in Fig. 2c. Note that all of the multiple bridges are derived from the delayed segregation of sister chromatids and that all originated at the subtelomeric region. Chromosome breakages at several sites could produce aberrant cells with multiple fragments (Fig. 2d).

The effect of the age of the culture on the frequency of aberrations

An examination of the frequencies of aberrations (Table 1) showed that most of the different types increased with time in culture. By the third month of culture the frequency of aberrant cells in embryogenic cultures was about 4% for both genotypes. From the sixth to the ninth month of culture, differences in the frequencies of chromosomal aberrations became much more pronounced. After 9 months in culture, the frequencies of aberrant cells increased to 7.0% and 8.3% in 'Dangjin' and 'Mexico Amber Kernel', respectively; in 12-month-old cultures, the number of abnormal anaphases increased to 10.1% and 10.6%, respectively. Variations in the frequencies of aberrations were due to the increasing relative numbers of cells with single and double bridges and chromosome fragments (for both genotypes) and also to the increasing numbers of cells with multiple bridges and fragments (for 'Mexico Amber Kernel'). However, the number of cells displaying evidence of the delayed segregation of sister chromatids apparently remained constant during the 1-year culture period. This



configuration was observed in about 1% of all the cultured cells of both genotypes, but most of the aberrant anaphases of 'Mexico Amber Kernel' cultures demonstrated the occurrence of multiple events (Fig. 2c).

Fig. 1a–f Photomicrographs of cultured cells of a maize inbred line (cv 'Dangjin'). **a,b** Early mitotic anaphases showing the primary event in the delay in separation of sister chromatids, **c,d** as the two sister chromatids move towards the opposite poles, breakages occur at the sub-telomeric region, **e,f** breakages give rise to chromosome fragments. Magnification: × 100 objective; × 7 ocular; × 1.6 additional lens

Discussion

Our results demonstrate that extensive mitotic abnormalities occur in friable and embryogenic callus cultures of maize mainly as a result of the formation of bridges that may lead to chromosome breakages. Moreover, the chromosome aberrations that occur in cultured cells tended to accumulate with time. These results are in agreement with earlier observations that the frequency of chromosomal variations detected in meiotic cells of regenerated plants increase with culture age (Lee and Phillips 1987; Benzion and Phillips 1988). It seems

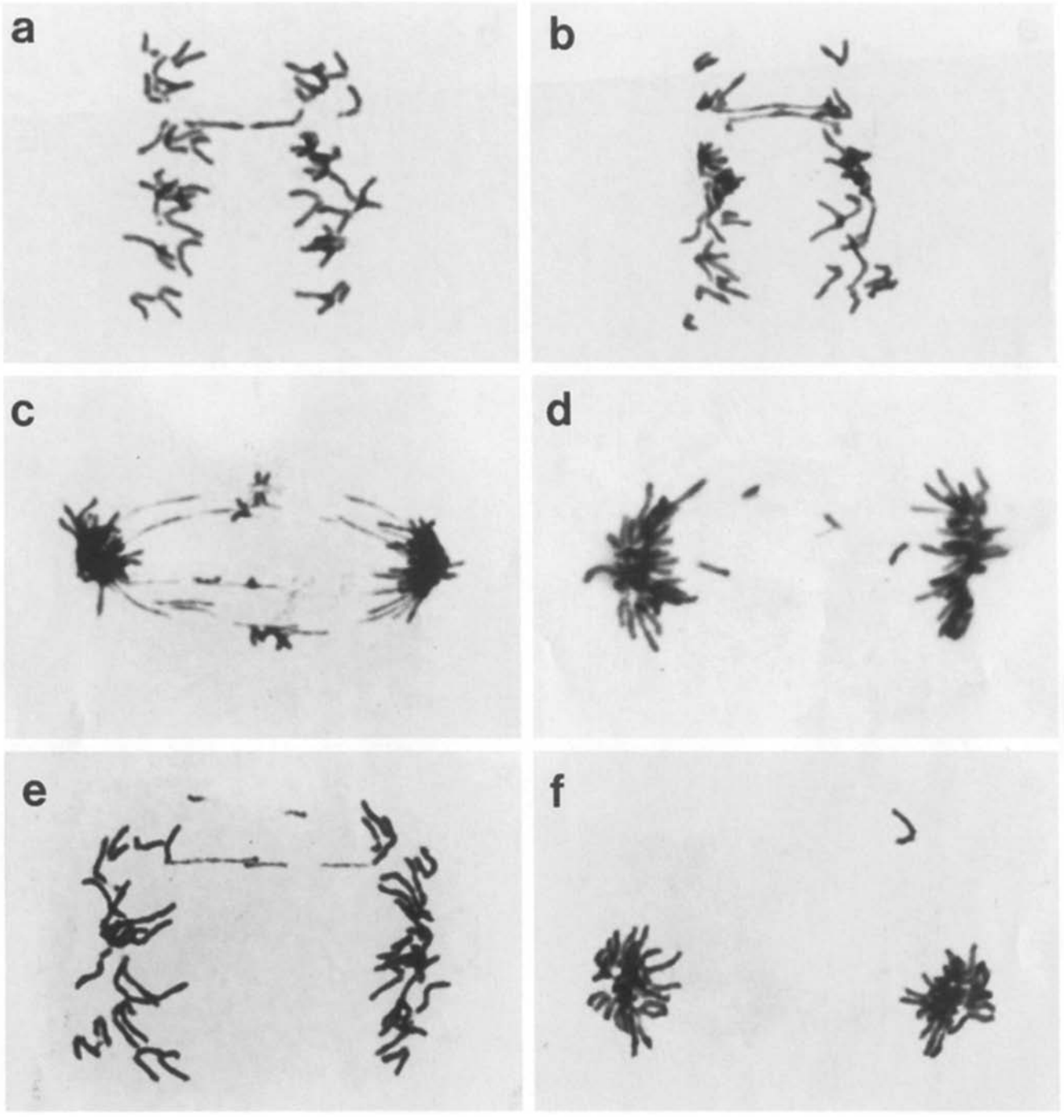
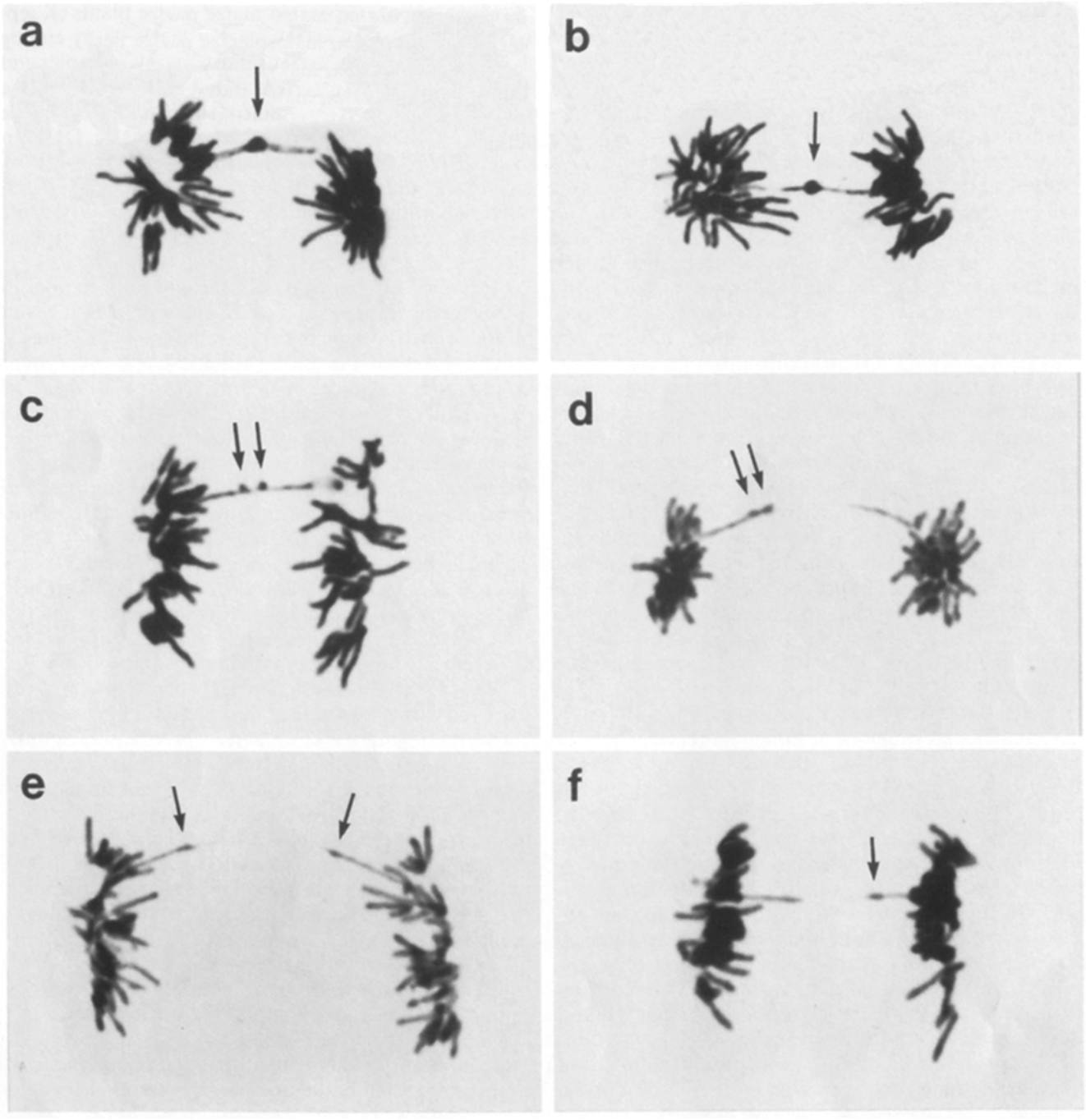


Fig 2a–f Photomicrographs of cultured cells of a maize inbred line (cv 'Mexico Amber Kernel'). **a** Typical anaphase showing a single bridge, **b** double bridges, **c** multiple bridges that apparently originated from primary events, and breakages at several locations, **d** late anaphase, showing multiple fragments, **e** typical anaphase showing chromosome fragments and an initial breakage between a knob and a centromere, **f** late anaphase with a lagging chromosome. Magnification: × 100 objective; × 7 ocular; × 1.6 additional lens

reasonable that the mechanism responsible (or mechanisms) for the generation of variations remains operative throughout the period of culture; this is reflected by the relatively constant number of mitotic figures showing evidence of the initial event – the delayed segregation of sister chromatids. The subsequent maintenance and accumulation of aberrant cells over time could be due to rearrangements that create reciprocal translocations of the chromosome fragments that are produced from breakage events. Such a mechanism could allow aberrant cells to survive, to proliferate and to compete with normal cells during differentiation. Lee and Phillips (1987) noted that interchanges were the most frequent structural alterations (42%) that they detected in meiotic cells of regenerated plants, followed by deficient chromosomes (35%) and heteromorphic pairs (19%). The interchange of segments was also recorded by Rho-



des et al. (1986). Benzion and Phillips (1988) and Armstrong and Phillips (1988).

In maize, the frequency of chromosomal deficiencies that have been observed in regenerated plants and in their progeny varies, being especially dependent upon the length of the deficient segments (for review, see Phillips et al. 1988). The present study demonstrates that the primary chromosome breakage in cultured maize cells occur preferentially within knobs or at junctions between the euchromatin and a heterochromatic knob. Since knobs have a sub-telomeric location, it is expected that deficient cells lack euchromatic regions between the knob and the correspondent telomere. The occurrence

Fig. 3a–f Photomicrographs of C-banded preparations of cultured cells of a maize inbred line (cv 'Dangjin'). **a,b**. Anaphases with single chromosome bridges that originated at the knob level, **c** typical anaphase showing the primary event, the delay in segregation of sister chromatids, and knobs under tension, which are a result of anaphase streching, **d** chromosome breakage at the junction of the euchromatin and the heterochromatic knob region, **e** typical anaphase showing broken or descondensed ends with knobs, **f** sister chromatids with apparently heteromorphic ends, with and without a knob. The *arrows* indicate the positions of heterochromatic knobs. Magnification: × 100 objective; × 7 ocular; × 1.6 additional lens

of breakage-fusion-bridge cycles (also reported by Fluminhan 1992 and by Fluminhan et al. in press) indicates that deficient cells can survive for several generations during callus development in vitro. Successive cycles would result in variable types of rearrangements as a result of the deletion and reduplication of segments. The lower rate of transmission of gross deficiencies in tissue culture might be attributable to the inability of deficient cells to compete with genetically balanced cells during differentiation (see Vasil 1986). Breakage-fusion-bridge cycles might be considered to be a mechanism for the maintenance and accumulation of aberrant cells in tissue culture. However, these cycles appear to be restricted to growth in vitro. According to observations by Lee and Phillips (1987), dicentric chromosomes are not detectable in meiotic cells of regenerated plants. The eventual elimination of deficient cells during the tissue differentiation or the possible healing of broken ends would prevent dicentric chromosomes from being detected in such analysis. It was stated by McClintock (1941) that broken chromosomes do not give rise to bridge configurations during successive nuclear divisions in sporophytic tissues. According to McClintock, "the broken end heals" and "healing of the broken end in the embryonic sporophyte is permanent". The possible healing of broken ends during growth in vitro or during differentiation of somatic embryos will be examined in a future study.

Cells displaying the delayed separation of sister chromatids were observed at similar frequencies in both of the genotypes that we analysed. This observation could be interpreted as evidence for the apparent absence of a correlation between levels of knobs and the frequency of mitotic abnormalities. In a previous study, Fluminhan and Aguiar-Perecin (in preparation) analysed callus cultures induced from 15 inbred lines with different levels of heterochromatic knobs, and they observed that levels of knobs and the frequencies of chromosome aberrations at anaphase were not directly correlated. Moreover, the authors did not find any correlation between callus type (morphogenic potential) and the frequency of mitotic abnormalities.

The predominance of abnormalities that involved knobbed chromosome arms with bridges formed at the C-band level (Fig. 3a–f) is evidence that knobs may have a role in the occurrence of tissue culture-induced breakages in maize chromosomes. These observations support the results of previous studies with callus cultures of genotypes of different origin (Fluminhan 1992; Fluminhan et al. in press), and they are consistent with a hyphothetical mechanism for the occurrence of such breakages (Lee and Phillips 1988; Phillips et al. 1988; 1990). Some problems associated with DNA replication, triggered by tissue culture conditions, could be the cause of the present phenomena. Deviations in the replication of late-replicating DNA could be related to changes in the extent or the pattern of DNA methylation (Phillips et al. 1990; Kaeppler and Phillips 1993a,b). A high frequency of variations in methylation was found in random sequences in regenerated maize plants (Kaeppler and Phillips 1993b), suggesting that many coding regions could be affected. As indicated by Kaeppler and Phillips, changes in DNA methylation could affect chromatin structure and could result in deviations in the timing of the replication of late-replicating DNA in heterochromatic regions. Our emphasis on the analysis of mitotic anaphase has focused attention on the delayed separation of sister chromatids as being a possible primary manifestation of deviations in DNA replication.

In addition to the proposed mechanism (variations in DNA methylation), it should be mentioned that many proteins have been identified that influence the segregation of chromosomes during cell division in eukaryotes. The majority of these proteins affect the synthesis of DNA (Hartwell and Smith 1985), the condensation and segregation of chromosomes (Peterson 1994) or the resolution of interlinked chromosomes at mitosis (Uemura et al. 1987; Holm et al. 1989). In the absence of some of these proteins, an attempt at mitotic segregation of chromosomes is still made, leading to non-disjunction or breakage, since the newly replicated chromosomes are still physically interlinked (reviewed by Watt and Hickson 1994). Future investigations may reveal whether such proteins have a role in cell culture-induced structural variations in plant chromosomes.

Most of the primary breakages observed in our study occurred within knobs or at the boundaries between the euchromatin and the knob heterochromatin. The molecular organization of transition zones between euchromatin and heterochromatin is still largely unknown. These zones are assumed to have special properties because the chromatin at such junctions exists in a state of flux between the condensed and the noncondensed form (for review, see John 1988). It has been argued that access to junction regions by repair enzymes might be impaired, with facilitation of exchanges between nonpartner DNA strands (Hatch and Mazrimas 1977), which might lead to sister chromatid exchange, translocation, fusion or the accumulation of heterochromatic DNA by unequal exchange.

These observations suggest that several mechanisms could be involved in the origin of structural changes in chromosomes, and such mechanisms might not neccessarily be limited to the environment in vitro. Some forms of culture-induced variation might result from mechanisms that are also operative in somatic tissue in vivo. One interesting issue that we are now investigating is the effect of cell ageing. It has been known that higher plants exhibit increased frequencies of spontaneous chromosomal and genetic changes with the increasing age of dried seeds (for review, see Priestley 1986; Murata 1991). Analysis of the frequencies and types of chromosomal aberrations in anaphase cells during the first mitotic divisions in root tips from artificially aged seeds of inbred lines derived from 'Mexico Amber Kernel' revealed that a chromosome bridge-type aberration was the most frequent event, followed by chromosome frag-

ments and combinations of bridges and fragments (Fluminhan and Kameya, submitted). The frequencies of aberrant anaphases ranged from about 3% (observed in freshly harvested materials or in seeds stored for short periods of time at low temperatures) to 16% (when the seeds had been stored for long periods at high temperatures and had an invariable moisture content). Similar results have been described in different species (Hang et al. 1994; see also review by Murata 1991). We have verified that the frequency of cells with mitotic abnormalities decreases progressively with increasing length of the root. The frequencies of aberrant anaphases in root tips after growth for 3, 6 and 10 weeks declined and appeared to be stabilized below the 1% level in all of the stocks analysed (Fluminhan and Kameya, submitted). This decreasing frequency has been considered to be an effect of diplontic selection during tissue differentiation (Murata et al. 1984). This lower frequency of abnormalities in the tissues of maize plants could be considered as a control for our experiments with cultured cells in vitro. The most interesting issue to be analysed in the future is the fact that both systems (cultures in vitro and storage of dried seeds) could be under the influence of common or related mechanisms of cellular senescence, which would lead to the occurrence of apparently identical cytological abnormalities at mitosis.

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