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Evidence for cytoplasmic control of in vitro microspore embryogenesis in the anther culture of wheat (*Triticum aestivum* L.)

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Summary. Anthers were cultured from two sets of seven lines of hexaploid wheat (*Triticum aestivum* L.) with different cytoplasms, the euplasmic nucleus donors, 'Siete Cerros 66' and 'Penjamo 62', as well as their six alloplasmic lines derived from wild relative species of the genera *Triticum* and *Aegilops*. Significant cytoplasmic and nuclear effects but no cytoplasmic-nuclear interaction were found for embryogenic anther response, with the best performance of 'Penjamo 62' in *Ae. kotschyi* cytoplasm. Plant regeneration was not affected significantly by the cytoplasmic background of the lines cultured. The possible genetic implications of the observed cytoplasmic and nuclear influences on the in vitro haploid induction of wheat are discussed.

Key words: Wheat – Cytoplasm – Anther culture – Microspore embryogenesis

Introduction

Parallel to the progress in wheat anther culture, there has been an increasing wealth of information on genotypic variation of in vitro microspore embryogenesis (Ouyang et al. 1973; Picard and De Buyser 1975, 1977; De Buyser and Henry 1979; Schaeffer et al. 1979; Andersen et al. 1987). The accumulated observations generated an interest in studying the genetic regulation of this process covering both nuclear and cytoplasmic (plasmon) effects (Bullock et al. 1982; Lazar et al. 1984b). Subsequently, further studies were carried out concerning the possible chromosomal basis of microspore embryogenesis (Lazar et al. 1987; Szakács et al. 1988; Agache et al. 1989).

However, results on the plasmon effects seem to be contradictory. Using reciprocal intervarietal crosses, weak or no maternal effects were found in wheat (Bullock et al. 1982; Henry and De Buyser 1985) and in barley (Foroughi-Wehr et al. 1982; Dunwell et al. 1987) whereas, by means of diallel analysis, a significant cytoplasmic influence was revealed in wheat (Lazar et al. 1984b) and in triticale (Charmet and Bernard 1984; Charmet et al. 1985). Nevertheless, most of the authors emphasised the need of further investigations on a wider genetic basis.

Numerous experimental data show that natural intraspecific variation in cereals is rather low in mitochondrial DNA (Quetier and Vedel 1977; Oro et al. 1985; Holwerda et al. 1986; Rines et al. 1988) and especially in chloroplast DNA (Ogihara and Tsunewaki 1982; Bowman et al. 1983; Terachi et al. 1984; Clegg et al. 1984).

Even anther culture conditions were found not to be connected with variations in chloroplast and mitochondrial DNA (Rode et al. 1985; Charmet et al. 1985) – except for albinos (Day and Ellis 1984, 1985) – in spite of the widespread nuclear DNA changes (De Paepe et al. 1982; Dhillon et al. 1983; Rode et al. 1987; Benslimane et al. 1988).

This suggests that cytoplasmic DNA is inherited conservatively (Palmer and Stein 1986; Ogihara and Tsunewaki 1988) and is less variable than nuclear DNA. Consequently, it is reasonable to suppose that lack of maternal effects in anther culture of reciprocal crosses may not only point to absence of cytoplasmic effects on microspore embryogenesis (as suggested previously) but it might also be based upon little or no cytoplasmic differences between the parents involved in the intervarietal crosses.

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To test this hypothesis, anther culture response in crosses of genotypes with great cytoplasmic differences should be investigated.

For this purpose, the use of reciprocal crosses of distant hybrids seems to be logical, but parents of these hybrids are usually incompatible in one of the crossing directions and they also show meiotic instability. Moreover, these hybrids have low in vitro microspore embryogenic capacity (unpublished results).

Since Kihara's pioneer work with wheat (1951), a number of cytoplasmic substitution lines (alloplasmic lines) were developed and studied recently in somatic tissue cultures (Mathias and Fukui 1986; Mathias et al. 1986; Felsenburg et al. 1987). In previous studies (Picard et al. 1978; Charmet and Bernard 1984; Charmet et al. 1985), the positive effect of *Triticum timopheevi* cytoplasm in anther cultures of wheat and triticale has already been demonstrated. In alloplasmic lines, the genome of a stable genotype is substituted into very different cytoplasms of wild relatives of wheat with repeated backcrosses, as opposed to placing a hybrid genome into similar cytoplasmic backgrounds, as in the case of reciprocal intervarietal crosses. Furthermore, it is known that alien cytoplasms can induce and control in vivo haploidy in wheat (Kihara and Tsunewaki 1962; Tsunewaki et al. 1974). Therefore, alien cytoplasmic substitution lines seem to offer a good possibility to decide whether cytoplasmic factors can also control in vitro haploid formation or not.

In this study we report on the significant cytoplasmic effects on in vitro microspore embryogenesis in two sets of alloplasmic lines of hexaploid wheat.

Materials and methods

Plant material. In the experiments, the following were used: two euplasmic lines of the nucleus donors, Siete Cerros 66 (SC) and Penjamo 62 (PJ), and their six self-fertile alloplasmic lines having the SC or PJ nucleus substituted into the cytoplasm of *Aegilops speltoides* [(*speltoides*)-SC or -PJ], *Aegilops longissima* [(*longissima*)-SC or -PJ], *Aegilops variabilis* [(*variabilis*)-SC or -PJ], *Aegilops ventricosa* [(*ventricosa*)-SC or -PJ], *Triticum aegilopoides* [(*aegilopides*]-SC only], *Aegilops bicornis* [(*bicornis*)-SC only], *Aegilops kotschyi* [(*kotschyi*)-PJ only] and *Aegilops juvenalis* [(*juvenalis*)-PJ only]. These lines were obtained from Mr. L. Purnhauser (Cereal Research Institute, Szeged, Hungary) and produced by Dr. I. Panayotov (Bulgaria). All these lines have had 12 backcrosses except for (*speltoides*)-PJ and (*aegilopoides*)-SC with 5 and 7 backcrosses, respectively. Seeds sown in March 1987 in the field provided suitable ears from the end of May.

Anther culture. Anthers containing mid-or late-uninucleate microspores from the middle 8–10 spikelets of each ear were excised. Microspore developmental stage was checked in each ear by an acetocarmine squash method prior to excision. Selected ears were immediately surface sterilised in 0.1% mercuric chloride for 7–8 min, followed by four to five sterile distilled water rinses. Anthers were placed on potato-2 agar induction medium containing 10% potato extract (Chuang et al. 1978) and supplemented with 1.5 mg/l 2,4-D, 0.5 mg/l kinetin, 90 g/l sucrose and 6 g/l agar. The pH was adjusted to 5.8 before autoclaving. Cultures were kept in darkness at 29 °C. One month after inoculation, the number of embryogenic anthers was recorded and induced embryos were transferred to the 190-2 regeneration medium (Zhuang et al. 1984), supplemented with 0.5 mg/l each of 1-naphthalene acetic acid and kinetin, 30 g/l sucrose and 6 g/l agar. Cultures were maintained at 26 °C under a 16-h illumination. After 1 month, the number of organised structures with shoots exceeding 0.5 cm in length was recorded.

Results

Anthers containing mid- or late-uninucleate microspores of the tested lines produced embryos with different efficiency. Embryo growth became visible during the 3rd week in culture, and there were no obvious differences among the lines in the morphology and time of appearance of the embryos. However, significant variation was observed among the lines in their frequency of embryo induction.

Cytoplasms of *Ae. longissima* and *Ae. variabilis* showed significant stimulatory effect on embryogenic anther response when compared with (*aestivum*)-SC. By contrast, *Ae. ventricosa* cytoplasm seemed to be ineffective. Cytoplasms of *T. aegilopoides, Ae. bicornis* and *Ae. speltoides* exerted a weak influence on microspore embryogenesis, and some of these differences between lines were significant (Table 1). In the case of Penjamo 62, (*kotschyi*)-PJ was significantly more efficient, whereas (*speltoides*)-PJ, (*variabilis*)-PJ and (*ventricosa*)-PJ produced significantly less embryogenic anthers than their euplasmic line. There was more significant variation between the Penjamo 62 lines than in the Siete Cerros 66

Table 1. Embryogenic anther response (% of anthers producing embryos on initiation medium) of seven cytoplasmic lines of Siete Cerros 66. Embryogenic anther responses followed by a particular letter are significantly ($P \leq 0.05$) different from the first one in the list with that letter (χ^2 analysis of the number of embryogenic anthers)

Cytoplasm	Anthers	
	Embryogenic, %	Plated
aestivum	0.0 a	180
aegilopoides	0.9 b	3,624
bicornis	1.0 c	621
speltoides	1.0 d	210
longissima	4.5 abcde	693
variabilis	2.7 abc	481
ventricosa	0.0 e	125
χ^2 homogeneity test *df* 6	65.11***	

Table 2. Embryogenic anther response (% of anthers producing embryos on initiation medium) of seven cytoplasmic lines of Penjamo 62. Embryogenic anther responses followed by a particular letter are significantly ($P \leq 0.05$) different from the first one in the list with that letter (χ^2 analysis of the number of embryogenic anthers)

Cytoplasm	Anthers	
	Embryogenic, %	Plated
aestivum	1.7 a	572
speltoides	0.0 ab	670
longissima	0.8 bc	390
variabilis	0.4 ad	711
kotschyi	8.9 abcde	335
ventricosa	0.0 acef	585
juvenalis	3.2 bcdef	253
χ^2 homogeneity test *df* 6	153.46***	

Table 3. Embryogenic anther response (% of anther producing embryos of initiation medium) of five cytoplasmic lines of Siete Cerros 66 (SC) and Penjamo 62 (PJ). Embryogenic anther responses followed by a particular letter are significantly ($P \leq 0.05$) different from the first one in the list with that letter. Rows followed by an asterisk represent cytoplasms significantly ($P \leq 0.05$) different between SC and PJ nucleus donors (χ^2 analysis of the number of embryogenic anthers). n = number of plated anthers per sample

Cytoplasm	Ploidy level 2n =	SC	*n*	PJ	n	
aestivum	6×	0.0 a	180	1.7 a	572	
speltoides	2×	1.0 b	210	0.0 ab	670	*
longissima	2×	4.5 abc	693	0.8 bc	390	*
variabilis	4×	2.7 a	481	0.4 a	711	*
ventricosa	4×	0.0 c	125	0.0 ac	585	
χ^2 homogeneity test	*df* 4		19.04***		22.66***	

χ^2 analysis of the embryogenic anther response of two sets of cytoplasmic lines with nucleus donors of Siete Cerros 66 (SC) and Penjamo 62 (PJ)

SC vs PJ	*df* 1	38.32***
Cytoplasms	*df* 4	44.32***
Interaction	*df* 4	3.04 NS
Total	*df* 9	85.68***

Table 4. Chi-square analysis of embryogenic anther response of cytoplasmic lines classified according to the nuclear ploidy level (NPL) of cytoplasm donor and the relatedness of their chloroplast DNAs (cp-DNA)

χ^2		NPL	cp-DNA
Total	*df* 4	44.32***	44.32***
Between groups	*df* 2	7.19*	33.35***
Within groups	*df* 2	37.13***	10.97**

Table 5. Green and total plant regeneration (% of embryos producing plantlets) of cytoplasmic lines of Siete Cerros 66 (SC) and Penjamo 62 (PJ). (Only lines with at least ten embryos are shown). Cytoplasms followed by a particular letter are significantly ($P \leq 0.05$) different from the first cytoplasm in the list with that letter

Cytoplasm	No. of embryos	Plant regeneration (%)	
		Green	Total
(*aegilopoides*)-SC	45	37.8	55.6 a
(*longissima*)-SC	53	32.0	45.2
(*variabilis*)-SC	22	27.2	27.2 a
Overall	120	33.3	45.8
χ^2 homogeneity test	*df* 2	0.80 NS	4.77 NS
(*aestivum*)-PJ	20	25.0 a	50.0 a
(*kotschyi*)-PJ	65	1.5 ab	21.5 a
(*juvenalis*)-PJ	12	25.0 b	33.3
Overall	97	9.3	28.9
χ^2 homogeneity test	*df* 2	14.02***	6.17*

ones (Table 2). Chi-square homogeneity tests were highly significant for both sets of alloplasmic lines.

When data for lines common to both nucleus donors were combined in order to estimate the cytoplasmic-nuclear interaction, Chi-square analysis revealed highly significant nuclear and cytoplasmic effects in each, but not any significant interaction. Cytoplasms of *Ae. speltoides*, *Ae. longissima* and *Ae. variabilis* with Siete Cerros 66 nucleus were significantly superior to those with Penjamo 62 nucleus (Table 3).

Similarly to Mathias et al. (1986), embryogenic response data combined again for common alloplasmic lines were evaluated according to two classification schemes, i.e. ploidy level of the cytoplasm donors and chloroplast DNA restriction pattern, in order to test if the degree of relatedness between the nuclear and cytoplasmic donors accounts for the observed differences. According to the first classification, alloplasmic lines of *Ae. speltoides* and *Ae. longissima* are diploids, lines of *Ae. variabilis* and *Ae. ventricosa* are tetraploids and euplasmic lines are hexaploids (Table 3).

Based upon the chloroplast DNA restriction patterns (Ogihara and Tsunewaki 1988), the investigated cytoplasmic donors differed by at least three restriction fragments. Cytoplasmic lines of *Ae. speltoides*, *Ae. variabilis* and *Ae. ventricosa* are separate groups, whereas those of *Ae. longissima* and *T. aestivum* belong to the same group.

When the lines were classified according to the nuclear ploidy level of the cytoplasmic donor, there was highly significant variation within the groups. The classification based on the cytoplasmic relationships, however, eliminated most of this variation (Table 4), showing that the observed effects on embryogenic anther response are due to the cytoplasm, and mainly the relatedness of the cytoplasms is responsible for the pattern of these effects.

Studies of cytoplasmic effects on plant regeneration were more limited because of the low number of lines producing enough embryos for calculation (Table 5). There was no significant cytoplasmic effect in the alloplasmic lines of Siete Cerros 66, neither on green nor on total plant regeneration frequency. (*Variabilis*)-SC, however, produced green plants only. Overall, lines with Siete Cerros 66 nucleus showed higher green and total plant regeneration percentage than those with Penjamo 62 nucleus. Among cytoplasmic lines of Penjamo 62, (*kotschyi*)-PJ embryos differentiated significantly less green and total plants than those of the euplasmic line. Thus, *Ae, kotschyi* cytoplasm showed an adverse effect on plant regeneration as compared to its embryogenic anther response.

Discussion

The cytoplasmic background of microspores considerably affects their embryogenic response in anther culture. Under the described conditions, the two euplasmic *T. aestivum* genotypes showed different embryogenic anther response, with the better genotype, Penjamo 62, being comparable with the range of results generally observed.

Cytoplasmic substitutions with embryogenic anther responses significantly superior or inferior to euplasmic lines were found. In general, alloplasmic lines showed an inverse effect on embryogenic anther response as euplasmic lines. In other words, most alloplasmic lines with Siete Cerros 66 nucleus increased the embryogenic anther response in contrast to those with Penjamo 62 nucleus, when compared to their respective euplasmic lines, which showed a reverse phenomenon in comparison to each other. The involvement of exotic African introduction and a local Palestinian durum variety in the pedigree of Siete Cerros 66 might be responsible for the different behaviour of the otherwise two closely related genotypes (Zeven and Zeven-Hissink 1976). Moreover, alloplasmic lines with Siete Cerros 66 nucleus always exceeded their counterparts with Penjamo 62 nucleus. These findings support the strong nuclear effects observed on embryogenic anther response. However, no significant cytoplasmic-nuclear interaction was found. This allows isolation of basic materials by selection of specific combinations of nucleus and cytoplasm donors producing high anther culture response. Such basic materials could serve as objects for transmission of tissue culturability into a genetically wide choice and would be suitable to investigate the molecular basis of cytoplasmic action on the in vitro haploid formation.

Chi-square analysis of embryogenic anther response data according to the relatedness of cytoplasms (Ogihara and Tsunewaki 1988) showed similar results to those found by Mathias et al. (1986), confirming that genetic relatedness between the cytoplasmic donors is responsible for the pattern of their effects. The only line breaking this tendency was euplasmic Siete Cerros 66 without an embryogenic anther response although, according to the behaviour of the nucleus in alloplasmic background, a better performance in the *T. aestivum* cytoplasm could be expected. Indeed, the optimal model which eliminates variation of the same experimental data within groups was achieved by increasing embryogenic anther response up to 10%–11% in this line, solely. This value is also suggested by embryogenic anther responses of alloplasmic lines of *Ae. longissima,* which is proposed to be the cytoplasmic donor of hexaploid wheat (Ogihara and Tsunewaki 1982). One reason for the observed deviation could be the limited number of anthers plated, although lack of embryogenic anther response would be very unlikely in such a supposedly strong embryogenic genotype, even in this relatively small anther population. Another reason might be the disturbed interaction between the *T. aestivum* cytoplasm and the imbalanced genome with exotic gene introductions in the genotype as suggested above.

Two further lines with surprisingly high embryogenic anther response were (*kotschyi*)-PJ and (*juvenalis*)-PJ. *Ae. kotschyi* and *Ae. variabilis* have the same genome constitution and plasma type, and no difference was found between their chloroplast and mitochondrial DNA restriction fragment patterns (Terachi and Tsunewaki 1985; Ogihara and Tsunewaki 1988). The different embryogenic anther response of (*kotschyi*)-PJ and (*variabilis*)-PJ reveals and predicts further possibilities of selecting intraspecific cytoplasmic variation in these species. Remarkably, this group of S^V plasma type which gives the highest in vivo parthenogenetic haploid induction (Kobayashi and Tsunewaki 1980), especially in combination with genomes containing 1BL/1RS translocation (Tsunewaki et al. 1984), showed a high overall embryogenic anther response. This concordance needs further clarification, also with the inclusion of studies on combinations between S^V plasma type and genomes with 1BL/1RS translocation. However, other plasma types might also be important, as indicated by the good performance of (*juvenalis*)-PJ having D^2 plasma type whose origin is still uncertain.

In contrast to the embryogenic anther response, cytoplasmic substitution lines did not show a notable effect on green and total plant regeneration. Lines with Siete Cerros 66 nucleus had higher overall green and total plant regeneration percentage and (*variabilis*)-SC produced green plants only, whereas cytoplasmically closely related (*kotschyi*)-PJ regenerated mostly albino plantlets. These facts show that nuclear effects and/or nuclear-cytoplasmic interaction could be rather responsible for plant regeneration capacity, similar to former results obtained in somatic tissue culture (Mathias and Fukui

1986; Mathias et al. 1986). This also indicates that embryo induction and plant regeneration in anther culture are under different and independent genetic control, as suggested previously (Foroughi-Wehr et al. 1982; Lazar et al. 1984a).

It was reported that in some cytoplasmic substitution lines, a preferentially transmitted chromosome was consecutively retained from the genome of the cytoplasmic donor (Maan 1975; Miller et al. 1982; Karim and Singh 1988). This alien chromosome may have a gametocidal action causing sterility, in a severe case, or meiotic instability and genome rearrangements in the progeny, in a less severe case (Endo 1988). Gene(s) responsible for this gametocidal action can be recombined into the wheat genome (Tsujimoto and Tsunewaki 1984) and can cause defective symptoms (seed shrivelling, morphological mutation) in F_1 progeny when crossed into some wheat cultivars as males only. This phenomenon, called 'hybrid dysgenesis', proved to be partially temperature-dependent (Tsujimoto and Tsunewaki 1985), and strongly resembles that found in *Drosophila melanogaster,* in which it is mediated by transposable elements (Engels 1983). Therefore, in studies on cytoplasmic effects on tissue culturability and on their molecular basis, it seems to be necessary to make a distinction between cytoplasmic effects (i.e. direct effects of cytoplasm and influence of cytoplasmic-nuclear interaction) and possible residual genomic effects of the cytoplasmic donor.

The results presented here demonstrate that the cytoplasm can significantly affect tissue culturability also at the haploid level, similar to the somatic level (Mathias and Fukui 1986; Mathias et al. 1986), and in vivo haploid inducing cytoplasmic background may be efficient under in vitro conditions, as well. As Harris et al. (1988) recently successfully regenerated wheat plantlets from protoplasts isolated from suspension cultures of anther culture origin, the present findings can contribute to more advanced genetic manipulation of cytoplasm in hexaploid wheat.

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