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The effect of the 4B chromosomes of hexaploid wheat on the growth and regeneration of callus cultures

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Summary. Calli were initiated from immature embryos of nine lines of hexaploid wheat (*Triticum aestivum* L. em. Thell). These were the euploid lines Chinese Spring and Cappelle-Desprez, a line of Chinese Spring ditelocentric for the long arm of 4B, four substitution lines of Chinese Spring in which chromosome 4B has been replaced by its homologues from different wheat varieties and substituted into Chinese Spring and a substitution line of Besostaya I 4B into Cappelle-Desprez. The calli from these lines were found to differ in their growth rates and morphogenic and regenerative activities. The substitution of different 4B chromosomes into Chinese Spring significantly increased morphogenesis and shoot regeneration from callus. The potential for developing wheat lines with improved culture characteristics is discussed.

Key words: Wheat – Callus – Regeneration – Tissue culture – Genetics

Introduction

The genetic modification of crop plants in vitro is dependent on the ability to regenerate plants from tissues in culture. The generally poor performance of the cereals in culture has prevented the exploitation of in vitro techniques in this major crop group.

In hexaploid wheat (*Triticum aestivum* 2n = 6x = 42), as in other species, the conventional approach to improving culture performance has been to screen a variety of explants on a range of media (Trione et al. 1968; Dudits et al. 1975; Chin and Scott 1977; O'Hara and Street 1978). Recently it has been recognised that tissue culture responses are affected by plant genotype and the efficiency of wheat callus induction (Sears and Deckard 1982), callus growth rate (Lazar et al. 1983) and plant regeneration frequencies (Shimada 1978; Sears and Deckard 1982; Maddock et al. 1983) have all been reported to be genotype dependent. Mathias and Simpson (1986) have assessed the relative contributions made by media additives and genotype in vitro and have suggested that the genotype may be more significant than the medium in affecting culture behaviour. The genes that control culture characteristics and their location within the genome have not been identified.

Mathias and Fukui (1986) demonstrated that the substitution of the 4B chromosome of the variety Cappelle-Desprez into the variety Chinese Spring improved the performance of calli in vitro. They concluded that a factor (or factors), which stimulate the growth, morphogenesis and regeneration of wheat calli is located on the 4B chromosome.

To analyse further the chromosomal effect reported by Mathias and Fukui (1986) we have examined the in vitro responses of Chinese Spring, Cappelle-Desprez and several substitution lines in which the 4B chromosomes of Chinese Spring and Cappelle-Desprez have been replaced by the 4B chromosomes of other varieties and a related hexaploid species, *T. spelta*.

Materials and methods

Genetic stocks

CSSears: Chinese Spring (a line of Chinese Spring obtained from Dr. E. R. Sears (USA)).

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CSPBI: Chinese Spring (PBI stock). Essentially the same as CSSears but with a small terminal deletion of the short arm of chromosome 1D which has removed the glucose phosphoisomerase (Gpi) isozyme locus.
CSDT4B^{L}: Chinese Spring di-telocentric stock in which the short arms of chromosome 4B are deleted. This line was developed by Dr. E. R. Sears (USA) and has been maintained at the PBI for over 20 years.
CS(Lut 4B): Chinese Spring substitution line in which the 4B chromosome of CS has been replaced by the 4B chromosome of the variety Lutescens 62, an old Russian land race.
CS(T.sp. 4B): Chinese Spring substitution line in which the 4B chromosomes of CS have been replaced by the 4B chromosomes of *T. spelta*.
CS(Cap4BI): Chinese Spring substitution line in which the 4B chromosome of Chinese Spring has been replaced by those of Cappelle-Desprez.
CS(Cap4BII): As above but derived from a separate series of backcrosses.
Cap(Bes 4B): Cappelle-Desprez substitution line in which the 4B chromosome of Cappelle-Desprez has been replaced by the 4B chromosomes of Besostaya I, a Russian bred variety.
Cap: Cappelle-Desprez, a French bred variety widely grown in Western Europe during the 1960's.

All the above mentioned inter-varietal substitution lines were developed at the PBI by Dr. C. N. Law and Mr. A. J. Worland.

Tissue culture

Plants were grown and tissue cultures initiated and maintained as described by Mathias et al. (1986). Fourteen days after anthesis grains from the outer florets of the middle spikelets of each ear were harvested and after surface sterilisation the immature embryos were removed. The basal medium was that specified by Sears and Deckard (1982). Initiation, and regeneration media contained 1.0, 0.5 and 0.1 mg/l of 2,4-D, respectively.

Approximately one third of the calli from each line were initiated and maintained in the dark, the remainder were cultured under continuous illumination. All cultures were maintained at 27° ± 1 °C.

The calli were subcultured at four week intervals.

Callus growth was measured as average callus fresh weight per petri dish with 3–4 calli per dish. Morphogenic/regenerative responses were recorded as the number of calli with green spots on initiation, maintenance and regeneration medium and with shoots on maintenance and regeneration medium.

Statistical analysis

The data was analysed using an analysis of deviance, this modified analysis of variance employs binomial weightings to compensate for unequal replication.

The Genstat program (Numerical Algorithms Group 1983) was used to produce the analysis of deviance using a logit transformation (Logit (p) = ln (p/(1–p) where p = number of calli with the scored character/total number of observations). The logit function transforms p from a range of (0, 1) to a range of (–infinity, + infinity). The discrepancy between the data and the corresponding fitted values derived from the model provides a measure of the deviance. The analysis produces deviance mean squared values for each of the effects included in the model. These values, when divided by the residual, produce a deviance ratio. The distribution of the deviance ratio closely approximates to the F distribution.

In the analysis of deviance the deviation amongst the nine lines can be broken down into deviations between the mean behaviour of four groups, (1) CSSears and CSPBI, (2) CSDT4B^{L}, (3). CS(Lut4B), CS(Tsp.4B), CS(Cap4BI) and CS(Cap4BII) and (4) Cap and Cap(Bes4B). This will take up three of the eight degrees of freedom in the analysis. The remaining five degrees of freedom can be apportioned to within group deviations, i.e. one degree of freedom for the deviation between CSPBI and CSSears, three degrees of freedom for deviations between the 4B substitutions into CS and one degree of freedom for the deviation between Cap and Cap(Bes4B). Since the analysis also compares the effect of light v dark growth conditions on callus growth and morphogenesis, the interactions between this treatment and the between and within group deviations can be defined in the analysis.

Generally the standard errors for the lines were similar and a pooled standard error was derived.

Comparisons between pairs of lines were made using *t*-tests.

Individual petri dishes were used as replicates for the analyses.

Results

Callus growth

At the time of dissection all the embryos were of similar size and appearance. Nearly all the cultured embryos produced callus and no effect of genotype or light regime on callus initiation was detected. In all cases the callus produced was friable with a yellow-white coloration.

The mean fresh weights of calli after one month on initiation and maintenance media (callus growth), are presented in Fig. 1, and the analysis of deviance in Table 1.

There were significant differences between lines and main groups on both media. On initiation medium neither the main effect of light v dark nor the interaction between lines and light versus dark was significant so the data between treatments could be pooled. There were significant differences between CSSears and CSPBI, between Cap and Cap(Bes4B) and between Cap and CSSears. On maintenance medium there were significant between line and group differences and a strong interaction of lines mainly attributable to group differences with light v dark conditions.

Morphogenesis

The analysis of deviance for the frequencies of shoot primordia formation on initiation, maintenance and regeneration media are presented in Table 2 and the mean per cent frequencies of morphogenic calli under light and dark conditions are depicted in Fig. 2.

The first indication of regenerative activity in cereal calli is the formation of "green spots" – shoot primordia

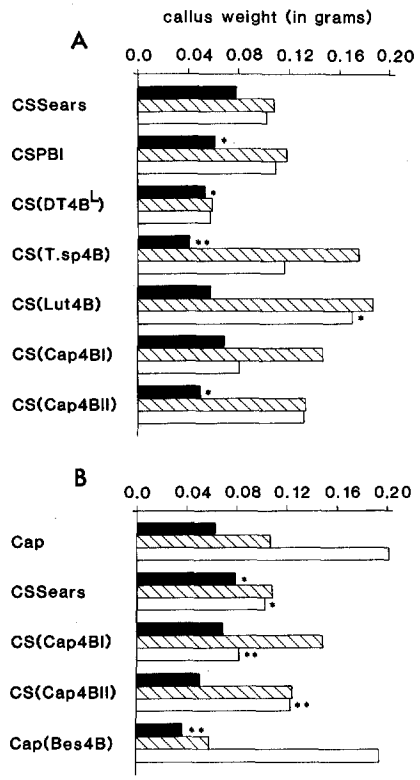

Fig. 1 A, B. Mean callus weight (in grams) after one month on initiation and maintenance medium in light and dark growth conditions. Black bars = initiation medium; hatched bars = maintenance medium, light conditions; white bars = maintenance medium, dark conditions. **A** Comparison of lines with CSSears; **B** Comparison of lines with Cap. Stars indicate where a line is significantly different from CSSears (**A**) or Cap (**B**) on a particular medium. *: 0.50%; **: 0.10%; ***: 0.01% probability levels. There was no significant effect of light on the response of calli growing on initiation medium, therefore the figures for callus weight in light and dark grown calli have been pooled and entered on the 'light' bar chart

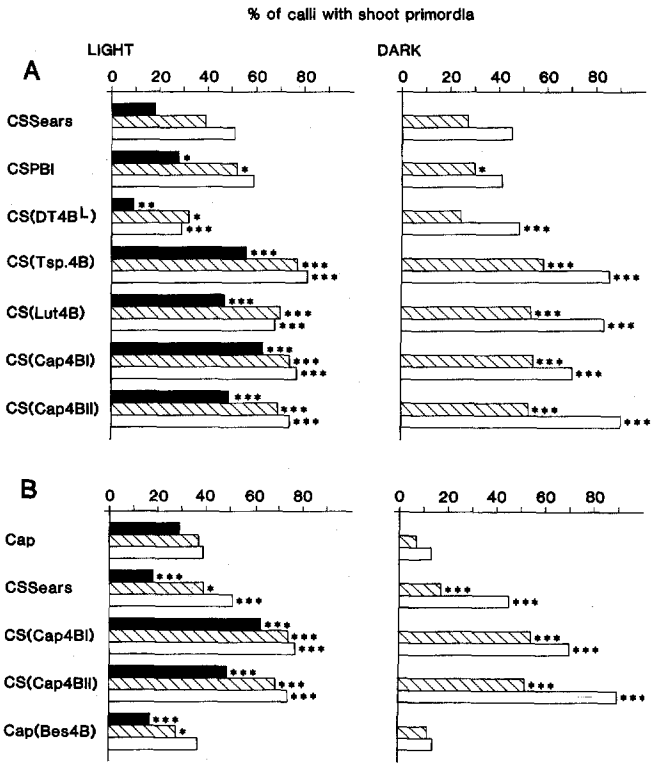

Fig. 2 A, B. Percent of light and dark grown calli producing shoot primordia on initiation, maintenance and regeneration medium. **A** Comparison of lines with CSSears; **B** Comparison of lines with Cap. Black bars = initiation medium; hatched bars = maintenance medium; white bars = regeneration medium. Stars indicate where a line is significantly different from CSSears (**A**) or Cap (**B**) on a particular medium. *: 0.50%; **: 0.10%; ***: 0.01% probability levels. There was no significant effect of light on the response of calli growing on initiation medium, therefore the figures for primordia formation in light and dark grown calli have been pooled and entered on the 'light' bar chart

(Sears and Deckard 1982; Ahloowalia 1982). Green spots were first visible about two weeks after callus induction on calli grown in the light. In all the lines some calli produced green spots on initiation medium in the light but calli grown in darkness only produced green spots after a period on maintenance medium. Very significant between group differences were seen on all three media.

The largest effect was due to the 4B substitution lines where primordia formation was increased in both light and dark grown calli. The only within group difference was between Cap and Cap(Bes4B) on initiation medium, where shoot primordia formation was inhibited on calli of the substitution line.

In the light the ditelosomic line was less morphogenic than CSSears on all three media. There was a significant main effect of light versus dark on all media and on initiation and regeneration media there was interaction between light v dark and between group differences.

Clearly the increased frequency of shoot primordia formation is largely associated with the 4B chromosome substitutions into Chinese Spring.

Regeneration

Formation of shoot primordia occurred in distinct 'meristematic' regions on the callus. Examination of the calli showed that shoots developed from shoot primordia and not from germinating somatic embryos.

The analysis of deviance for shoot regeneration on maintenance and regeneration media is presented in Table 3 and the mean per cent frequencies of regenerating calli under light and dark conditions are de-

Table 1. Analysis of deviance for callus weight after one month on initiation and maintenance medium. Group 1 = CCSears, CSPBI. Group 3 = CS(Cap4BI), CS(Cap4BII), CS(Lut4B), CS(T.sp4B). Group 4 = Cap, Cap(Bes4B)

Effect	d.f.	Initiation medium mean change	Maintenance medium mean change
Lines	8	0.011***	0.081***
Groups	3	0.999***	0.219***
Group 1	1	0.011*	0.004
Group 3	3	0.006	0.064**
Group 4	1	0.023**	0.056*
LVD	1	0.001	0.004
Interaction	8	0.002	0.055***
Groups LVD	3	0.004	0.123***
Group 1/LVD	1	0.003	0.000
Group 3/LVD	3	0.001	0.015
Group 4/LVD	1	0.002	0.003
Residual	536	0.002	0.009

* 0.50%; ** 0.10%; *** 0.01%. Significance levels of effects

Table 3. Analysis of deviance for shoot regeneration on maintenance and regeneration medium. Group 1 = CCSears, CSPBI. Group 3 = CS(Cap4BI), CS(Cap4BII), CS(Lut4B), CS(T.sp4B). Group 4 = Cap, Cap(Bes4B)

Effect	d.f.	Maintenance medium mean change	Regeneration medium mean change
Lines	8	8.31***	34.75***
Groups	3	15.49***	88.48***
Group 1	1	1.54*	0.11
Group 3	3	4.97***	3.32*
Group 4	1	3.59***	2.50*
LVD	1	7.72***	32.55***
Interaction	8	1.59	6.76**
Groups LVD	3	0.87	10.19**
Group 1/LVD	1	0.00	0.48
Group 3/LVD	3	3.37***	6.96***
Group 4/LVD	1	0.00	0.04
Residual	536	0.47	1.42

* 0.5%; **0.1%; *** 0.01%. Significance levels of effects

Table 2. Analysis of deviance for the frequency of shoot primordia formation on initiation, maintenance and regeneration medium. Group 1 = CCSears, CSPBI. Group 3 = CS(Cap4BI), CS(Cap4BII), CS(Lut4B), CS(T.sp4B). Group 4 = Cap, Cap(Bes4B)

Effect	d.f.	Initiation medium mean change	Maintenance medium mean change	Regeneration medium mean change
Lines	8	17.25***	27.92***	37.77***
Groups	3	4.02***	72.16***	99.32***
Group 1	1	2.29	1.69	0.22
Group 3	3	2.54	1.05	1.26
Group 4	1	7.42**	2.04	0.23
LVD	1	4.06*	55.15***	3.21
Interaction	8	4.02***	1.19	2.65**
Groups LVD	3	4.21**	2.21	10.94**
Group 1/LVD	1	4.58*	0.46	1.03
Group 3/LVD	3	2.85	0.08	3.06*
Group 4/LVD	1	6.41*	2.19	0.04
Residual	536	9.32	1.42	1.31

* 0.5%; ** 0.1%; *** 0.01%. Significance level of effects

picted in Fig. 3. Differences between lines were found on both media although the overall frequency of shoot formation on maintenance medium was very low. The differences between the four major groups were highly significant, the largest effect being due to the 4B substitution lines. There were also differences within groups and this was particularly so amongst the 4B substitution lines. The interactions of the main groups with light versus dark growth conditions were significant as was the interaction with the different 4B substitution lines.

Discussion

The conditions described for callus induction were optimal as greater than 99% of the cultured embryos produced calli. There have been several reports of differences in the callus initiation frequencies among hexaploid wheat varieties (Shimada 1978; Ahloowalia 1982; Lazar et al. 1983) and when using similar conditions to those described here Sears and Deckard (1982) reported callus induction frequencies ranging from 0–90% among 39 varieties. Shimada and Makimo (1975) reported that a factor on the β-arm of the 4A chromosome inhibited callus formation from CS anthers but no genotypic effects on callus initiation were observed among the lines tested in this experiment.

There were significant differences among the lines for callus growth, shoot primordia formation and shoot regeneration. The most significant effects on these characters were associated with the substitution of the 4B chromosomes of Chinese Spring by homologues from other varieties. Our results confirm the previous report (Mathias and Fukui 1986) that morphogenesis and regeneration from CS calli was stimulated by the 4B chromosome of Cappelle-Desprez.

We have also demonstrated that the regeneration promoting effect of the 4B chromosome substitutions in CS is not unique to the Cap 4B chromosomes. The 4B chromosomes of two distantly related varieties and a related hexaploid species had similar effects. However, the substitution of the Bes 4B chromosome into the

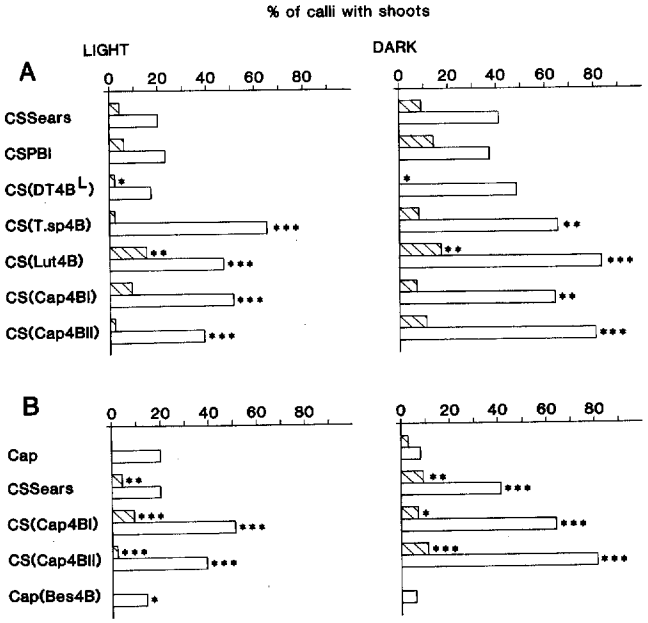

Fig. 3A, B. Percent of light and dark grown calli regenerating shoots on maintenance and regeneration medium. **A** Comparison of lines with CSSears; **B** Comparison of lines with Cap. Hatched bars = maintenance medium; white bars = regeneration medium. Stars indicate where a line is significantly different from CSSears (**A**) or Cap (**B**) on a particular medium. *: 0.50%; **: 0.10%; ***: 0.01% probability levels

variety Cap did not promote callus morphogenesis and regeneration.

There were significant differences for callus growth among the lines, in particular the growth of the ditelosomic and several substitution lines was reduced on initiation medium. This may result from non-specific reduction of 'cell fitness' in modified genotypes or from the loss of specific factors on the 4B short arm which affect callus growth. The former interpretation agrees with the report of Baroncelli et al. (1978) that callus growth of ditelocentric lines of CS was typically reduced in comparison to the euploid. Improved callus growth of a CS(Cap4B) line has been reported (Mathias and Fukui 1986) but neither CS(Cap4B) line tested in this experiment showed better growth than either of the parental varieties.

The genotype not only affects callus growth directly but also through its interaction with the light regime. On maintenance medium there was a significant line × light versus dark interaction. Growth of CSSears was unaffected by darkness but growth of Cap was stimulated. As there was no interaction on initiation medium the light effect appears to be conditioned by the age of the callus or by the concentration of 2,4-D in the medium. Similar line × light versus dark interactions occur for primordia formation on initiation and regeneration media and for shoot production on regeneration medium.

As the initiation, maintenance and regeneration media contained the same basal medium and different concentrations of 2,4-D, the different responses of the lines can be simply interpreted as the result of interactions between genotypes and 2,4-D, although the mechanism may be complex.

Mathias and Fukui (1986) suggested that the 4B substitution modifies cellular hormone metabolism and changes the sensitivity of cells to applied growth regulators. As a result the capacity of calli to organise shoot primordia and regenerate shoots in the presence of particular concentrations of 2,4-D is modified. They noted that the reduced height/GA insensitivity (Rht) alleles and the "grass clump dwarfness" (D3) allele are located on the group 4 chromosomes of wheat (Hermsen 1963; McVittie et al. 1978) and suggested that these genes, which have major effects on plant phenotype as a result of altered hormone metabolism (Gale and Youreffian 1985), might be involved in the 4B effect in vitro. However, the effect may not necessarily be related to hormone metabolism. Galiba and Erdei (1986) have reported that the exogenous carbon source/osmotic potential affects the growth of wheat calli. Therefore a genetically determined shift in sugar metabolism or stress resistance might alter regeneration activity. The 4B chromosome also carries a locus for alcohol dehydrogenase (Hart 1970). Allelic variation of this (Adh) locus might result in differences in the tolerance of tissues to the slightly anaerobic conditions of culture.

The differences between the lines can be described by a simple model of the relative 'potency' of the 4B chromosomes in affecting callus response;

Cap4B ≡ T.sp4B ≡ Lut4B > CS4B ≡ CS4B^L
also Cap4B > Bes4B
and either Cap4B > Bes4B > CS4B
or Cap4B > Bes4B ≡ CS4B + interactions.

As the parental varieties Cap and CSSears were similar in their in vitro responses the simplest explanation of our results would be that Cap is + – and CSSears – + for relative allelic expression of two genes that affect the tissue culture response. If the genes are promoters then Cap would be + –, with the promoter on the 4B chromosome and the second gene, a neutral or inhibitory allele, an another chromosome. CSSears would be – +, with a neutral or inhibitory allele on 4B. T.sp4B and Lut4B would also carry an allele for the promoting gene. The variation in response between substitution lines may reflect allelic variation at the locus on 4B and/or background effects.

The Bes4B substitution into Cap typically had little or no effect on culture performance suggesting that

Bes4B carries a factor which is similar in its action to that on Cap4B. Alternatively it may indicate that the cytoplasmic background of the recipient has some role in the effect. Mathias and Fukui (1986) have shown that the regeneration promoting effect of the Cap4B chromosome in CS operates in the cytoplasms of *T. aestivum* and *Aegilops ovata* and that with Cap4B there is no interaction with the cytoplasm.

The lines show the same general trends, in relation to one another, for growth, morphogenesis and regeneration and this suggests that the response may be controlled by the same gene(s). If so these gene(s) must operate at a fundamental level of cellular metabolism, perhaps altering hormone or stress metabolism.

We have demonstrated that major changes in in vitro behaviour result from single chromosome substitutions. This suggests that one or a few genes with major effects are involved in determination of culture response, rather than many genes all with minor effects. Diallel tests have demonstrated that callus regeneration in maize is highly heritable (Beckert and Ming-Qing 1984) and may be controlled by as few as two genes (Hodges et al. 1985). The existence of major effects on in vitro response suggest that through conventional breeding and selection strategies it may be possible to develop special genotypes for tissue culture, as has been achieved in alfalfa (Bingham et al. 1975) and tomato (Koorneef et al. 1986).

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