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Sister chromatid exchanges in cultured immature embryos of wheat species and regenerants

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Abstract Immature embryos of *Triticum aestivum* (ten cultivars and lines), *T. durum*, *T. dicoccum* and *T. monococcum* were cultured in vitro on MS medium supplemented with 1 or 2 mg/l of 2,4-D and 20 or 30 g/l of sucrose for 3 days and processed to score sister chromatid exchanges (SCEs) per chromosome. Media components affect DNA replication from the start of the culture. The SCE frequencies were dependent on the genotype and were not correlated with the degree of ploidy. They increased after doubling of the concentration of 2,4-D and/or sucrose, except in one cultivar of *T. aestivum*. The mean numbers were lower than observed in root meristems of *T. aestivum* (two cultivars) and *T. dicoccum*. Immature embryos of regenerants of *T. aestivum* (one cultivar) and *T. durum* demonstrated variable SCE frequencies, which may have been caused by mutations in the parental cell cultures. In the *T. aestivum* embryos the lowest frequencies were found in regenerants obtained from explants with the highest frequencies.

Key words Auxin · Callus · Regenerants · Sister chromatid exchange · Sucrose · *Triticum*

Introduction

In-vitro plant cell and callus cultures are subject to chromosome instability. The occurrence of polyploidy, aneuploidy, and structural chromosome rearrangements are influenced by genotypic, nutritional, and other environmental factors (reviews Bayliss 1980; Karp 1991). Cultures of wheat (*Triticum aestivum* L.) and related species also do not always escape this karyotypic variation, which can be followed up to and within regenerated plants and their progenies (Shimada et al. 1969; Ahloowalia 1982; Ozias-Akins and Vasil 1982; Karp and Maddock 1984; Davies et al. 1986; Chen et al. 1987; Bennici et al. 1988; De Buyser et al. 1988; He et al. 1992; Winfield et al. 1993).

Sister chromatid exchanges (SCEs) are a very sensitive tool to detect potential DNA damage caused by chemicals when compared with genome and chromosome mutations (Kato 1977; Wolff 1977; Latt 1981; Kihlman and Andersson 1984). Their occurrence in plant cell cultures has been little studied. Doležel and Novák (1986) observed more SCEs in chromosomes of callus cultures than in root-tip chromosomes of garlic (*Allium sativum* L.) and Murata (1989) found that in cell suspensions of wheat a higher concentration of the auxin 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) increased the SCE frequency, and that the auxins NAA (α-naphtylacetic acid) and 2,4-D (2,4-dichlorophenoxyacetic acid), and the cytokinin kinetin, had no effect.

It may be expected that components of the culture medium exert their influence on the karyotype from the onset of a culture. Hardly anything is known about their mutagenic effect in tissue explants during the very first mitotic divisions. Endoreduplication, nuclear fragmentation, and DNA amplification can all be induced by hormones (Libbenga and Torrey 1973; Cionini et al. 1978; Durante et al. 1983; review D'Amato 1985) and endoreduplication also by sucrose (Pijnacker and Ferwerda 1990). In the present study we investigated whether 2,4-D and sucrose had an influence on SCE frequencies during the first mitoses in explants of immature embryos from wheats. Various cultivars and breeding lines of the hexaploid *T. aestivum* L. em Thell., the tetraploids *T. durum* Desf. and *T. dicoccum* Schrank, and the diploid *T. monococcum* L. were used to establish genotypic predisposition, while regenerants of *T. aestivum* and *T. durum* were employed to examine culture-related variation.

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Materials and methods

Plants

The following self-pollinating wheats, grown in the glasshouse under controlled conditions, were used: hexaploid *T. aestivum* (genomic constitution AABBDD, 6x = 42) spring wheats cv Minaret, cv Sicco, cv Baldus, line 2002, line 9009, line 89-3331 and winter wheats cv Fletum, cv LM, cv Konsul, cv Obelisk; tetraploid *T. dicoccum* (AABB, 4x = 28); tetraploid *T. durum* (AABB, 4x = 28) and diploid *T. monococcum* (AA, 2x = 14).

Embryo culture and regeneration

Immature embryos of about 2 mm were collected according to standard procedures (Maddock et al. 1983; Borrelli et al. 1991; Qureshi et al. 1992) and 15 embryos per genotype were cultured, with the scutellum exposed, at 23 °C in the dark on the following media: MS basal medium (Murashige and Skoog 1962) + 1 mg/l 2,4-D + 20 g/l sucrose = w120; MS + 1 mg/l 2, 4-D + 30 g/l sucrose = w130; MS + 2 mg/l 2,4-D + 20g/l sucrose = w220; MS + 2mg/l 2,4-D + 30 g/l sucrose = w230. All media were adjusted to pH 5.8 and solidified with 8 g/l of agar.

For plant regeneration of Sicco, *T. durum*, and *T. dicoccum* 1-month-old calli from the four media were divided into small portions and subcultured on MS medium with 10 g/l of sucrose at 23 °C and with a 16/8 h light/dark regime. After 3–4 weeks, shoots were transferred to containers with MS10 and after another 2–3 weeks transferred to soil and grown to maturity in the glasshouse. Depending on the initial medium, the regenerants were designated 120-R1, R2, etc, 130-R1, R2, etc.

Cytology

For SCE analysis, immature embryos were cultured on the four media with 5-bromodeoxycytidine (BrdC) according to Pijnacker et al. (1986) and immature embryos of the regenerants were cultured on medium w120 + BrdC only. After 3 days, that is after two cell cycles with BrdC incorporation, the embryos were collected, pretreated in α-bromonaphtalene for 24 h at 4 °C and fixed in cold 3:1 alcohol-glacial acetic acid for at least 24 h. After enzymatic digestion in 3% cellulase (w/v) + 30% pectinase (v/v) in citrate buffer, pH 4.8, for 3.5 h at 37 °C, air-dried slides were prepared. To detect SCEs, the slides were differentially stained by the fluorescent plus Giemsa technique (cf. Pijnacker et al. 1986). SCEs of a least 100 chromosomes per embryo of minimally six embryos per genotype were counted.

Results

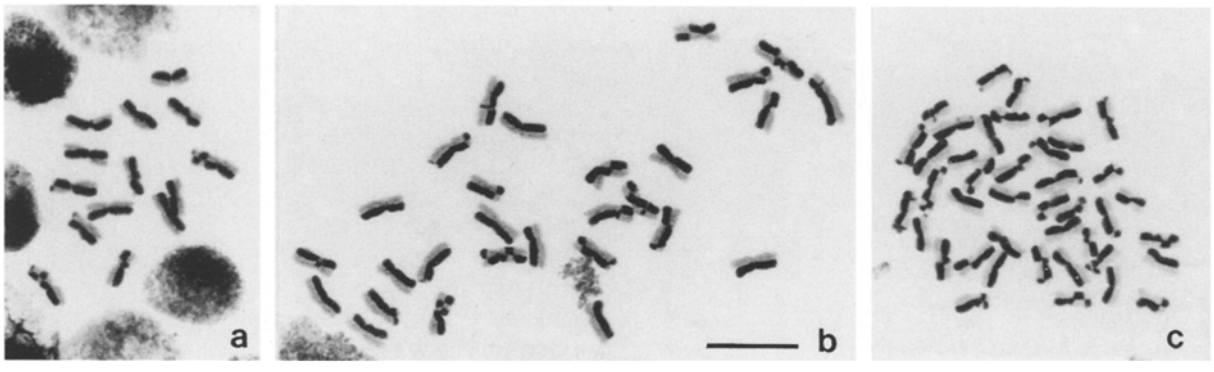
General

Differentially-stained metaphases were found in the immature embryos (with scutellum) of all genotypes cultured for 3 days on the four callus-inducing media (Fig. 1). The number of SCEs per chromosome varied from 0 to 8. The highest number was always found in embryos cultured on 30 g/l of sucrose: i.e., six SCEs in LM, Minaret, Sicco and *T. monococcum*, seven SCEs in Konsul, Baldus, 89-3331, *T. durum* and *T. dicoccum*, and eight SCEs in Obelisk. The frequency distributions of chromosomes with a different number of SCEs corresponded with Poisson distributions (Fig. 2).

Means

The mean number of SCEs per chromosome, calculated from the distribution frequencies of the chromosomes of all the immature embryos, are given for each genotype and each medium in Fig. 3 and the differences between the means (t-tests) are summarised in Table 1. The lowest mean is found in *T. monococcum* on medium with the lowest concentration, and the highest in 89-3331 on medium with the highest concentration. Minaret reacts most uniformly, and 89-3331 most variably, to the various media. The standard deviations are not given: they are equal to the square root of the means. The differences between the means reveal that an increase in 1 mg/l of 2,4-D induces significantly more SCEs in Fletum, Konsul, 9009, 89.3331, *T. durum* and *T. dicoccum* on media with 20 and 30 g/l of sucrose, and in LM, Sicco, Baldus, 2002 and *T. monococcum* on media with either 20 or 30 g/l of sucrose, but not in Obelisk and Minaret. The effect of sugar is less pronounced. More SCEs were induced in Baldus, 89-3331 and *T. dicoccum* on media with 1 and 2 mg/l of 2,4-D, in LM, Sicco, 2002 and *T. monococcum* on media with either 1 or 2 mg/l of 2,4-D, but not in Fletum, Konsul, Obelisk, Minaret, 9009 and *T. durum*. The influence of the medium components on SCE frequencies is affected by the genotype which is especially clear in 89-3331, Obelisk, Minaret and *T. dicoccum*. The effects of 2,4-D and sucrose are additive, for the highest frequencies are found on medium w230,

Fig. 1a–c Differentially-stained metaphases showing sister chromatid exchanges. **a** *T. monococcum*, 2x = 14; **b** *T. dicoccum*, 4x = 28; **c** *T. aestivum*, cv Sicco, regenerant 130–R8 with 6x = 41; bar = 10 μm



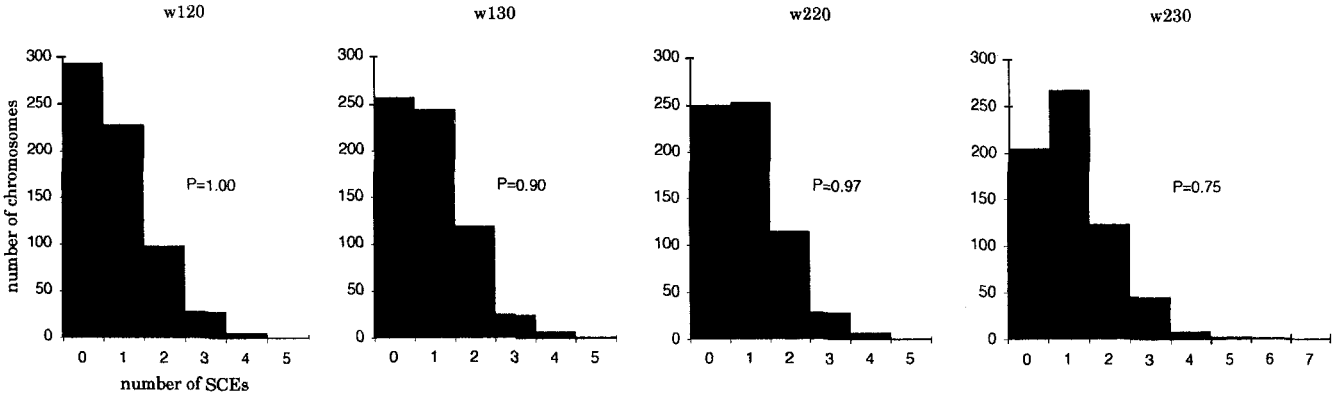
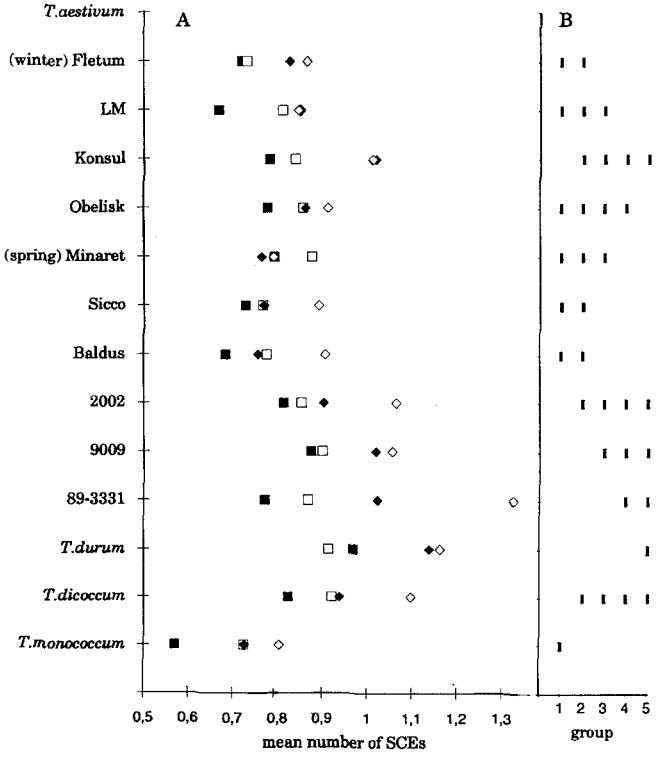


Fig. 2 Frequency distributions of 656 chromosomes with 1–7 SCEs from immature embryos of *T. dicoccum* cultured on media w120, w130, w220, w230 for 3 days, compared with Poisson distributions (*P*-values)

with Minaret as an exception. The means of the pooled data of the spring and winter wheats did not differ except when the medium contained 2 mg/l of 2,4-D and 30 g/l of sucrose ($P < 0.01$).

Analysis of variance on the means of all genotypes reveals that the addition of 1 mg/l of 2,4-D as well as 10 g/l of sucrose induces significantly ($P < 0.01$) more SCEs per chromosome, and that the influence of the genotype is also highly significant ($P < 0.01$). Figure 3 shows that there are five groups of genotypes in which the mean numbers of SCEs per chromosome, calculated from all the chromosomes found in the embryos on the four media, are not significantly different from one another ($P > 0.05$). The tetraploids *T. durum* and *T. dicoccum* belong to the genotypes with the highest mean SCE frequencies and the diploid *T. monococcum* to the lowest; the hexaploids cover the whole range.

Fig. 3 **A** Effect of media on the mean number of SCEs per chromosome in cultured immature embryos of various wheat genotypes. ■, □, ◆, ◇ = medium w120, w130, w220, w230, respectively. **B** Genotypes divided into five groups in which the mean numbers of SCEs per chromosome, based on all the chromosomes, do not differ significantly ($P > 0.05$) according to Tukey's test; to the right the groups with higher means



Calli

The embryos had changed into a callus after 14 days of culture. Calli of Sicco, cultured on fresh media with BrdC for another 3 days, demonstrated a significant increase in the mean numbers of SCEs per chromosome compared to the means found after 3 days of culture ($Ps < 0.01$). The means were 1.40, 1.39, 1.50 and 1.79 SCEs and the maximum numbers 6, 7, 7 and 9 SCEs on media w120, w130, w220 and w230, respectively (Poisson distributions).

Roots

Seeds of Minaret, Sicco, and *T. dicoccum* germinated on wet filter paper and with roots of about 1 cm were grown on MS medium without hormones and sucrose and with BrdC, like the immature embryos. The chromosomes of six roots of each genotype had a significantly higher mean number of SCEs per chromosome than the chromosomes of their immature embryos ($Ps < 0.01$). The mean numbers were 1.01, 1.05 and 1.71 SCEs and the maximum numbers 5, 4 and 8 SCEs (Poisson distributions), respectively.

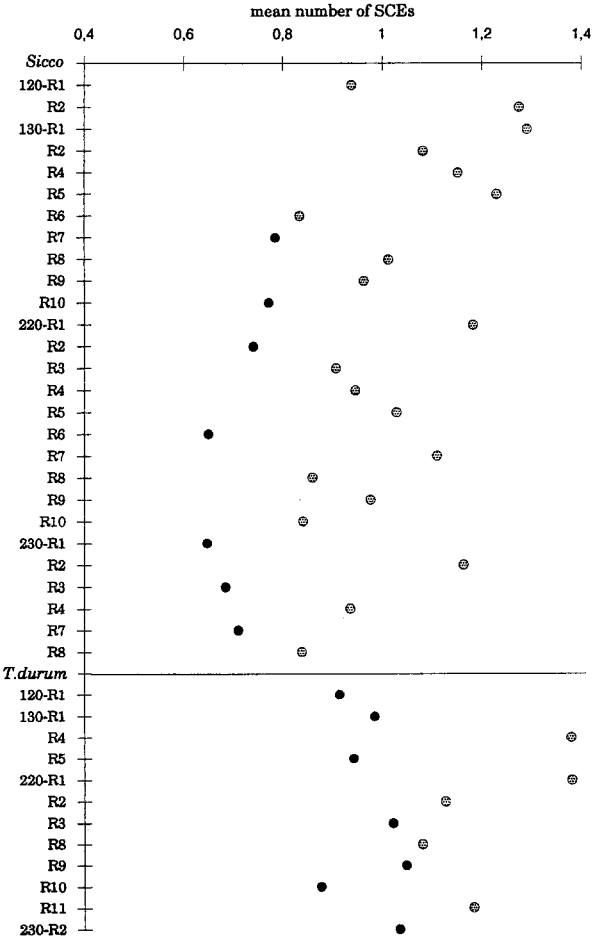
Embryos of regenerants

Fertile regenerants were obtained from Sicco and *T. durum* calli (for numbers see Fig. 4), but it was not possible to induce regeneration in calli of *T. dicoccum*

Table 1 Differences between the means (t-tests) of SCEs of chromosomes of immature embryos of various wheat genotypes cultured on different media for 3 days: −, $P > 0.05$; ±, $0.05 > P > 0.01$; +, $P < 0.01$

Genotype	Media					
	w120 vs w130	w220 vs w230	w120 vs w220	w130 vs w220	w130 vs w230	w120 vs w230
T. aestivum						
Fletum	−	−	±	±	+	+
LM	+	−	+	−	−	+
Konsul	−	−	+	+	±	+
Obelisk	−	−	−	−	−	+
Minaret	−	−	−	±	−	−
Sicco	−	+	−	−	+	+
Baldus	±	+	−	−	+	+
2002	−	+	−	−	+	+
9009	−	−	+	±	+	+
89-3331	±	+	+	+	+	+
T. durum	−	−	+	+	+	+
T. dicoccum	±	+	±	−	+	+
T. monococcum	+	−	+	−	−	+

Fig. 4 Mean number of SCEs of chromosomes from immature embryos of regenerants (*R*1, 2, etc.) of *T. aestivum* cv Sicco and *T. durum* from callus cultures on media w120 (*120*), w130 (*130*), w220 (*220*), w230 (*230*); ●, are mean numbers which are not significantly ($P > 0.05$) different from those from parental embryos cultured on w120; the others are significantly higher



and *T. monococcum*. The embryos of the regenerants had the parental chromosome number except for two out of six embryos of regenerant 130-R8 of Sicco which were mosaic for 41 (Fig. 1c) and 42 chromosmes.

The frequency distributions of the chromosomes with different numbers of SCEs in the immature embryos of these regenerants, cultured on medium w120 with BrdC for 3 days, had a Poisson pattern as did the parental embryos. The maximum number of SCEs per chromosome was seven in Sicco and eight in *T. durum*. The mean number of SCEs per chromosome (standard deviations, = square root of the means, are not given) varied from regenerant to regenerant in both species (Fig. 4). The mean number of SCEs of a regenerant was either significantly higher ($P < 0.05$) than, or equal to, the mean of the parental embryos cultured on medium w120 (Fig. 4). In particular, regenerants of Sicco had higher frequencies. Embryos of regenerants of Sicco from cultures with more sugar generate significantly fewer SCEs (total 120R versus total 130R, $P < 0.01$; total 220R versus total 230R, $P < 0.01$) and the same applies to cultures with more 2,4-D (total 120R versus total 220R, $P < 0.01$; total 130R versus total 230R, $P < 0.01$). A similar comparison for *T. durum* gives either no differences (total 220R or total 130R versus 230R, $Ps \geq 0.16$) or the opposite result (120R versus total 130R or total 220R, $Ps < 0.01$). These latter conclusions are rather unreliable because they are based on only one regenerant from medium w120 and one from medium w230.

Discussion

The results demonstrate that media components may affect DNA replication in cell cultures from the start of the culture. Either more 2,4-D and/or more sucrose resulted in more SCEs per chromosome in the diploid, tetraploid, as well as hexaploid wheats, except for Minaret. Murata (1989) did not find a significant increase in the number of SCEs per metaphase in cell suspensions of the hexaploid Chinese Spring when increasing the con-

centration of 2,4-D from 0.5 mg/l to 10 mg/l. From his data it can be deduced that the mean number of SCEs per chromosome increased considerably, from 0.412 to 0.667. How 2,4-D and sucrose produce alterations in DNA replication is not known. Their influence may be indirect, rather than direct, because 2,4-D, as a growth regulator, and sucrose, as a carbon energy source, are involved in many different kinds of metabolic processes. The effects of 2,4-D and sucrose were additive and the exchanges occurred randomly within the chromosomes as demonstrated by the Poisson distributions (cf. Friebe 1978). These two medium components thus induce SCEs at different sites on the chromosomes, most likely by two different processes.

The tetraploids *T. dicoccum* and *T. durum* have relatively-high mean numbers of SCEs and the diploid *T. monococcum* low ones. These differences cannot be explained by differences in the length of the chromosomes (Kato 1977; Wolff 1977; Latt 1981) because the differences between the mean length of the genomes/chromosomes of the two tetraploids (A + A + B + B/4) and that of the diploid (A + A/2) amounts to only 4.8% (Table 1 in Gill et al. 1991), which is much lower than the differences between the mean numbers of SCEs. The same applies when the amounts of DNA are considered (Rees and Walters 1965; Bennett and Smith 1976). The level of SCE frequency is thus determined by the genetic constitution (cf. Kato 1977; Wolff 1977; Latt 1981). This explains the differences in SCE frequencies between the hexaploid genotypes of *T. aestivum* and perhaps also the low numbers of SCEs found by Murata (1989) in the primitive cultivar Chinese Spring. The range of SCE frequencies of the hexaploid genotypes covers that of the diploid and tetraploids. The frequencies are thus not correlated with the degrees of ploidy.

The mean number of SCEs per chromosome in the roots of seeds of Minaret, Sicco, and *T. dicoccum* was higher than in the immature embryo cultures. In contrast, root meristems of *Allium sativum* have lower frequencies than callus cultures (Doležel and Novák 1986), which applies also to root meristem and stem-explant cultures of *Vicia faba* (unpublished). However, after 17 days of culture of immature embryos, the chromosomes of Sicco demonstrated more SCEs than the roots. Though several causes may underlie these differences (Kato 1977; Wolff 1977; Latt 1981), the age of the culture is an influential factor. It is well-known that genetic variation in cell cultures increases with the duration of the culture (D' Amato 1985; Lee and Phillips 1988; Karp 1991).

The SCE frequencies in the immature embryos of the regenerants of Sicco and *T. durum* varied notwithstanding culturing under similar circumstances. Within the species, differences were not expected because genome mutations were not found (except for two embryos). One explanation could be that heritable factors of the same kind that cause differences in SCE frequencies between species and between hexaploid genotypes underwent mutation during the cell culture period before regeneration. Point mutations can occur at high frequencies in cell cultures (D'Amato 1985; Karp 1991). Embryos from regenerants of Sicco which originated from explants with the highest SCE frequencies had a lower mean number than embryos from regenerants which originated from explants with the lowest frequencies. It thus seems as if the plants regenerated from cells with high SCE frequencies built up a resistance against the induction of SCEs. Whether this effect has an epigenetic or genetic basis is not yet known.

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