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STRESS AS A TRIGGER FOR ANDROGENESIS INDUCTION IN ISOLATED MICROSPORE CULTURE OF WHEAT (*Triticum aestivum* L.)

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Introduction

Isolated microspore cultures can be used as a perfect model system in many areas of basic research in plant developmental biology, plant breeding and biotechnology. The recent discovery that stress is a main trigger in inducing androgenic development allows for considerable progress in application of this method for practical purposes. The list of the most effective stress treatments consists of: cold shock [HUANG, SUNDERLAND 1982; GAILLARD et al. 1991; GUSTAFSON et al. 1995], heat shock [TOURAEV et al. 1996a] and carbohydrate/nitrogen starvation [KYO, HARADA 1986; TOURAEV et al. 1996b]. The stresses could be applied to spikes, anthers or isolated microspores. Despite great progress, the effectiveness of the androgenic process is highly genotype-depended and still inefficient and/or limited to selected genotypes in many economically important crops, among others in wheat.

The aim of the present work was to study the effect of various stress treatments applied in several combinations on androgenic response in isolated microspore cultures of spring hexaploid wheat (*Triticum aestivum* L.).

Materials and methods

Spring wheat genotype (*Triticum aestivum* L.) 'Minaret' used in the experiment was selected on the basis of our earlier studies as having a high androgenic response.

Ten to twelve seeds were sown in each pot filled with premixed soil (3 : 2 : 1; peat : soil : sand v/v/v). Plants were grown in temperature-controlled greenhouse at 20°C/18°C (day/night) under a 16 h/8 h (day/night) photoperiod, and were fertilized twice a week with liquid Hoagland's medium. Tillers with still

ensheathed spikes were harvested and immediately placed in water-containing jars, and kept for 9–14 days at 4°C. Freshly cut or cold-pretreated tillers that contained microspores at the mid- to late-uninucleate stage were disinfected with 70% ethanol for 1 min, then with 5,6% sodium hypochlorite solution for 15 min, followed by five rinses with sterile water. Anthers from three tillers were aseptically excised, and put into 5 ml liquid culture medium AT3 (TOURAEV, personal communication) or into starvation, inducing liquid medium B [KYO, HARADA 1986] in 60 x 15 mm petri dishes. Anther cultures were incubated at 5, 26 or 33°C for 4 days (3 petri dishes for an object). Then, microspores were mechanically isolated by squashing anthers with a ceramic rod followed by filtration of microspore suspensions through a 70 μm metal sieve. The suspensions were pelleted (100 x g, 7 min) and washed by density gradient centrifugation (mannitol 0,3 mol·dm^{-3}/21% maltose; 80 x g, 10 min). Finally microspores were plated at a density 1 x 10^5 microspores per cm^3 in 35 x 15 mm petri dishes. Cultures were incubated in medium AT3, at 26°C, in the dark. Ten ovaries (dissected simultaneously with anthers isolation and cultured in medium AT3 until use) were placed into 1 cm^3 microspore suspension. Well-developed embryos ($\varnothing \geq$ 3mm) were transferred successively onto solid regeneration medium 190-2 [ZHUANG, HU 1983] and cultured at 26°C and light intensity of 80 μmol (hγ)·m^{-2}·s^{-1} (PAR). Every two weeks, cytological observations were done with light, inverted microscope (Olympus ATM3).

Results

The final estimation of the effectiveness of androgenic induction was highly consistent with characterisation of the cultures based on observations done after 4 days of anthers pre-culture (tab. 1). When the number of spontaneously shed microspores was high and the percentage of microspores with induction symptoms ('star-like' structures 'SLS' or microspores after symmetrical nucleus divisions) was considerable, high frequency of multicellular structures was noted later. However, in some cases the process of regeneration was inhibited on stage of embryo-like structures ('ELS') formation and finally all produced structures degenerated.

In isolated microspore cultures of studied wheat genotype, stress proved to be an indispensable factor for androgenesis induction (Tab. 1, 2). In cultures not subjected to any stress treatment, microspores continued their normal gametophytic developmental pathway. The majority of microspores accumulated starch and finally degenerated (Tab. 1). The type and sequence of stress treatment affected considerably the effectiveness of androgenesis and the regenerability of produced ELS. Although, cold pre-treatment of spikes was not an indispensable trigger for androgenesis induction, cold-shock applied to spikes or/and anthers enhanced androgenic ability and plant regeneration. The most effective in androgenesis induction was the combination of cold pretreatment of tillers with heat and carbohydrate/nitrogen starvation shocks applied to anthers. This treatment resulted in high number of ELS development (about 100 ELS per dish). The percentage of regenerating ELS ranged from 0 to 50%. However, all regenerates were chlorophyll-lacking (albino) plants.

Table 1; Tabela 1

Effectiveness of androgenesis induction after 4-day isolated anthers pre-culture (1) and 14-day isolated microspore culture on AT3 medium at 26°C (2)

Ocena indukcji androgenezy po 4 dniach prekultury izolowanych pylników (1) oraz wstępna ocena efektywności androgenezy w kulturach izolowanych mikrospor po 14 dniach hodowli na pożywce hodowlanej AT3 w 26°C (2)

Kłosy Tillers	Isolated anthers Izolowane pylniki		Effectiveness of induction; Efektywność indukcji	
	medium pożywka	temperature temperatura	(1)	(2)
No pretreatments Bez traktowania wstępnego	AT3	5°C	single shed mks, in majority degenerated, occasional 'sls' pojedyncze mks uwolnione z pylników, większość zdegenerowana, pojedyncze „sls"	numerous multicellular structures liczne struktury wielokomórkowe
		26°C	single shed mks, in majority starch accumulating pojedyncze mikrospory uwolnione z pylników, wypełnione skrobią	no cell divisions brak podziałów komórkowych
		33°C	numerous shed mks, occasional 'sls' liczne mikrospory uwolnione z pylników, pojedyncze struktury „sls"	no cell divisions brak podziałów komórkowych
	B	5°C	single shed mks, in majority degenerated, occasional 'sls' pojedyncze mikrospory uwolnione z pylników, większość zdegenerowana, pojedyncze „sls"	occasional multicellular structures nieliczne struktury wielokomórkowe
		26°C	single shed mks, in majority starch accumulating pojedyncze mikrospory uwolnione z pylników, wypełnione skrobią	no cell divisions brak podziałów komórkowych
		33°C	numerous shed mks, in majority degenerated, occasional 'sls' liczne mikrospory uwolnione z pylników, większość zdegenerowana, pojedyncze „sls"	occasional multicellular structures nieliczne struktury wielokomórkowe

Ciąg dalszy tabeli 1; Table 1 continued

Cold pretreatment (9–14 days at 4°C) Traktowane chłodem (9–14 dni w 4°C)	AT3	5°C	no shed mks brak uwolnionych mikrospor z pylników	no cell divisions brak podziałów komórkowych
		26°C	numerous shed mks, in majority starch accumulating liczne mikrospory uwolnione z pylników, wypełnione skrobią	no cell divisions brak podziałów komórkowych
		33°C	single shed mks, in majority degenerated pojedyncze mikrospory uwolnione z pylników, większość zdegenerowana	no cell divisions brak podziałów komórkowych
	B	5°C	single shed mks, in majority starch accumulating pojedyncze mikrospory uwolnione z pylników, wypełnione skrobią	no cell divisions brak podziałów komórkowych
		26°C	numerous shed mks, in majority degenerated, occasional after symmetrical nucleus division liczne mikrospory uwolnione z pylników, większość zdegenerowana, pojedyncze po symetrycznych podziałach	numerous multicellular structures liczne struktury wielokomórkowe
		33°C	numerous shed mks after symmetrical nucleus division bardzo liczne mikrospory uwolnione z pylników, po symetrycznych podziałach	numerous multicellular structures bardzo liczne struktury wielokomórkowe

AT3 – rich culture medium (according to Touraev, personal communication); pełna pożywka hodowla (Touraev, doniesienia ustne)
B – starvation medium lacking nitrogen substances and carbohydrates which could be metabolized by plant cells; pożywka głodowa pozbawiona substancji azotowych i metabolizowalnych cukrów [KYO, HARADA 1986]
„sls" – (ang. 'star-like-structures') – first stage microspore androgenic development; mikrospory we wstępnej fazie indukcji androgenezy
„els" – (ang. embryo-like-structures) – multicellular androgenic structures; wielokomórkowe sruktury androgeniczne

Table 2; Tabela 2

The effectiveness of embryo induction and regeneration as a result of various stress treatments in isolated wheat microspore cultures. Presented data are the means from at least four replicates ± S_d

Efektywność indukcji androgenezy oraz zdolności regeneracyjne zarodków zaindukowanych pod wpływem różnego typu stresów w kulturach izolowanych mikrospor pszenicy. Prezentowane dane są wartościami uśrednionymi dla przynajmniej 4 powtórzeń ± S_d

Tillers Kłosy	Isolated anthers; Izolowane pylniki		Effectiveness of induction [A] Efektywność indukcji [A]	Effectiveness of regeneration; Efektywność regeneracji	
	medium pożywka	temperature temperatura		mean number of embryos [B] średnia liczba zarodków [B]	percentage of regenerating embryos [C] procent zarodków regenerujących [C]
No stress pretreatment Bez traktowania wstępnego	AT3	5°C	100 ±25	0	–
		26°C	0	0	–
		33°C	0	0	–
	B	5°C	20 ±5	50 ±12	18
		26°C	0	0	–
		33°C	20 ±8	7 ±3	0
Cold pretreatment (9–14 days at 4 °C) Traktowane chłodem (9–14 dni w 4°C)	AT3	5°C	0	0	–
		26°C	0	0	–
		33°C	0	0	–
	B	5°C	0	0	–
		26°C	50 ±19	0	–
		33°C	100 ±37	42 ±11	50

A – mean number of embryo-like-structures per dish scored after 40 days culture on AT3 medium; średnia liczba zarodków na szalkę po 40 dniach hodowli na pożywce AT3

B – mean number of embryos per dish transferred onto regeneration medium 190-2; średnia liczba zarodków na szalkę pasażowanych na pożywkę regeneracyjną 190-2

C – the number of regenerating embryos : total number of embryos transferred onto regenerating medium 190-2; liczba regenerujących zarodków : całkowita liczba zarodków pasażowanych na pożywkę regeneracyjną 190-2

Discussion

Our results suggest, that on the basis of pre-cultured anthers response the androgenic potential of isolated microspore cultures could be anticipated. The analyses of the number of spontaneously shed microspores and its cytological characterization allows for evaluation of the final effect of culture with high approximation. The appearance of 'star-like' microspores (with centrally positioned nucleus or nuclei, and vacuole cross-passed by cytoplasmic strands) was followed by multicellular embryo-like structure formation, which is in agreement with the assumption that this type of microspore (SLS) is the first step of induction of gametic embryogenesis [TOURAEV et al. 1997]. The effectiveness of androgenesis induction was depended on the type and sequence of stress treatments. The highest intensity of stress treatment induced the most effective androgenic development and as a result, great number i.e. about 100 ELS per dish were formed. However, the final effect (the number of well-developed embryos ready for transfer onto regeneration medium) was not satisfactory as only 50 embryos were obtained as a maximum. Such abortion effect could be an effect of shortage of some nutrients and/or other disadvantageous changes of medium parameters. It cannot be excluded that it is genetically programmed.

Cold shock applied to both tillers and anthers enhanced androgenic development and plant regeneration. The mechanism of cold temperature action is still not fully explained although it is known that it causes polarisation of cytoplasm, arrests microspores in the first mitosis and blocks starch production [OHNOUTKOVÁ et al. 2000]. As a result these stresses enhance the rate of switch from the gametophytic to the sporophytic development [ATANASSOV et al. 1995]. Cold treatment could also delay degeneration of microspores, accelerate and synchronise nuclear divisions [RAINA 1997]. Efficient cold treatment was described in many reports [KASHA et al. 1990; LAZAR et al. 1990; HU, KASHA 1997].

Although high accumulation of stresses was very effective in androgenesis induction, it caused detrimental influence on green plant regeneration. Similar effect was observed by other authors [INDRIANTO et al. 1999; KUNZ et al. 2000; TORP et al. 2000]. Molecular studies revealed that albinism is usually associated with deletions in the chloroplast DNA, possibly coding functions of some enzymes involved in chlorophyll synthesis [DAY, ELLIS 1984, 1985; HARADA et al. 1991]. Recent studies suggest that plastid genome deletions could be the secondary effect [HOFINGER et al. 2000] since an altered transcription and translation pattern is common to all albino plant. There are also suggestions that environment could be involved in the formation of albino plants apart from genetic factors. Especially in cereals, the stability of plastid ribosomes is temperature-depended [MOIENI, SARRAFI 1995].

Conclusions

1. The main trigger factor influencing the androgenic development in isolated microspore cultures of tested genotype of spring wheat was stress; the type and sequence of stress treatments considerably influenced the effectiveness of androgenesis induction.
2. Cytological analyses of microspore response on the conditions of anther pre-culture could be used as indicator of culture androgenic potential.

References

ATANASSOV A., ZAGORSKA N., BOYADJIEV P., DJILIANOV D. 1995. *In vitro production of haploid plants*. World J. Microb. Biot. 11: 400–408.

DAY A., ELLIS T.H.N. 1984. *Chloroplast DNA deletions associated with wheat plants regenerated from pollen: possible basis for maternal inheritance of chloroplasts*. Cell 39: 359–368.

DAY A., ELLIS T.H.N. 1985. *Deleted forms of plastid DDNA in albino plants from cereal anther culture*. Curr. Genet. 9: 671–678.

GAILLARD A., VERGNE P., BECKERT M. 1991. *Optimization of maize isolation and conditions for reliable plant regeneration*. Plant Cell Rep. 10: 55–58.

GUSTAFSON V., BAENZIGER P.S., WRIGHT M.S., STROUP W.W., YEN Y. 1995. *Isolated wheat microspore culture*. Plant Cell Tissue Org. Cult. 42: 207–213.

HARADA T., SATO T., ASAKA T., MATSUKAWA I. 1991. *Large-scale deletions of plastid DNA in the rice anther culture*. Theor. Appl. Genet. 81: 157–161.

HOFINGER B.J., ANKELE E., GÜLLY CH., HEBERLE-BORS E., PFOSSER M.F. 2000. *The involvement of the plastid genome in albino plant regeneration from microspores in wheat*. 'Biotechnological approaches for utilisation of gametic cells'. Materials from COST 824 final meeting, Bled, Slovenia, 1–5 VII 2000: 215–228.

HU T.C., KASHA K.J. 1997. *Improvement of isolated microspore cultures of wheat (Triticum aestivum L.) through ovary co-culture*. Plant Cell Rep. 16: 520–525.

HUANG B., SUNDERLAND N. 1982. *Temperature-stress pretreatment in barley anther culture*. Ann. Bot. 49: 77–88.

INDRIANTO A., HEBERLE-BORS E., TOURAEV A. 1999. *Assessment of various stresses and carbohydrates for their effect on the induction of embryogenesis in isolated wheat microspores*. Plant Sci. 143: 71–79.

KASHA K.J., ZIAUDDIN A., SIMON E., SIMON A.A., CHEN YU RONG 1990. *Barley and wheat microspore culture*. Plant Cell Rep. 9: 69–72

KUNZ C., ISLAM S.M.S., BERBERAT J., PETER S.O., BÜTER B., STAMP P., SCHMID J.E. 2000. *Assessment and improvement of wheat microspore derided embryo induction and regeneration*. J. Plant Physiol. 156: 190–196.

KYO M., HARADA H. 1986. *Control of the developmental pathway of tobacco pollen in vitro*. Planta 168: 427–432.

LAZAR M.D., SCHAEFFER G.W., BAENZIGER P.S. 1990. *The effects of interactions of culture environment with genotype on wheat (Triticum aestivum L.) anther culture response*. Plant Cell Rep. 8: 525–529.

MOIENI A., SARRAFI A. 1995. *Genetic analysis for haploid-regeneration responses of hexaploid-wheat anther cultures*. Plant Breeding 114: 247–249.

OHNOUTKOVÁ L., NOVOTNÝ J., MÜLLEROVÁ E., VAGERA J., KUČERA L. 2000. *Is a cold pretreatment really needed for induction of in vitro androgenesis in barley and wheat?* „Biotechnological approaches for utilisation of gametic cells". Materials from COST 824 final meeting, Bled, Slovenia, 1–5 VII 2000: 33–37.

RAINA S.K. 1997. *Doubled haploid breeding in cereals*. Plant Breeding Reviews 15: 141–186.

TORP A.M., HANSEN A.L., HOLME I.B., ANDERSEN S.B. 2000. *Genetic markers for haploid formation in wheat anther culture*. „Biotechnological approaches for utilisation of gametic cells". Materials from COST 824 final meeting, Bled, Slovenia, 1–5 VII 2000: 269–273.

TOURAEV A., ILHAM A., VICENTE O., HEBERLE-BORS E. 1996a. *Stress induced microspore embryogenesis from tobacco microspores: an optimized system for molecular studies*. Plant Cell Rep. 15: 561–565.

TOURAEV A., INDRIANTO I., WRATSCHKO I., VICENTE O., HEBERLE-BORS E. 1996b. *Efficient microspore embryogenesis in wheat (Triticum aestivum L.) induced by starvation at high temperature*. Sex. Plant Reprod. 9: 209–215.

TOURAEV A., VICENTE O., HEBERLE-BORS E. 1997. *Initiation of microspore embryogenesis by stress (a review)*. Trends Plant Sci. 2: 285–323.

ZHUANG J.J., XU J. 1983. *Increasing differentiation frequencies in wheat pollen callus*, in: *Cell and tissue culture techniques for cereal crop*. Hu H., Vega M.R. (eds). Improvement Science Press, Beijing: 431.

Key words: androgenesis effectiveness, isolated microspore cultures, spring wheat, stress treatment

Summary

The effect of various stress treatments (cold, heat, starvation) and its combinations on androgenesis induction in isolated microspore cultures of spring wheat (*Triticum aestivum* L.) was under the study. On the basis of our preliminary experiments, spring wheat cultivar Minaret recognized as responsive for androgenic development was selected and used in the study. The anthers were aseptically isolated from freshly cut or cold-pretreated (9–14 days at 4°C) tillers. The anthers were precultured in starvation B medium or rich AT3 medium at 5, 26 or 33°C. After 4 days, microspores were mechanically isolated and cultured in AT3 medium at a density 1 x 10^5 microspores per cm^3, at 26°C, in the dark. Well-developed embryos were transferred to solid 190-2 medium and cultured at 26°C, in the dim light. Cytological observations were done in 2-weeks intervals with inverted light microscope.

The analysis of the number of spontaneously shed microspores and its cytological characterization conducted after 4-day anther pre-culture was a good indicator of culture androgenic potential. The effectiveness of androgenesis induction was depended on the type and sequence of stress treatments. The highest intensity of stress treatment (combination of cold pretreatment of tillers with heat and carbohydrate/nitrogen starvation stresses applied to anthers) induced the most effective androgenic development and as result a great number (about 100 per dish) of multicellular androgenic structures were formed. However, the final effect (the number of globular embryos big enough to be transferred onto regeneration medium) was not satisfying (max 50 embryos per dish). Cold shock applied both to tillers or anthers enhanced androgenic development and plant regeneration. The percentage of regenerating embryos ranging from 0 to 50%. However all regenerates were chlorophyll-lacking (albino) plants. In the

control cultures (no stress treatment) microspores continued their normal gametophytic development.

WPŁYW STRESU NA INDUKCJĘ ANDROGENEZY W KULTURACH IZOLOWANYCH MIKROSPOR PSZENICY (*Triticum aestivum* L.)

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Słowa kluczowe: efektywność androgenezy, kultury izolowanych mikrospor, pszenica jara, stres

Streszczenie

W przedstawionej pracy badano zdolności do rozwoju androgenicznego w kulturach izolowanych mikrospor pszenicy jarej poddanej działaniu różnych czynników stresowych.

Ze świeżo ściętych lub uprzednio traktowanych chłodem kłosów (9–14 dni w 4°C) izolowano pylniki wykładając je na płynną pożywkę hodowlaną AT3 lub płynną pożywkę „głodową" B. Kultury umieszczano w temperaturze 5, 26 lub 33°C. Mikrospory izolowano mechanicznie i hodowano w pożywce AT3, w ciemności, w 26°C. Zregenerowane zarodki przenoszono na stałą pożywkę 190-2 i hodowano w 26°C. Hodowli towarzyszyły obserwacje cytologiczne wykonywane przy użyciu świetlnego mikroskopu odwróconego.

W kulturach izolowanych mikrospor badanego genotypu pszenicy jarej, stres okazał się być czynnikiem niezbędnym dla zaindukowania rozwoju androgenicznego; mikrospory niepoddane działaniu stresu kontynuowały gametofityczną drogę rozwoju. Dobór czynnika indukującego stres wpływał znacząco na efektywność androgenezy i zdolności regeneracyjne zarodków. Wstępne traktowanie chłodem kłosów nie było czynnikiem niezbędnym dla zaindukowania androgenezy, jednakże niska temperatura działająca na kłosy i/lub izolowane pylniki wyraźnie podnosiła efektywność indukcji i zdolności regeneracyjne zarodków. Najbardziej efektywnym w indukowaniu androgenezy było połączenie działania stresu temperaturowego (33°C) ze stresem głodowym (20–100 wielokomórkowych struktur na obiekt). Liczba zarodków zdolnych do regeneracji wynosiła od 0 do 50%, jednakże nie uzyskano roślin zielonych.

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