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Chromosomal location of genes controlling short-term and long-term somatic embryogenesis in wheat revealed by immature embryo culture of aneuploid lines

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Abstract The expression of essential genes during somatic embryogenesis can be analysed by inducing aneuploid cells to undergo embryogenesis during immature embryo culture and then determining whether defects occur. *Triticum aestivum* disomic and aneuploid stocks, including 36 ditelosomics and 7 nullitetrasomic 'Chinese Spring' wheats, were compared for their ability to undergo somatic embryogenesis after 2 months of in vitro immature embryo culture. Their regeneration capacity was observed after 4 and 14 months of in vitro culture to determine which chromosome arms influence the process. The large range of variation found among the tested aneuploids suggested that genetic control of the somatic tissue culture ability is polygenic. Our results indicate that genes affecting somatic embryogenesis and regeneration are located in all of the homoeologous chromosome groups. The lack of chromosome arms 1AL (DT 1AS) and 3DL (DT 3DS) practically suppresses somatic embryogenesis, demonstrating that major genes on wheat chromosome arms 1AL and 3DL control regeneration capacity. Results suggest that plants were mainly produced from somatic embryo development. Although the control of somatic embryogenesis and regeneration is polygenic, the genes located on the long arms of homoeologous group 3 chromosomes have a major effect. We also have evidence of chromosome arms that determine the time required for regeneration.

Key words Somatic embryogenesis · Regeneration · Wheat · Aneuploid analysis

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Introduction

In wheat, embryo production can be induced in a wide range of cells through in vitro tissue culture. Nevertheless, manipulation at the cellular level is frequently limited by unsuccessful attempts to regenerate whole fertile plants. Evidence that anther culture is under genetic control has been provided by a diallel study (Charmet and Bernard 1984) and genetic (Raquin 1982; Henry et al. 1984; Henry and De Buyser 1985) and aneuploid analyses (Szakacs et al. 1988; Agache et al. 1988). A few papers have been published that describe the behaviour of wheat ditelocentric lines during somatic tissue culture. First results showed that different chromosome arms are involved in the control of callus growth (Shimada and Makino 1975; Baronchelli et al. 1978). Monosomic (Kaleilau et al. 1989a) and substitution analyses (Galiba et al. 1986; Mathias and Fukui 1986; Mathias et al. 1988) have identified chromosomes that appear to be involved in the regeneration of shoots from short-term wheat callus cultures. This suggests that a polygenic system is responsible for somatic embryogenesis, defined here as the ability of the cultured somatic tissue to produce embryos. Several groups are presently working on the localization of the genes that control tissue culture response on specific chromosome arms using several ditelosomic lines (Felsenburg et al. 1987; Kaleilau et al. 1989b).

Reciprocal crosses have already been performed between the embryogenic variety 'Chinese Spring' and a nonembryogenic line 'Aquila'. The embryogenic ability of the F_2 genotypes was found to depend on recessive alleles with a two to four loci difference between 'CS' and 'Aquila' for short- and long-term somatic embryogenesis ability, respectively (De Buyser et al. 1992). The mode of inheritance of somatic embryogenesis ability appears to be mostly nuclear.

Aneuploid genetic stocks of 'Chinese Spring' were employed to ascertain the homoeologous group and chromosomal arm locations of the genes acting on

somatic tissue culture ability. In theory, the loss of structural or regulatory genes located on a missing chromosome arm would be expressed by the qualitative or quantitative reduction or increase of the character controlled. In the investigation reported here our aim was to determine the genetic basis of somatic embryogenesis and regeneration capacity by identifying chromosome arms that modify the intensity of the characters.

Materials and methods

Plant material

The hexaploid wheat (*Triticum aestivum* L., 2n = 6x = 42) var 'Chinese Spring' ('CS') used as the control genotype has a high somatic embryogenesis capacity. The ditelosomic (DT) lines (2n = 40' + 2t') and all of the nullisomic-tetrasomic (NT) series derived from 'Chinese Spring' that were used were provided by S. M. Reader (AFRC, Cambridge Laboratory, UK). The 'CS' DT 6BL was obtained from Dr. Y. Furata (Gifu University, Japan). Each DT line is deficient for one arm of both chromosomes of a homologous chromosome pair. DT lines are designated by their homoeologous chromosome group (1–7), their genome (A, B or D) and the chromosome arm length (*L* = long, *S* = short). The chromosome nomenclature used is that agreed to by the Seventh International Wheat Genetic Symposium.

The dimonotelos (2n = 40' + 3t') of 'Chinese Spring' provided by G. Kimber (University of Missouri, USA) were used to produce the following ditelosomics in their progeny: DT 1DS, DT 2AL, DT 2BS, DT 3BS, DT 6AL and DT 7DL.

DT 2DL, DT 4BL, DT 5BS and DT 5DS, missing as a result of sterility or reduced fertility, were complemented through the use of their corresponding nullisomic-tetrasomic (NT) lines (2n = 42): N2DT2A, N4BT4D, N5BT5A, N5BT5D, N5DT5A, N5DT5B. This enables the tissue culture parameters of 2DS, 4BS, 5BL and 5DL arms to be calculated. N6BT6A was used as a reference in order to confirm the results of the DTs. Each NT line lacks a given chromosome pair and compensates with an extra pair of one of their homoeologues. The effect of the 4AL and 5AL arms was not studied.

Caryopses of the controls and DT and NT lines were germinated in petri dishes. All seedlings were identified cytologically by counting chromosomes in root-tip cells by the Feulgen technique to ensure that plants carried the correct chromosome complement. From 3 to 6 plants from every checked control and DT and NT genotype were grown to maturity in 25-cm diameter pots containing compost, in a growth cabinet under controlled environmental conditions: a 16-h photoperiod with a light intensity of 200 μE $m^{-2}s^{-1}$ provided by sodium lights at day/night temperatures of 20 °C and 16 °C, respectively. The spikes were bagged to ensure self-pollination.

The experiment was separated into groups of plants in order to make tissue culture work easier: respectively, homoeologous group 1, 3, 7, 6, 4, 2, 5, two groups involving NT and lastly two complementary DT groups. Each group also included two controls: 'CS' and a di short mono long telocentric (DSMT) or a di long mono short telocentric (DLMT) double ditelosomic progeny possessing 40' + 4t'.

Tissue culture

The establishment of wheat somatic tissue cultures which are competent for somatic embryogenesis requires an explant containing totipotent cells and a simple medium providing 2,4-D. Fourteen days after anthesis the 'CS', DT and NT immature seeds were harvested and surface sterilized in 2% Ca-hypochlorite for 1-2 min. The young embryos were excised under a dissecting microscope and placed, 10 per 9-cm petri dish, with the scutellum exposed and the meristem and the epiblast embedded in the medium. A total number of 7897 "immature embryos" were cultured, including 1777 from 'CS' and 1039 from the controls having four telocentrics. A mean number of 124 (and as many as 226 for DT 6DS) immature embryos from a minimum of 6 spikes were cultured for every DT genotype. Because of poor fertility very limited numbers of immature embryos were used for DT 6AL (32), DT 2BS (11) and DT 2AL (5). The mean number of cultured immature embryos was 94 for the NT lines, ranging from 23 in N5DT5A to 278 for N2DT2A.

The culture medium contained Murashige and Skoog (1962) inorganic salts and vitamins, 20 g l^{-1} sucrose, 2 mg l^{-1} 2,4-D and 5.5 g l^{-1} agarose. The pH was adjusted to 5.8 before autoclaving. Individual calli were observed after 2 months of in vitro culture and classified as nonembryogenic (1), possessing green spots but without embryos (2) and possessing somatic embryos (3). Calli were subcultured at 2-month intervals onto the same medium for a total of 14 months. The capacity for "somatic embryogenesis" was maintained over a long period by selecting the organized callus pieces at subculture (De Buyser et al. 1988). SC_0 refers to the initial embryo culture phase and SC_1–SC_n to subsequent subcultures 1 to *n*, with SC_1, i.e. 4 months in vitro, classifed as a short-term culture and SC_6, i.e. 14 months in vitro, classified as a long-term culture.

In order to regenerate plants, pieces of callus were transferred onto the same medium without 2,4-D. The regeneration capacity was tested after 4 and 14 months of culture, on the basis of 100 subcultured callus pieces for every genotype (only 70 for the DT 1AS, DT 1BS and DT 3BS lines after short-term culture). All cultures were maintained in the same culture room at day-night temperatures of 27 °C ± 1 °C and 24 °C ± 1 °C, respectively, under low illumination (10 μE $m^{-2}s^{-1}$) with a 16-h daylength.

On the basis of the 64 tested genotypes (11 'CS', 38 DT, 8 DSMT or DLMT and 7NT), correlation coefficients were calculated between frequency of SC_0 callus possessing embryos, frequency of SC_0 callus with green spots, frequency of short-term regeneration and frequency of long-term regeneration.

Results

The absence of significant differences between 'CS' and DSMT or DLMT lines revealed that for the tissue culture characteristics observed here, a chromosome pair or both DTL and DTS arm pairs have similar effects. The chromosomal control of somatic tissue culture characteristics was inferred from their absent, reduced or increased expression in the aneuploid (DT and NT) lines deficient for a given chromosome arm or chromosome. The results for the ditelosomic lines of all homoeologous groups are presented in Figs. 1–4. The effect of the absence of the chromosome arms is shown in Table 1 (e.g. effect of the absence of 1AL arm using DT 1AS).

In our experiments, the absence of many chromosome arms has consequences on the tissue culture phenotype. As an example, for homoeologous group 1, the ditelosomic lines DT 1AS and DT 1BS (i.e. the lines deficient for the pair of chromosome arms 1AL or 1BL) were different from the euploid 'CS' line; the frequency of SC_0 callus with embryos was decreased (Fig. 1) in these DT lines. On the contrary, DT 1DS and DT 1DL seemed to be better than the 'CS' line. When we observed the frequency of SC_0 callus with green spots (Fig. 2), these 4 ditelosomic lines had similar effects in reducing the frequency. The other group 1 ditelosomic lines were similar to 'CS'. Regeneration after 4 months of in vitro culture (Fig. 3) was also affected for several DT

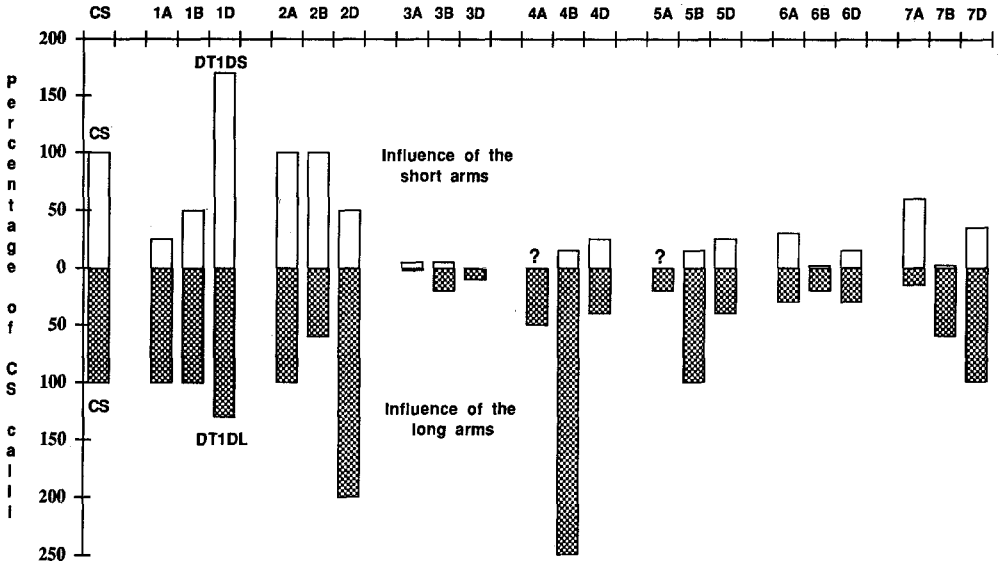

Fig. 1 Ditelosomic analysis of the frequency of SC_0 calli with embryos

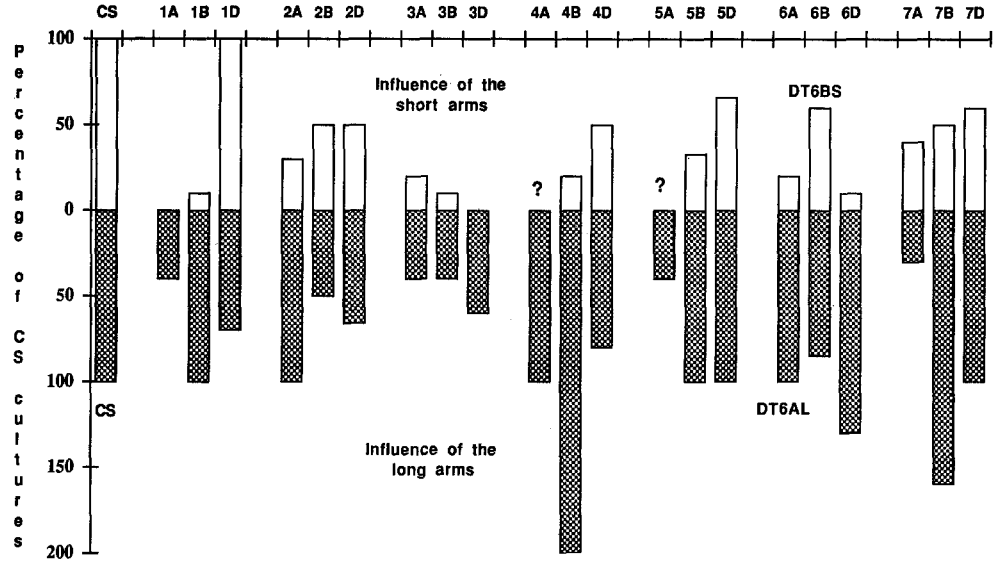

Fig. 2 Ditelosomic analysis of the frequency of SC_0 calli with green spots

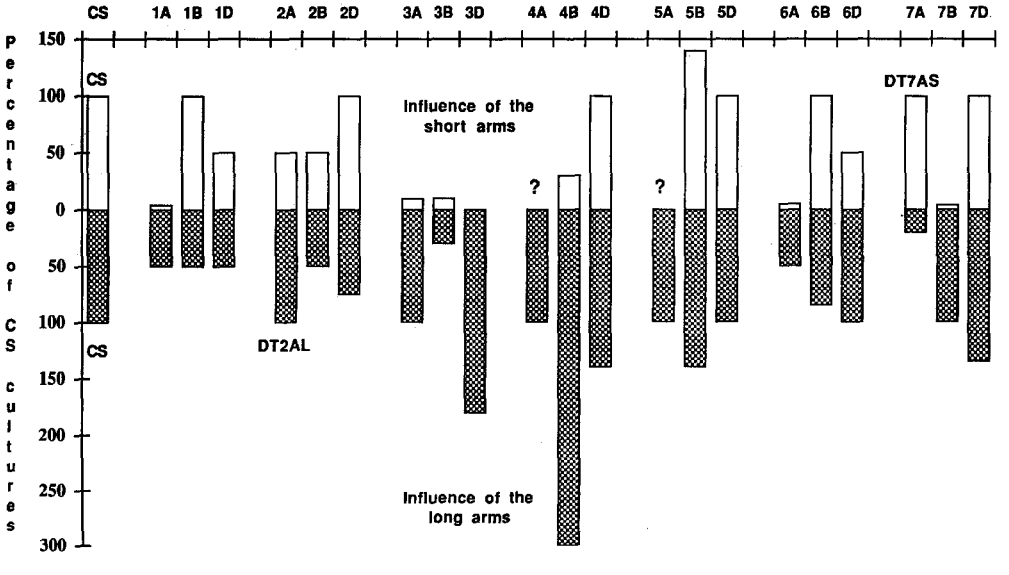

Fig 3 Ditelosomic analysis of the regeneration frequency after short-term culture

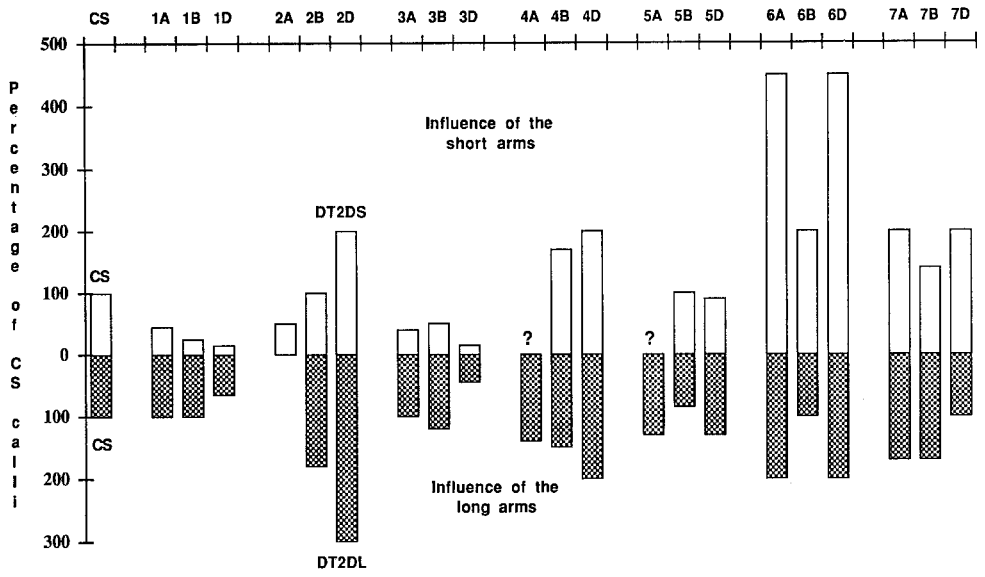

Fig. 4 Ditelosomic analysis of the regeneration frequency after long-term culture

lines, most significantly DT 1AS and DT 1BS. After 14 months (Fig. 4) a regeneration frequency similar to that of 'CS' was restored for the DT 1BS line, whereas the regeneration capacity remained limited for DT 1AS and decreased for the other group 1 DT lines.

For homoeologous group 3 (Table 1 and Figs. 1–4) important differences were observed for each of the ditelosomic lines. The frequency of SC_0 callus with embryos was very low and in most cases was not accompanied by a high level of green spot formation. So it seems that homoeologous group 3 chromosome arms affected both characteristics of short-term somatic embryogenesis. After short- or long-term somatic embryogenesis, a better regeneration frequency was observed from long-arm ditelosomic lines than from short-arm ditelosomic lines. Some of them, i.e. DT 3AL and DT 3DL, reached a level equal to or better than that of 'CS'. Short-arm ditelosomic lines have a limited regeneration frequency that does not improve after long-term culture.

A decrease in the frequency of any tissue culture characteristic in the absence of a particular chromosome arm means that the presence of this arm could have a positive effect. Many chromosome arms modified the frequency of callus with somatic embryos during the initiation of the culture (Table 1 and Fig. 1). Only 7 arms of the 40 tested had no effect on the first step of somatic embryogenesis (embryos and green spots). The absence of 29 different chromosome arms resulted in a reduction in the frequency of embryos: 19 also revealed an increase in the frequency of green spots, 4 did not affect the frequency of green spots and 6 had a similar effect on embryo and green spot formation. In most cases the genes controlling the first step of somatic embryogenesis have a positive effect on embryo formation. This suggests that most of the arms are needed for high levels of embryogenesis. Several arms, mainly those from homoeologous group 3, had strong effects. Chromosome arms 1DS, 1DL, 2DS and 4BS seemed to reduce the frequency of callus with somatic embryos, since the absence of the short or long arm pairs increased the frequency of SC_0 callus with embryos.

The frequency of SC_0 callus with green spots but without embryos (Table 1 and Fig. 2), was inversely related to the frequency of callus with embryos. Many of the chromosome arms had an inhibiting effect on the formation of green spots on SC_0 callus. Only the separate absence of 6 arms (1AL, 1BL, 3AL, 3BL, 3DS, 3DL) reduced both the frequencies of SC_0 callus with embryos and green spots. The presence of the 1-D chromosome reduced embryo formation but increased green spot formation. Experimental results concerned with the frequency of SC_0 callus possessing embryos or green spots showed a significant negative correlation coefficient (-0.41) between these two traits of somatic embryogenesis (Table 2). This suggests that a decrease in the frequency of embryos is often associated with an increase in green spot frequency on SC_0 callus.

In the second part of the experiment regeneration ability after short-(4 months) or long-term (14 months) *in vitro* culture was examined. In 29 of the 40 ditelosomics the absence of a chromosome arm decreased the frequency of green plant regeneration from short-term cultures (Fig. 3). Several arms had no effect, but only arms 4BS, 6DS and 7BS seemed to have had a negative effect on short-term regeneration capacity since their absence in the corresponding DT increased the regeneration potential.

Analysis of the results showed positive correlation coefficients (0.39 and 0.34) between embryos on SC_0 callus and regeneration capacities, but a lack of any significant correlation coefficient between green spots and regeneration (Table 2). This suggests that plant regeneration occurs from somatic embryo germination and has no clear relationship with the presence of green spots. The absence of chromosome arm 3DL suppressed

Table 1 Effect[a] of the absence of chromosome arms on the appearance of somatic embryos and meristems on SC_0 callus and on the regeneration capacity after short- and long-term somatic embryogensis

Missing arm	Embryos on SC_0 callus	Green spots on SC_0 callus	Regeneration capacity short-term	long-term
1AS	0	0	−	−
1BS	0	0	0	−
1DS	+	−	−	−
1AL	−	−	− −	− −
1BL	−	− −	− −	0
1DL	+	− −	0	−
2AS	0	− −	0	0
2BS	−	+	−	−
2DS	+	+ +	0	−
2AL	0	−	−	−
2BL	0	0	−	−
2DL	−	+	−	0
3AS	− −	0	−	0
3BS	−	+	−	−
3DS	− −	−	−	+
3AL	− −	−	− −	− −
3BL	− −	−	− −	− −
3DL	− −	− −	− −	− −
4AS	−	0	0	0
4BS	+	0	+	+ +
4DS	−	+	0	+
4BL	−	+	−	−
4DL	− −	+	−	0
5AS	− −	+	−	0
5BS	0	0	0	+
5DS	−	+	0	0
5BL	−	+	−	+
5DL	−	0	−	0
6AS	−	+	0	−
6BS	−	0	−	−
6DS	−	+	+	0
6AL	−	+ +	− −	− −
6BL	− −	+	−	0
6DL	− −	+ +	− −	−
7AS	− −	+	−	− −
7BS	−	+	+	0
7DS	0	0	0	+
7AL	−	+	−	0
7BL	− −	+	−	− −
7DL	−	+	−	0

[a] − −and −, Important and limited significant decrease, respectively, compared to CS; + + and +, important and limited significant increase, respectively, compared to CS

the production of embryos on SC_0 callus, whereas the absence of the 1AL arm strongly reduced it (Fig. 1). The 3DL and 1AL arms also controlled the regeneration capacity (Figs. 3 and 4). Of the 29 chromosome arms whose absence was associated with decreased embryo formation, 23 were also associated with a reduced frequency of short-term regeneration. Most of the regeneration capacity seemed to depend upon the ability to produce somatic embryos during the initial tissue culture step. With the other 6 arms, genes acted by inhibiting regeneration since in their absence a regeneration rate at least equivalent to that of 'CS' was observed.

The long-term culture (14 months) revealed more contrasting results (Fig. 4). For several DT lines it was possible to recover a normal regeneration rate during successive subcultures and even to obtain a higher regeneration capacity than that of 'CS'. Results from 19 arms of 40 tested were not significantly different for short- and long-term regeneration capacity. When the embryogenic callus pieces in some DT lines were selected for at subculture it was possible to recover a regeneration ability at least similar to that of 'CS' (15 arms). These results could explain the observation of similar regeneration frequencies from short-(45.7%) and long-term (46.3%) cultures for most of the missing arms. Moreover, the correlation coefficient (0.68) between these two traits was highly significant (Table 2). Most of the chromosome arms acted in the same way for short- and long-term regeneration capacity.

Mean results showed that the process of somatic embryogenesis was equally affected by the genomes A, B and D. There was no relationship between the level of heterochromatin (highest in the B genome) and any of the tissue culture characteristics measured. However, there were differences between short and long chromosome arms for the different traits. Results from Table 1 show that none of the test aneuploid lines behaved in the same way as 'Chinese Spring'. All of the tested arms modified one or more characteristics of the somatic embryogenesis process, i.e. embryo formation, green spots or short- and long-term regeneration capacity. Most of the chromosome arms were needed, but a few seemed to have deleterious effects.

Our last observation had to do with the time needed for regeneration. After short-term culture, only the 7AS arm increased. In long-term cultures, two groups appeared: chromosome arms that significantly sped up the

Table 2 Correlation coefficient between somatic tissue culture characteristics

		Percentage of SC_0 callus with green spots	Regeneration capacity	
			SC_1	SC_6
Percentage of SC_0 callus	With embryos	−0.41**	0.39*	0.34*
	With green spots		−0.10	−0.13
Regeneration capacity	SC_1			0.68**

*,** Significantly different from 0 at 1% and 0.1%, respectively

time needed for regeneration with respect to 'CS', and others that slowed it down. In the first group 7 arms were relevant (3DS, 4AS, 4BL, 4DS, 7BS, 7DS, 7DL), and in the second only 4 (1DL, 3BS, 6AS, 6DL).

Discussion

The control of tissue culture characteristics (embryo formation, meristem development and regeneration capacity) by various chromosomes was either confirmed or, in most cases, established. When our results are compared to previously published results on the effect of the B genome chromosomal arms on regeneration capacity from short-term cultures (Felsenburg et al. 1987), our ditelosomic analysis confirms that 1BL, 2BS, 6BS, 6BL and 6DL arms promote regeneration; however under our conditions the absence of 1BS and 5BS arms has no effect. Using substitution lines Mathias and Fukui (1986) and Mathias et al. (1988) found that the 'Cappelle' 4B chromosome has a significant effect on regeneration compared to the 'CS' chromosome 4B. Our results further clarify this point since the lack of the 4BS arm is associated with an increased somatic embryo formation and short-term regeneration, whereas the absence of 4BL is associated with a decrease for both traits. Significant differences have been observed between homoeologous group 2 DT lines and 'CS' (Kaleikau et al. 1989b). Our results confirm that the 2BS and 2DL arms promote both embryo formation and regeneration from short-term cultures, whereas the 2AL and 2BL only promote short-term regeneration. The previously described decrease in the regeneration capacity after short-term culture using the 'Cheyenne' 1D, 2D, 4D, 6D and 7D substitution lines into 'CS' (Galiba et al. 1986) was similar to that attributable to the absence of chromosome arms 1DS, 2DL, 4DL, 6DL and 7DL from our experiments.

The ability of cultured plants cells to produce embryos without a sexual process suggests that signals for embryogenesis are supplied in vitro to a wide range of somatic or gametic cells that have the potential to express an egg-cell gene expression programme. Somatic embryogenesis involves several steps:

- The induction of cells towards embryogenesis, limited to scutellar cells during a phase that needs auxin, involves a reprogramming of gene expression. There could be a major control of 4 chromosome arms: 1AL, 3AL, 3BL and 3DL. Their absence reduces the induction of embryos and green spots, and limits regeneration. The other 41 chromosome arms do not fully compensate for the absence of genes regulating embyogenesis located on these arms.
- The second step is concerned with the differentiation of the induced cells into embryos or green spots. Many genes (i.e. chromosome arms) play a role during this step. Most of them seem to be required for embryo formation. Only the 1DL and 1DS arms have a negative effect on this trait because their absence allows improved embryo formation. The 4AS and 2AS arms are concerned with only one trait: embryo or green spot formation, respectively.
- Regeneration capacity from short-term cultures depends mostly on the formation of embryos susceptible to germination. Evidence for normal development was gathered here from morphological observations and germination of the embryos. It seems that in some cases regeneration also occurs from green spots that look like leaf meristems. The occurrence of leaf meristems and embryogenesis from the same explant raises the question of their relationship. There are a few chromosome arms, 1AS, 2AL and 2BL, that only control the germination mechanism. Their absence reduces regeneration capacity both in short- and long-term cultures. At least 2 other arms, 6DS and 7BS, seem to be involved in the regeneration process. In their absence a limited frequency of embryo and a high level of green spots were observed, associated with a high level of short-term regeneration. The gene effect of these arms could be to act on the quality of the embryos, i.e. on regeneration from green spots. Perhaps the 4DS, 5DS and 6AS arms have a similar effect.
- There was evidence that some chromosome arms have an effect on long-term culture. The absence of 4BS, 5BS or 7DS improves the regeneration frequency only after long-term culture and has no effect on the other traits involved in somatic embryogenesis. On the other hand, 1BS and 2DS arms are needed to maintain embryogenic capacity and the ability to regenerate plants after 14 months of culture. The genes of these arms could have more or less favourable effects on the maintenance of somatic embryogenesis through successive subcultures.

Our experimental results allow the identification of chromosome arms acting during the different steps of somatic embryogenesis. Many arms are involved in this process, but it was possible to show that particular steps are controlled by a few arms and therefore a limited number of genes. The capacity of regeneration during in vitro cereal tissue culture depends on the plant genotype. For example, a previous paper reported the nuclear inheritance of responsiveness to somatic wheat tissue culture (De Buyser et al. 1992). The control of somatic and microspore tissue culture mostly results from the action of nuclear genes, but little is known about the nature of this control (Pechan et al. 1991). Some genes are probably concerned with the response to plant growth regulators (Close and Gallager-Ludeman 1989, Komamine et al. 1990). Hence, knowledge of the genetic control of somatic embryogenesis could lead to the identification of genes affecting sexual embryogenesis.

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