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ORIGINAL PAPER

A brief temperature pulse enhances the competency of microspores for androgenesis in *Datura metel*

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Abstract Androgenesis may be induced in plants by a stress application on microspores or anthers. Temperature stress treatments have generally been confined to a single temperature regime (above or below ambient) lasting from a few hours to days. We introduced a gradient with two temperature pulses (30 s each) in the stress application on anthers of *Datura metel* L. by stepping the temperature up and down for a total period of 60 s. Anthers were immersed in sterile water preheated and cooled to the desired temperature and cultured on Nitsch medium. The temperature pulse gradient significantly improved androgenesis compared to single temperature treatments, resulting in increased mean embryogenesis of 128% over control for 45°/15°C, 110% for 45°/10°C, 113% for 40°/10°C and 96% for 45°/5°C. The 45°/10°C gradient also significantly increased the number of dividing microspores observed, after 14 days of anther culture. Besides the differential of the gradient, the temperature limit was important, with anthers not tolerating temperatures beyond 45°C. The temperature pulse gradient applied at an early stage of culture may increase the window of competency of microspores for androgenesis.

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Introduction

During microsporogenesis, many species have the ability to switch from gametophytic to sporophytic development of microspores under specific conditions in vitro. This switch in development for angiosperms is generally induced by imposing some type of stress on anthers or isolated microspores just before culture. Androgenesis can be induced or enhanced by temperature pre-treatments of anthers or microspores. A single temperature regime is generally applied either above or below ambient, extending from a few hours to days and weeks. The application of a temperature pulse and gradient (combination of temperatures applied successively) as a pre-treatment in the range of seconds, has not been reported previously.

Many abiotic and biotic stress treatments have been reported to induce androgenesis, including gamma rays and ethanol (Pechan and Keller 1989), colchicine (Barnábas et al. 1991; Möllers et al. 1994), mannitol (Hoekstra et al.1992), nitrogen starvation (Kyo and Harada 1986), and cold treatment (Gaillard et al. 1991). In wheat and tobacco, a combination of starvation and heat shock induced androgenesis (Touraev et al. 1996).

In spite of these empirical findings, however, the nature of embryogenic competence remains elusive. Ever since flower buds of *Datura innoxia* were first cold treated to induce embryogenesis (Nitsch and Norreel 1973), external stress factors have been a deciding factor in inducing or enhancing androgenesis in many species. Temperature stress has taken the form of cold stress or heat shock (Sangwan and Sangwan-Norreel 1996). Cold stress has been routinely applied to wheat spikes (Kunz et al. 2000) and heat stress to microspores of *Brassica napus* (Lichter 1982). In *Nicotiana tabacum*, starvation of microspores by substituting a non-metabolizable sugar in the culture medium induced androgenesis (Kyo and Harada 1986; Heberle-Bors 1989). In barley, pretreatment with mannitol and calcium, which increased the osmolality, improved production of both embryo-like structures and plants (Hoekstra et al. 1992, 1997). Disorienting the microtubular architecture of the microspore to produce a symmetric first division of the nucleus has also enhanced embryogenesis in *B. napus* (Zaki and Dickinson 1991; Iqbal et al. 1994).

A combination of different stresses has also been used to induce androgenesis in some species. Isolated microspores of *N. tabacum* responded to heat shock at 33°C or 37°C by undergoing androgenetic divisions and additional stress by sucrose-starvation at 25°C improved the response (Touraev et al. 1996). Similarly Bueno et al. (1997) induced androgenesis in *Quercus suberus* L. by a combination of starvation treatment and heat shock at 33°C for 5 days. Since many stress factors can trigger androgenesis, Maraschin et al. (2005) suggested that converging signaling pathways responding to different stress signals, may trigger the same downstream response. Thus in addition to selection of the appropriate stage of microspore development for androgenesis, stress is generally necessary for induction.

Temperature stress as a pre-treatment has ranged from 5–35°C extending from a few hours to days or weeks. Our objective was to stress the cellular metabolism within the microspores, by switching between two different temperature pulses in quick succession, to which microspores are not subjected to in their normal course of development. Here, we report the use of a temperature pulse as a pre-treatment on anthers of *Datura metel* where the high and low temperature extremes were applied in quick succession for a duration of 30 s before incubating cultures at 25°C.

Materials and methods

Materials

Donor plants

Plants were collected in the Kandy district, established in the greenhouse and identified as *Datura metel* L. from the description in the Flora of Ceylon Vol. IV (Dassanayake and Fosberg 1987) and comparison with herbarium specimens at the Royal Botanic Gardens, Peradeniya. The flowers were self-pollinated and a population of 45 seedlings was established in the greenhouse in clay pots (diam 30 cm). The plants were fertilized fortnightly with a commercial garden fertilizer (Bayer Chem. Co.) and watered daily. Daytime temperature was 26–30°C and relative humidity 70%. The plants flowered at a height of 1 m, 3 months after planting. Unused flowers were removed to avoid pod setting and prevent the bushes from senescing. In each experiment, flower buds were collected from plants of the same age.

Methods

Staging of microspores

Anthers were fixed in a mixture of ethanol (90 ml), glacial acetic acid (5 ml) and formalin (5 ml) and kept at 4°C for 72 h. The anthers were squashed in Alexander's stain (Alexander 1969) or acetocarmine on a microscope slide, the debris removed and the stage of microspore development correlated to the length of the unopened bud and anther length. Cultured anthers were similarly stained after 2 weeks of culture to determine the division of microspores and photographed under an Olympus BH2 light microscope with a camera attachment. Five slides were prepared

from each anther and five fields of observation were randomly made for each slide to determine the frequency of dividing microspores.

Anther and microspore culture

Unopened flower buds (length 3.5–6 cm, corresponding to the mid-late uninucleate stage) were washed with two drops of a commercial detergent and rinsed in tap water. Under the laminar flow hood the washed buds were placed in 95% ethanol for 2 min in a sterile bottle followed by 2% (w/v) calcium hypochlorite for 5 min and then rinsed in three changes of sterile distilled water.

Flower buds were aseptically opened and the anthers separated from their filaments. Of the five anthers from a single bud, three were treated with the temperature pulse and the other two were controls. The anthers were first immersed in a 50 ml sterile beaker with sterilized distilled water pre-heated to the desired temperature. Immediately thereafter, the anthers were immersed in a second beaker with sterilized distilled water at the cool temperature for another 30 s. The water was warmed and cooled with a Bunsen flame and ice cubes respectively. The control anthers remained in sterile water at 24°C for 60 s. The combinations of the temperature pulse treatments are given in Table 1. The three treated and two control anthers were plated into the same petri-dish. Anthers were cultured on Nitsch (1969) medium with 3% sucrose and 4.65 μM kinetin. Microspores were isolated after treatment by cutting the anthers lengthwise with a sharp scalpel and squeezing out the microspores into liquid Nitsch medium. Anther debris was removed under a stereo-microscope. Microspores were cultured in plastic petri-dishes (5.5 cm diam) in 2.5 ml medium per anther. The pH was adjusted to 5.8 using 1N NaOH or 1N HCl, the medium solidified with 0.42% agar gel (Sigma Chem. Co.) and autoclaved at 121°C for 20 min at 105 kpa pressure. Approximately, 20 ml of medium was poured into 100 mm diam glass petri-dishes. The culture plates were incubated at 25 ± 2°C under 16 h photoperiod provided by fluorescence bulbs (Thorn, 40 W tropical daylight). Results were recorded 8 weeks after initial culture by releasing the embryos formed on and within the anthers into water in a petri-dish and counting them under a stereomicroscope. Developmental stages beyond heart were scored as embryos.

Regeneration of embryos to plants

Cotyledonary stage embryos were placed on MS (Murashige and Skoog 1962) basal medium solidified with 0.42% agar gel and 3% sucrose for conversion. To improve conversion, (i) embryos were partially desiccated by transfer to sterile filter paper in a laminar air flow cabinet or (ii) inclusion

Table 1 Response of the anthers, mean difference in embryos (treatment–control), and the range of embryos produced per anther in *Datura metel* to the temperature combinations tested

Temperature (°C)	% response	Mean difference in embryos	Range (mean embryo number)	
			Treatment	Control
55/10	–	−42.7	0–4	9.5–33
50/10	–	−50.0	0–20	57–159
45/15	128.1	18.2*	9–56.3	0–36.5
40/15	76.7	20.5*	3.6–94	2–74
35/15	17.0	6.1	23–65	19.5–54.5
45/10	110.6	44.7***	25–253.3	0–103
40/10	113.7	33.1***	83–106.3	0–55
35/10	15.8	3.9	7.3–95	4.5–81.5
45/5	94.6	24.9***	14–130.6	1–114.5
40/5	34.0	8.2	10–78	0–91
35/5	81.3	15.3*	113–117.3	0–93.5

*** $P < 0.001$.

* $P < 0.05$.

of 2.89 μM gibberellic acid in the MS medium. Plantlets with 2–3 secondary leaves were hardened by transfer to liquid MS medium with 2% sucrose in glass tubes, decreasing the sucrose content in the medium to zero over 3 weeks and exposing them to sunlight under humid conditions. Robust plantlets with a well-developed root system were transferred to 9 × 9 cm pots containing a mixture of loam and coir-dust.

Ploidy determination

Ploidy of anther-derived plantlets was determined by flow cytometry (Möllers et al. 1994) using a Partec Cell Analyzer CA ll (Partec GmbH, Münster). Briefly, a piece of a newly emerged leaf, 1–2 cm^2, from potted plants was chopped in a few drops of DAPI (4′, 6-diamidino-2-phenylindole) diluted to 80% with water (v/v), a buffer added (Partec GmbH, Münster) and filtered through a 40 μm nylon gauze. The filtrate was analyzed in the flow-cytometer. Leaf tissue from a seed-raised diploid plant was the control.

Data analysis

The five anthers (two control and three treated) of a *D. metel* flower bud comprised a replication. The experiment was designed as a randomized complete block with four blocks. Within blocks, each treatment was repeated at least three times and the petri-dishes were arranged in a completely randomized design. ANOVA was conducted for anther culture response as the difference between treated and untreated control anthers for each bud. Computations were done with PROC MIXED in SAS (SAS Institute 2000) with blocks treated as random factor and response as fixed factor. Percentage anther response = [(Treatment−Control)/Control] × 100. Treatments were compared by paired *t*-tests for cell division of microspores.

Results

Cytological examination showed all five anthers of a bud at similar stages of microsporogenesis. Cytological staging of the microspores revealed that the mid to late uninucleate stages of development occurred in buds of 3.5–6 cm and anthers of 1.2–1.5 cm in length. Such anthers were chosen for culture.

Combination of temperature pulses on androgenesis

Of the 11 combinations of high and low temperature pulses tested, each pulse for a duration of 30 s, the temperature combination 45°/15°C increased mean embryogenesis by 128% over the untreated controls ($P < 0.05$). The temperature combinations of 45°/10°C (110% increase), 40°/10°C (113% increase) and 45°/5°C (96% increase) were significantly different at $P < 0.001$ (Table 1). In the significant treatments, more embryogenesis was observed than in the untreated controls (Fig. 2d and e). This is shown by the skewed frequency distribution of experiments producing over 300 embryos under treatment (Fig. 1) whereas their respective controls produced less than 300 embryos. One of the buds submitted to the 45°/10°C temperature combination produced 760 embryos from its three treated anthers (253 embryos per anther, see range, Table 1) while the two control anthers yielded 206 embryos (103 embryos per anther). This shows the empirical advantage of the control and treated anthers explanted from the same bud. All the cultures under temperature treatments less than 50°C produced embryos whereas five of the control experiments from anthers of the same flower bud did not (see range, Table 1).

The significant temperature combinations had a gradient (high minus low) of 30, 35 and 40°C (Table 1). When temperatures were increased beyond 45°C, the higher temperatures of 50° and 55°C in combination with 10°C produced fewer embryos than their controls (Table 1). Treating the anthers with a single temperature pulse (45, 40, 35, 10 and 5°C) did not produce a significant difference in androgenesis compared to untreated anthers (data not shown). Isolated microspores from anthers treated with and without temperature pulse treatment did not undergo androgenesis.

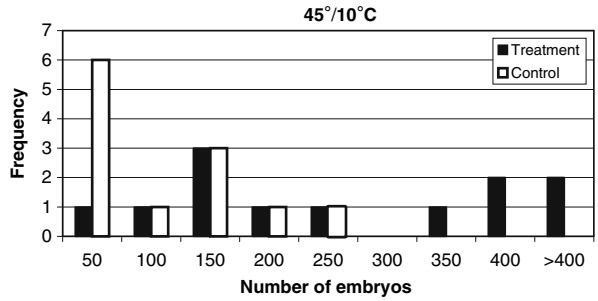

Fig. 1 Frequency distribution of the experiments and the total number of *Datura metel* embryos produced in each experiment by the treated and control anthers under the temperature combination of 45°/10°C

Effect of temperature pulse on microspore division

We examined microspores in anthers of the best temperature combination of 45°/10°C to determine the effect on microspore division 14 days after culture. Microspores treated with the temperature pulse underwent a significantly greater number of cell divisions than those treated at single temperatures of 45 or 10°C (Table 2). The first cell division in the microspores was observed 72–96 h after anther culture with proembryos emerging after 12 days while embryos emerged from the anthers after 5–6 weeks (Fig. 2).

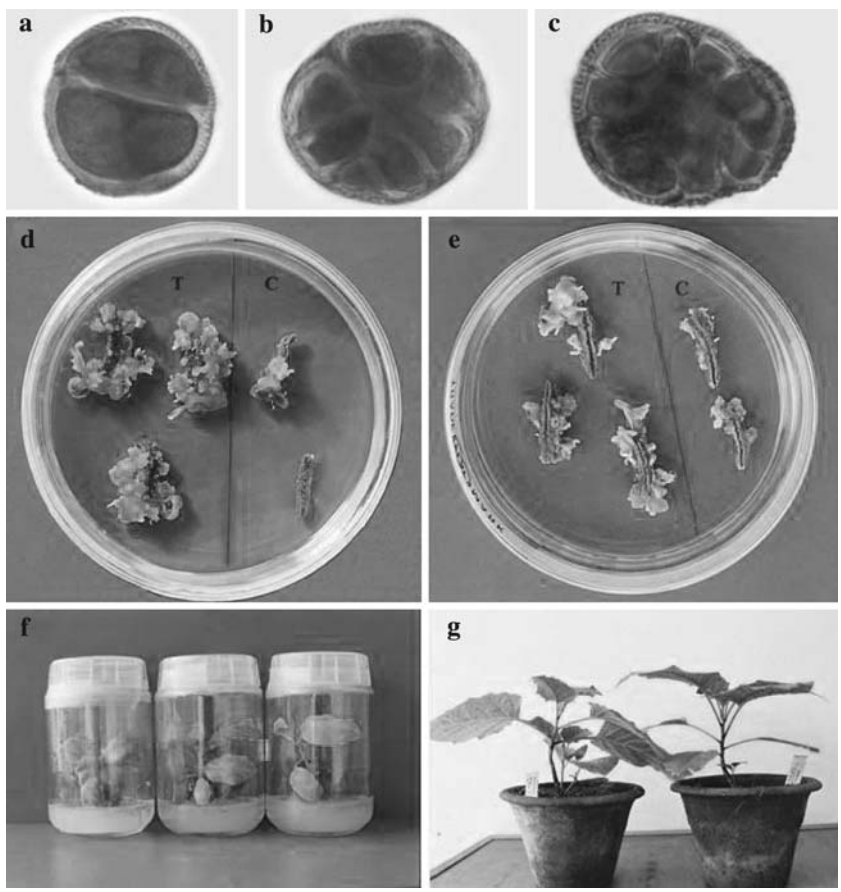

Fig. 2 Division of microspores, androgenesis and regeneration of plants under a temperature gradient of 45°C/10°C in *Datura metel*: (**a**) First division after 4 days of culture; (**b**) and (**c**) Multicellular pollen within the exine (**b**) and with proembryo just emerging (**c**) after 12 days of culture; (**d**) and (**e**) Embryos emerging from the three treated (T) anthers and two control (C) anthers, all five anthers from the same bud; (**f**) Plantlets in vitro; (**g**) Hardened, potted plants

Table 2 Cell division in microspores of *Datura metel* after treatment with the temperature gradient of 45°/10°C, single temperatures of 45°C and 10°C, and untreated control anthers, 14 days after anther culture

Treatment	Mean no. of divisions in microspores	Treatment comparison
1. 45°/10°C	13.7	1–4*
		1–2*
		1–3*
2. 45°C only	3.7	2–4
		2–3*
		2–1
3. 10°C only	5.3	3–4
		3–1*
		3–2
4. Control	3.0	–

*Significant at 0.05 level.

Regeneration to plants

Morphological differences or abnormalities were not observed in embryos recovered from temperature treatments or control. Gibberellic acid at 2.89 μM was most effective in embryo conversion to plantlets followed by partial desiccation on the laminar flow hood for 30 min (Table 3). Increasing desiccation beyond 30 min reduced embryo conversion. Reducing the sucrose in the medium to 2% and finally to zero was necessary to induce growth of secondary leaves and roots, to enable transfer of the plantlets to soil. On transfer to soil, 80% of the plantlets survived. Flow cytometric analysis confirmed that the anther-derived plantlets were haploid (Fig. 3). Spontaneous diploidization occurred in 20% of the plants, which on self-pollination produced pods and seeds.

Table 3 Germination of androgenic embryos of *Datura metel* in Murashige and Skoog basal medium following gibberellic acid treatment and desiccation

Treatment	Number of embryos treated	% germination
Basal medium (BM)	82	41
BM + 2.89 μM gibberelic acid	85	84
BM + 30 min desiccation	85	69
BM + 45 min desiccation	84	30

Discussion

The imposition of stress on anthers or microspores to induce or enhance androgenesis is a *sine qua non* for most plant species. Temperatures above and below ambient are generally used at durations of a few hours to weeks. We investigated the role of a temperature pulse, close to the physiologically possible limits of tolerance, to induce androgenesis in anthers of *D. metel*. Exposure of the anthers of *D. metel* to 45 or 40°C for 30 s followed immediately by 10°C for 30 s produced a highly significant ($P < 0.001$) increase in embryogenesis. Such a combination of temperatures for a brief period has not been reported previously. In *Brassica* species temperatures of 30–35°C for 24–72 h induced androgenesis (Palmer et al. 1996) while Custers et al. (1994) reduced this to 8 h at 32°C. In *B. napus*, reducing the temperature from 32°C within 4 h of culture initiation interrupted the induction process in potentially androgenic microspores (Pechan and Smykal 2001). However, the exposure of *D. metel* anthers in this study to a total of 60 s temperature gradient stress induced the onset of androgenesis. The androgenic response while temperature dependent was also determined by duration of exposure. Microspores and pollen grains of *B. napus* died above 35–37°C (Pechan and Smykal 2001; Smykal and Pechan 2000) whereas microspores of *D. metel* protected by their anther wall withstood temperatures of 45°C for a period of 30 s.

The exposure of anthers to 50 and 55°C for 30 s significantly reduced embryogenesis in *D. metel*. The microspores within the anthers apparently cannot physiologically withstand even this short temperature shock. In *D. innoxia* a linear increase in androgenesis occurred when the temperature was raised from 22 to 30°C (Sopory and Maheswari 1976). However, more significant is the fact that in this study the lower temperature in conjunction with a higher temperature (less than 45°C) induced significantly greater embryogenesis. The maximum recorded temperature known to induce androgenesis was 41°C for 1–2 h in *B. napus* (Binarova et al. 1997). Here, androgenesis was shown in late bicellular pollen associated with rearrangements of the microtubular cytoskeleton and the presence of heat shock proteins in the nucleus of the microspore. In the present experiments the

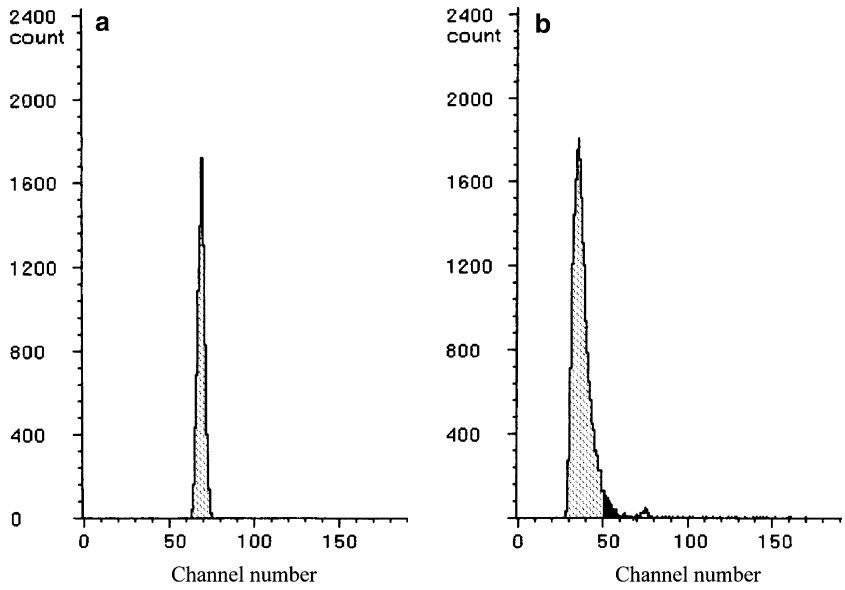

Fig. 3 Flow cytometry histogram (number of stained nuclei vs. channel number) from newly emerged leaves of (**a**) seed-raised and (**b**) anther culture derived plants of *Datura metel*. In (**b**) the haploid peak occurs at a peak positive value corresponding to half that of the diploid peak in (**a**). Hence, the DNA content per cell of the haploid is half that of the diploid

magnitude of the gradient alone (30–35°C), however, was not the deciding factor. The sudden switch between high and low temperatures had to be restricted to physiologically acceptable limits of temperature exposure.

While some plants require a low and others a high temperature pre-treatment, none are known that respond to both low and high temperatures. In *D. innoxia* a cold treatment of 3°C for 48 h promoted androgenesis (Nitsch and Norreel 1973). While isolated microspores from fresh anthers of *D. innoxia* failed to undergo androgenesis, there was a dramatic increase in response when anthers were taken from cold treated buds (Tyagi et al. 1979). Cold treatment was not only beneficial but also obligatory for success in ab initio pollen cultures of *D. innoxia*. In *T. aestivum*, spikes are routinely pretreated at 4°C for 3–14 days (Redha et al. 2000) and in microspore culture of japonica rice *O. sativa*, panicles are pre-treated at 6 ± 1°C for 17–27 days (Xie et al. 1997). *Brassica napus*, however, does not undergo androgenesis when cultured below 25°C (Pechan and Smykal 2001) and small heat shock proteins were not transcribed in the microspores and pollen grains. Anthers of *D. metel* were responsive to temperatures as low as 5°C, particularly when used as a component of a temperature gradient. It would be interesting to determine if small heat shock proteins are also transcribed in microspores of *D. metel* from low temperature stress. Thus *D. metel* appears responsive to a high temperature as well as a cool temperature.

In this study, androgenesis from the anthers treated with a temperature gradient increased significantly and in five of the eleven treatments, the untreated anthers did not show androgenesis, while all the treated anthers underwent androgenesis (Table 1). This suggests that the temperature treatments increased the frequency of microspores that are competent to undergo embryogenesis. Maraschin et al. (2005) reviewed the cellular and molecular mechanisms of haploid induction. They suggested that stress-induced reprogramming of cellular metabolism (repression of gene expression, induction of proteolytic genes and stress-related proteins) followed by activation of key regulators of embryogenesis occurs during the process. Thus, exposure of anthers to an extreme temperature gradient for a brief period was apparently able to induce the cellular events that invoke embryogenesis. Our results show that a gradient change enhanced embryogenesis more than the component temperatures of the gradient applied alone. The anthers were treated by immersing in water maintained at the specified temperatures for 30 s. During this brief period, it was unlikely that the temperature reached the microspores within the anther sac. It is possible that the inducing factors were formed or enhanced in the anther wall and subsequently transferred to the microspores. This is

supported by the fact that attempts to culture microspores in isolation and exposing the isolated microspores to a temperature gradient failed to induce embryogenesis in *D. metel*. Furthermore, Nitsch (1974) was able to induce embryogenesis of isolated microspores of *D. innoxia* in a medium conditioned with an anther wall extract of the same species. However, such a chain of events requiring the anther wall for androgenesis conflicts with isolated microspore cultures of tobacco and *B. napus*. In tobacco, androgenic response by starvation stress causes an accumulation of mRNAs in embryogenic pollen, apparently translated only upon transfer to a sugar rich medium (Garrido et al. 1993), while in *B. napus*, specific mRNAs and proteins were synthesized de novo during heat stress induced androgenesis (Cordewener et al. 1998). It was unclear whether stress-induced proteins were necessary for androgenesis or required for the microspores to survive the stress treatment (Touraev et al. 1997). The present study, while extending the types of stress application (temperature pulse and gradient) for inducing androgenesis, has also shown that the duration of stress can be reduced. Thus other forms of extreme stress applied briefly may be worthwhile treatments in other culture systems (e.g., induction of somatic embryogenesis) to redirect cellular events.

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