Protoplasma (2006) 228: 95–100
DOI 10.1007/s00709-006-0179-x

PROTOPLASMA
Printed in Austria

Androgenic response to preculture stress in microspore cultures of barley

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Received March 22, 2005; accepted June 8, 2005; published online August 31, 2006

Summary. Various stresses such as starvation and cold or heat shocks have been identified as triggers in the induction of the microspore embryogenesis. This study attempts to quantify the effects of different pretreatment conditions for successful microspore culture of malting barley (cv. Scarlett). While the sporophytic microspore development could be induced from treated and nontreated microspores, abiotic stress was essential for embryo formation and plant regeneration. The type of stress treatment applied affected the numbers and the ratios of albino and green plants regenerated, as well as their fertility. The highest number of green plants was obtained after the treatment of anthers in 0.3 M mannitol at 32 °C for 24 h before microspore culture.

Keywords: Androgenesis; Microspore culture; Doubled haploid.

Introduction

Embryogenesis is a process that can be initiated not only from a fertilised egg cell (a zygote) but also from cells of the haploid generation, such as microspores. Under specific in vitro conditions, microspores can be induced to switch their developmental pathway from gametophytic to sporophytic, resulting in the formation of androgenic embryos (the process of androgenesis). These embryos germinate into plants. Along the way, spontaneous or induced doubling of the chromosome number may lead to production of doubled-haploid lines. This process makes it possible to obtain completely homozygous breeding lines within a shorter time frame than in conventional plant breeding. Efficient systems of isolated-microspore cultures leading to pure breeding lines have progressed considerably during the last decade and in some genotypes they were found to be better than anther culture especially in barley (Hoekstra et al. 1992, Davies and Morton 1998).

It has been demonstrated that optimisation of the preculture and culture conditions is essential for a successful induction of microspores to switch to the embryogenic pattern of development (Jähne and Lörz 1995, Touraev et al. 1997). The manipulation of conditions includes various pretreatments applied to tillers, tassels, excised inflorescences, flower buds, anthers, or microspores. Cold treatment improves the androgenic response of microspores and has been routinely used in cultures of many cereals such as maize (Gaillard et al. 1991), wheat (Gustafson et al. 1995), barley (Davies and Morton 1998), and rice (Cho and Zapata 1988). Heat shock combined with starvation (Touraev et al. 1996) or with cold (Reddy et al. 1985) has also been reported to reprogram the gametophytic pathway. Other protocols include treatments with oligosaccharides (Penhuizic et al. 2001), antimicrotubular drugs such as colchicine (Smykal and Pechan 2000), ethanol, gamma irradiation (Pechan and Keller 1989), and chemicals (Liu et al. 2002a, b).

In microspore culture, only some proportion of the cultured cells from a microspore population enters the pathway of embryo formation. Any pretreatment method or conditions that increase the proportion of cells undergoing androgenesis is of considerable importance, particularly in the improvement of plant regeneration rates in recalcitrant genotypes. Understanding the mechanisms inducing the sporophytic pathway would be very helpful in optimising such culture conditions.

Spike cooling as the main preculture stress has been used widely in barley microspore cultures (Jähne and Lörz 1995, 1999). To induce androgenesis in barley anther culture, cold treatment of tillers for two weeks at 4 °C was routinely used in our laboratory. In this study we attempted to quantify the

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effects of other preculture treatments applied to the microspores, anthers, and tillers on the efficiency of the androgenesis process and plant regeneration.

Material and methods

Plant material

Plants of spring malting barley (*Hordeum vulgare* L.) cv. Scarlett were grown in a growth chamber at a photoperiod of 16 h light and 8 h day at 16 and 12 °C, respectively. Cultivar Scarlett was selected for this experiment because it is quite widely grown in Central Europe. The spikes were harvested when microspores were at the mid to late developmental stage. To estimate the microspore developmental stage, anthers were excised from the centre of a spike, squashed on a microscope slide to release the microspore, stained with acetocarmine, and examined under an inverted microscope (Nikon Diaphot-TMD). In all experiments, the anthers were excised only from the well developed section of each spike; no anthers were excised from the poorly developed spikelets at the top and the bottom parts of each spike.

Pretreatment conditions

Various treatment methods, and their combinations, were tested: cold pretreatment of tillers at 4 °C for two weeks (pretreatment A); incubation of isolated anthers in 0.3 M mannitol at 32 °C for 24 h (pretreatment B), at 26 °C for three days (pretreatment C), and at 4 °C for three days (pretreatment D); cold pretreatment of tillers at 4 °C for two weeks followed by preculturing of anthers for three days in mannitol at 26 °C (pretreatment E); preculture of freshly isolated microspores in mannitol at 32 °C for 24 h (pretreatment F) and at 4 °C for three days (pretreatment G). Microspores isolated from spikes without pretreatment were considered as a control. Anthers were excised with fine forceps from florets and placed on petri dishes containing 5 ml of 0.3 M mannitol. By adding nurse (N) (one-week-old isolated microspores of barley cv. Igri in Millicell-CM; Millipore) to the culture we aimed to improve the most commonly used pretreatment of tillers for two weeks at 4 °C (pretreatment A plus N) and incubation of isolated anthers at 32 °C for 24 h giving the best results in previous experiments (pretreatment B plus N). Each pretreatment was tested on 40 spikes.

Isolation and culture

After the pretreatment, microspores were isolated by blending according to Mordhorst and Lörz (1993) with minor modifications. Isolated microspores were cultured at a density of 8×10^6 microspores per ml in the K99 induction medium (Deutsch et al. 2004) supplemented with 1 mg of benzylaminopurine, 100 mg of myo-inositol, and 250 mg of casein hydrolysate per ml as recommended by J. Kumlehn (Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, Federal Republic of Germany, pers. commun.).

Culture plates were sealed with Parafilm and incubated at 27 °C in the dark. After 10 days, 0.5 ml of fresh induction medium was added to each plate. To regenerate plants, the embryo-like structures of about 1 mm in diameter were aseptically transferred onto the solid 190-2 medium (Zhuang and Xu 1983) supplemented with plant growth regulators according to Pauk et al. (1991) and incubated under light at 27 °C. Green plants with well developed roots and shoots were potted in soil. Plant fertility was evaluated on the basis of seed set.

The following parameters were monitored: the frequency of the initial division defined as the percentage of divided and swollen microspores in the total number of living microspores after one week of culture; the efficiency of androgenesis defined as the ratio of embryos larger than 1 mm in diameter after four weeks of culture to the number of viable microspores; the number of green and albino plants regenerated; and the ratio of doubled haploids to haploids. Ploidy levels of regenerated plants were determined cytologically on squashed preparations of root tips collected from microspore-derived plants.

Results

The development of embryogenic microspores and the effects of different pretreatments were monitored microscopically during the preculture, microspore isolation, and the culture itself. The difference between treatments in the ability to induce androgenesis was visible during the first two weeks of culture. A portion of the microspores started to differentiate already during the pretreatment (Fig. 1A). With the exception of pretreatments F and G, all treatments applied prior to the microspore culture stimulated the embryo formation.

The cold treatment of tillers (A) induced 18% of microspores to divide. Similar effects were observed after starvation at 26 °C (C) and at 4 °C (D), and after cooling at 4 °C followed by starvation at room temperature (E). However, pretreatment B (anthers in 0.3 M mannitol at 32 °C for 24 h) gave the highest frequency of initial microspore division (35%) and the highest efficiency of androgenesis (0.03%) (Table 1). The stress applied directly to the freshly isolated microspores also led to sporophytic development. In cultures in which microspores were starved in mannitol for 24 h at 32 °C (F), only a few divisions were observed (0.03%) and the multicellular structures did not develop any further. The frequency of microspore division after starvation at 4 °C for three days (G) was also low (5%) and no plants were regenerated. In the control (nontreated), about 3% of the embryogenic microspores underwent divisions without embryo formation. In this case, most of the viable microspores started to accumulate starch after one week of culture.

The duration of stress treatment affected the pace of the androgenesis process. The time needed for embryo formation after mannitol with heat shock (B) was shorter than that after the cold treatment (A). Pretreatment B (0.3 M mannitol at 32 °C for 24 h) produced the first embryos after 13–14 days (Fig. 1C, D), while with pretreatment A they appeared after 17–18 days. Consequently, the first regenerated plants (Fig. 1E, F) in pretreatment B were obtained two weeks earlier than with any other pretreatment.

The type of stress treatment applied had an effect on the number of regenerated albino and green plants and on the fertility of these plants (Table 1). The rate of green-plant regeneration ranged from 0.2 to 1 per spike, depending on the pretreatment variant. Overall, 107 green plants grew to maturity. Starvation of anthers at 32 °C for 24 h (B) also had a significant effect on plant regeneration. In this pretreatment,

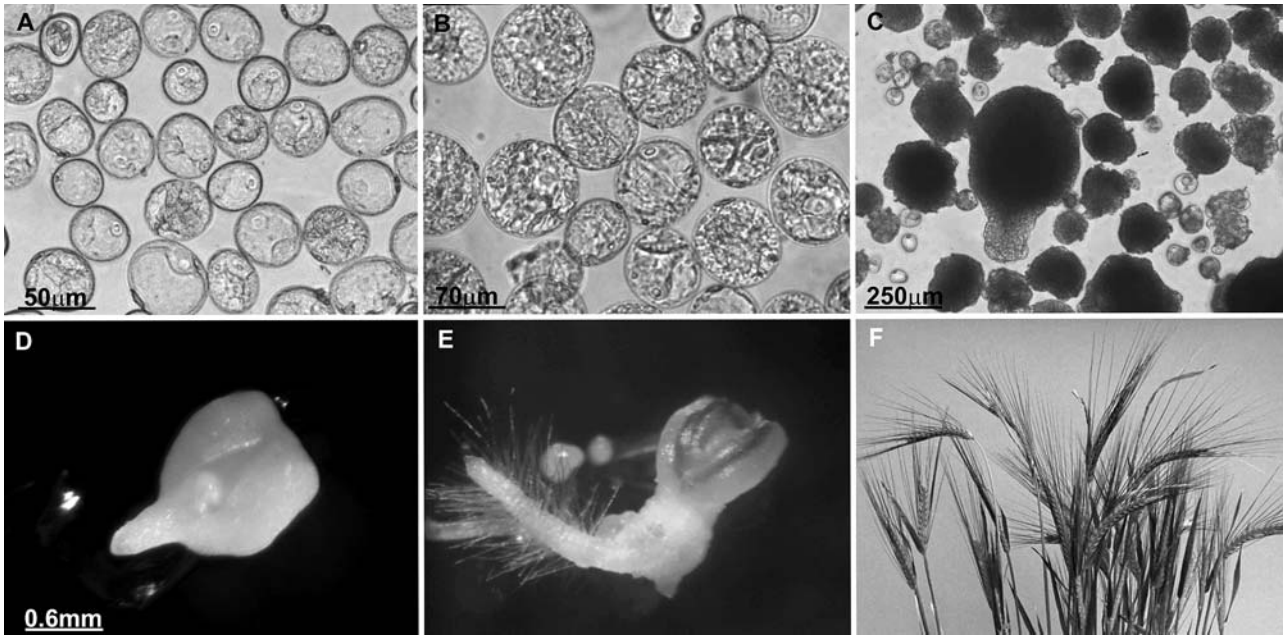

Fig. 1 A–F. Microspore embryogenesis in barley cv. Scarlett after pretreatment of anthers in 0.3 M mannitol at 32 °C for 24 h. **A** Freshly isolated microspores; **B** dividing cells after seven days in culture; **C** cell aggregates and proembryos; **D** well developed androgenic embryo at coleoptile stage; **E** germinating embryo on regeneration medium; **F** fertile microspore-derived plants

Table 1. Effect of pretreatment method on androgenesis efficiency and plant regeneration

Pretreatment	Androgenesis efficiency (%)	Nr. of plants regenerated		Ratio of green to albino plants	Nr. of plants grown to maturity		Ratio of double haploid to haploid plants
		Green	Albino		Double haploid	Haploid	
A, spikes for 2 weeks at 4 °C	0.0062	15	38	1:2.5	13	2	6.5:1
B, anthers in mannitol, 32 °C, 24 h	0.03	40	16	2.5:1	15	21	1:1.4
C, anthers in mannitol, 26 °C, 3 d	0.013	28	1	28:1	12	14	1:1.2
D, anthers in mannitol, 4 °C, 3 d	0.011	23	7	3.2:1	12	10	1.2:1
E, spikes for 2 weeks at 4 °C plus anthers in mannitol, 26 °C, 3 d	0.01	9	20	1:2.2	7	1	7:1

Table 2. Effect of feeder cells after pretreatment by spike cooling and anther starvation in mannitol solution

Pretreatment	Nr. of anthers	Frequency of initial division (%)	Androgenesis efficiency (%)	Nr. of regenerants/anther		Nr. of plants regenerated		Ratio of green to albino plants	Nr. of plants grown to maturity		Ratio of double haploid to haploid plants
				All	Green	Green	Albino		Double haploid	Haploid	
A+N, spikes for 2 weeks at 4 °C	1170	25.0	0.018	0.037	0.013	15	28	1:1.9	10	3	3.3:1
B+N, anthers in mannitol, 32 °C, 24 h	1365	28.3	0.026	0.047	0.034	47	17	2.8:1	16	28	1:1.75

of 56 regenerants produced, 16 were albino. However, among green plants, only 41% set seeds. The ability to set seed is a good indicator of spontaneous chromosome doubling. Cold pretreatment of tillers (pretreatments A and E) increased the rate of albino plantlet regeneration but the frequency of spontaneous chromosome doubling among green plants was almost 100%. Regeneration frequencies of green plants (both doubled-haploid and haploid) obtained after starvation of anthers at room (C) and low temperature (D) were similar, although low temperature resulted in more plants with chlorophyll defects.

Further improvement of culture by the addition of 'Igri' microspores as feeder cells resulted in a slightly higher frequency of initial divisions in pretreatment B plus N than in A plus N (Table 2). The population of microspores continuing to develop to the embryo stage, expressed as the androgenesis efficiency, was also higher in pretreatment B plus N. This pretreatment also gave more green regenerants per anther (0.034). However, the rate of green-plant regeneration from microspores after cooling was less and the ratio of double haploids to haploids was greater relative to mannitol starvation (Table 2).

Discussion

The efficiency of androgenesis and the number of regenerated green plants can be improved by specific preculture and culture conditions. Kasha et al. (2001a) speculated that the proper application of an appropriate treatment may be the main solution to overcome the genotypic dependency. The nuclear development pathway in barley and wheat microspores depends upon the pretreatment method applied (Hu and Kasha 1999, Kasha et al. 2001a). As the pretreatment conditions can affect the timing of nuclear divisions, embryo formation, and plant regeneration, it can be speculated that the timing of the initiation of sporophytic development depends on the method of treatment and its duration. In this study we observed that with mannitol at 32 °C for 24 h as the pretreatment, the process of embryo formation and plant regeneration was faster relative to any low-temperature treatments. This is in agreement with the observation of Kasha et al. (2001a). Liu et al. (2002a) also found that chemical treatment at 33 °C in wheat microspore cultures was more efficient than in low temperatures.

For many crops, low temperature is a factor that disturbs the stability inside anthers that finally leads to changes in microspore development to form embryos. Cold pretreatment for several days before isolation was reported as an important factor in barley cultures (Davies and Morton 1998, Mordhorst and Lörz 1993). Moreover, a cold shock promoted the androgenic response in recalcitrant genotypes of barley (Devaux et al. 1993). In some genotypes, a period of cooling from a few days up to a few weeks may be optimal and appears to be genotype-dependent (Jähne and Lörz 1995). Cold pretreatment applied in our experiments resulted in high numbers of regenerants; however, a large portion of them were albino (Tables 1 and 2).

Another factor that may change microspore development from gametophytic to sporophytic and enhance the induction of androgenesis is starvation. This is usually accomplished by incubation of anthers or whole spikes in nitrogen- or sugar-free medium. The use of mannitol by Li et al. (1995) gave 19% pollen viability relative to 8.4% after a cold treatment and 6.6% for the control. In some less responsive barley genotypes, starvation in mannitol was more effective than cold pretreatment (Cistué et al. 1999). Other reports on barley confirmed mannitol to be advantageous in inducing androgenesis (Castillo et al. 2000; Cistué et al. 1995; Hoekstra et al. 1992, 1996). Positive effects of mannitol have also been shown in cultures of wheat (Hu and Kasha 1999) and rice (Raina and Ifran 1998). On the other hand, some reports indicate that preisolation conditioning with mannitol reduces the initial microspore viability and is not an effective step in the wheat microspore system (Gustafson et al. 1995). Moreover, a pretreatment with mannitol was ineffective in embryo induction in recalcitrant barley genotypes (Li and Devaux 2001). In our study, mannitol starvation combined with cold or heat shock was more efficient in reprogramming the gametophytic pathway, and induction of androgenesis, than cooling alone. Combinations of the two stress factors had an additive effect on the initiation of androgenesis in other studies also. Touraev et al. (1996) has shown that a pretreatment of anthers under heat shock and starvation induced microspore embryogenesis in wheat genotypes considered recalcitrant in anther culture. Another combination of heat with cold shock has been reported to reprogram the gametophytic pathway (Immonen and Robinson 2000, Reddy et al. 1985).

The role of stress to which the microspores are subjected in the preparation for culture is not easy to define, particularly taking into account the number of experiments that have attempted to elucidate the actual mechanisms of this phenomenon. Our experiments indicate that the separation of the microspores from the mother plants itself, without any treatment, can lead to reprogramming of the microspore development. Although embryos have not been produced, the reprogramming of some cells must have been initiated as evidenced by swelling of microspores and the formation of multicellular structures. Most of the cells in culture could not divide as they started to accumulate starch. Although it

seems to be possible to enhance the androgenic response by different stresses, in many reports, the stress treatment was not required to tip the microspore development to the sporophytic program. An initiation of androgenesis even without cold pretreatment in wheat and barley was reported by Ohnoutková et al. (2000). The results of Indrianto et al. (1999) showed that although in most cases different pretreatments increased plant production compared with nonpretreated microspores, cultures without stress treatment were also effective.

Although the mechanism of the nurse culture effect in microspore culture is unknown, it was found that ovaries from barley release hormone-type substances that promoted microspores division (Köhler and Wenzel 1985). Other explants like glumes, florets, and anthers were also successfully used as nurse culture (Puolimatka and Pauk 1999). The positive feeding effect of microspores has been demonstrated in barley protoplasts (Salmenkallio-Martilla and Kauppinen 1995). In our study, the addition of nurse culture improved the androgenesis efficiency almost three times when microspores were isolated from spikes that had been stored for two weeks at 4 °C relative to cultures without nurse (Tables 1 and 2). However, plant regeneration was not enhanced. No improvement in culture with nurse was observed after mannitol starvation.

The high frequency of fertile double-haploid regenerants eliminates the extra time required to treat plants with agents for chromosome doubling. There are some results showing the essential influence of the type of stress treatment on the ploidy level of microspore-derived plants (Li and Devaux 2003). Our data demonstrate that the type of the preculture stress treatment directly affects the ploidy level of the regenerants. Microspores isolated from spikes treated by low temperature more frequently developed into diploids (Table 2). This is similar to observations reported by Indrianto et al. (1999) that androgenic progeny obtained from cold-treated tillers were mostly diploid compared with regenerants derived from nonpretreated and stressed by starvation and heat. It suggests that low temperature can be an important factor leading to the doubling of the chromosome numbers. Prolonged cold pretreatment is known to significantly increase the spontaneous doubling of the chromosome number (Immonen et al. 1999, Immonen and Robinson 2000). However, the proportion of spontaneous chromosome doubling among the microspore-derived plants varies between genotypes (Immonen et al. 1999, Liu et al. 2002a). Kasha et al. (2001b) observed that the combination of cold and mannitol pretreatments produced a frequency of double haploids similar to that after cooling of spikes. Heat shock combined with starvation used in our experiments enhanced the production of green plants but reduced the number of plants with spontaneous chromosome doubling. The stress of low temperature used on spikes had a positive effect on the number of double-haploid regenerants (ratio of double haploids to haploids of 6.5 : 1), although the cold-pretreated anthers produced a similar number of doubled-haploid and haploid plants, perhaps because the time of incubation was too short.

A high proportion of regenerated albino plants is a serious problem in the androgenic culture of cereals (Caredda and Clement 1999). In this study, the ratio of green to albino plants varied depending on pretreatments. Cold pretreatment not only increased the spontaneous diploidisation but also promoted the undesirable albinism. It seems that the occurrence of albino plants could be reduced by modifications of culture protocols. Results of Liu et al. (2002b) indicate that providing available nutrients during embryo initiation is beneficial for plant regeneration and promotes development of green regenerants in a genotype with a tendency for albinism. As Hu and Kasha (1999) reported, the ratio of green to albino regenerants may be affected by factors such as temperature pretreatment.

The results presented here suggest that an abiotic stress is beneficial to the effective induction of androgenesis. However, taking into account the variables in the microspore culture (such as the embryo yield, the ratio of albino plants, and the frequency of chromosome doubling, etc.), it is difficult to claim that there exists a universal stress level or type with a general positive effect on androgenesis and plant regeneration. Neither in the published reports nor in our data is it easy to distinguish between the contribution of a stress treatment and the in vitro condition in induction of androgenesis. Moreover, the question whether abiotic stress is the only factor in tipping the developmental pathway of microspores to androgenesis still remains without a clear answer.

Acknowledgments

We thank Marianna Dobrzyńska, Halina Świątek, and Aleksandra Zimny for technical assistance.

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