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Correlations between low-temperature tolerance of anther donor clones of potato and the production of anther-derived embryos and calli at low temperatures

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Abstract The main purpose of this study was to investigate whether the degree of tolerance to low non-freezing temperatures of immature microspores in anther culture was correlated to the degree of low-temperature tolerance, measured by chlorophyll fluorescene, in the anther donor clone. Anther cultures of six tetraploids and eight dihaploids, derived from anther cultures of clone 199.13, were incubated at 10, 15, 20, 25 or 30 °C respectively. The embryo and callus production were determined and subsequently two quotients/clone, designated "temperature-related embryo and callus production," were established. The quotients were defined as embryo and callus production at 10 or 15 °C divided by the embryo and callus production, for the individuals clone, at the optimal temperature (20 or 25 °C) for the same production. These quotients were thereafter correlated to the low-temperature tolerances of the anther donors. The tetraploid and dihaploid group were treated separately and significant positive correlations were found in both cases. This indicates that tolerance to low temperatures is expressed in the anther donor plant as well as in the microspores grown in anther culture. It is suggested that in vitro selection through anther culture may be a useful tool for breeding for increased tolerance to low temperatures in potato.

Key words Anther culture · In vitro selection · Low-temperature tolerance · Potato · Temperature-related embryo and callus production

Introduction

Mulcahy (1979) was the first to suggest gametophytic selection as a possible tool for breeding, a condition being that there is a genetic overlap between the sporophyte and the gametophyte. Isozymes, which were investigated in pollen and sporophytes of tomato, maize, poplar, and barley respectively (Tanskey et al. 1981; Rajora and Zsuffa 1986; Sari-Gorla et al. 1986; Pederson et al. 1987), revealed that 60–80% of the expression in the sporophyte was found also in the pollen. Furthermore, Willing and Mascarenhas (1984) and Willing et al. (1988) studied the mRNA of shoots and pollen from *Tradescantia* and *Zea mays* and similarity found that about 60% of the genes overlapped. Accordingly, it has been suggested that several traits expressed in the sporophyte can be modified through gametophytic selection (for a review see Ottaviano and Sari-Gorla 1993).

The expression of low-temperature tolerance in the sporophyte and the gametophyte has been investigated by Zamir et al. (1981, 1982), Zamir and Vallejos (1983). They suggested that the same genetic factor(s) were responsible for chilling tolerance in the sporophyte and the gametophyte. Pollen selection for low-temperature tolerance in tomato has been performed by Zamir and Gadish (1987), who noted that root elongation of the progeny from crosses at low temperatures was less inhibited by low temperatures compared with progeny from crosses at a normal temperature. Furthermore, Kovacs and Barnabas (1992) and Lyakh and Soroka (1993) demonstrated gametophytic selection in maize since low-temperature treatment of pollen donors resulted in a progeny with a significantly higher cold tolerance compared with the control. Kristjansdottir (1990) performed pollen assays on potato and noted that the low-temperature-tolerant Andean potato clones maintained their germination capacity better than the less tolerant European clones at lower temperatures. In this case, however, it was not possible to conclude whether the low-temperature tolerance was under gametophytic or sporophytic control. Most of the studies mentioned above focused on the expression of low-temperature tolerance during fertilization or during pollen-tube growth. Zamir and Vallejos (1983), how-

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ever, studied gametophytic selection on pollen grains of tomato at two distinct developmental stages, i.e. during pollen formation (from meiosis to mature gametophyte) and pollen function (from pollination to fertilization). They reported that haploid selection for chilling tolerance was more pronounced during the pollen-function stage than during pollen formation. Patterson et al. (1987) noted that two stages of sensitivity to cold during pollen development of tomato could be identified (11 and 5–6 days prior to anthesis). Furthermore, Bedinger and Edgerton (1990) and Mandaron et al. (1990) investigated the protein synthesis during pollen development in *Z. mays* and reported a variation, indicating that different characters may be expressed at different stages of development.

While several studies suggest that gametophytic selection for chilling tolerance in vivo has a significant potential, the purpose of the present study was to investigate the potential for in vitro selection for tolerance to low temperatures through anther culture of potato. According to Lashermes (1991) the correspondence of gene expression in microspores during anther culture may be even higher than during normal pollen development, since the microspores in anther culture will develop via an embryogenic, rather than via a normal gametophytic, pathway. In the present investigation the degree of low-temperature tolerance of the anther donor clone (sporophyte) was correlated with embryo and callus production at low temperatures of the same clone. A possible correlation would indicate that gene expression for low-temperature tolerance in the two developmental phases is partly similar.

Materials and methods

Plant material

The following tetraploid clones were used: 199.13, 201.5, and 201.12, originating from breeding material established in a programme for frost resistance at the International Potato Center (CIP) Lima, Peru (Landeo 1980). These clones are derived from several tuber-bearing *Solanum* species, namely *S. phureja*, *S. stenotomum*, *S. curtilobum*, *S. ajanhuiri*, *S. juzepczukii*, and *S. tuberosum* ssp. *andigena*, as well as *S. tuberosum* from South America. Details of the clone pedigrees are presented by Kristjansdottir (1989). Furthermore, the Swedish cultivar Elin, S12, and clone 73 were also included in the study. S12 is a clone originating from a seed population created by a selfing of the Swedish cultivar Annika (Kristjansdottir 1989), while clone 73 originates from a tetraploid seed population created from a cross between 201.5 and S12 (Kristjansdottir 1991a). In addition, eight anther derived dihaploids (HA13.1, HA13.2, HA13.4, HA13.6, HA13.7, HA13.9, HA13.11, HA13.18) originating from clone 199.13 were used.

Anther culture

Buds with microspores in the uninuclear stage were harvested and thereafter pre-treated and inoculated according to Calleberg et al. (1989). A MS-based medium (Murashige and Skoog 1962) containing 6.0% sucrose and 45.0 mg/l of L-cysteine-HCl (Merck, biopur), prepared according to the double-layer technique (Johansson et al. 1982), were used in all experiments. The solid phase of the double-layer media, however, was modified in that Gelrite (0.35%) (Merck and Co. Inc Rahway, N.J., Kelco Div, USA) was supplemented with 3% potato starch (Merck, Germany) (Calleberg and Johansson 1993).

Immediately after inoculation, the anther cultures were incubated at the following temperatures: 10, 15, 20, 25, or 30 °C. The light regime was 16 h and the light was 5–7 W^{-2} at all temperatures.

Determination of embryo and callus frequencies

Due to the difference in growth-rate at the various temperatures, the number of embryos and calli were determined after 90 days at 10, 15 and 20 °C and after 60 days at 25 and 30 °C. The frequencies were expressed as embryos and calli/100 anthers. No increase in the numbers of embryos and calli were found at 25 or 30 °C beyond 60 days. Embryo and callus production frequencies from two identical experiments including the temperatures 10, 15, 20, 25, or 30 °C were summarized. The lower temperatures (10 and 15 °C) were considered to be sub-optimal for the production of embryos and calli, while 20 and 25 °C were considered to be optimal (see Discussion). Two quotients, designated "temperature-related embryo and callus production", were established for each clone. In the first quotient embryo and callus production at 10 °C was divided by production at the optimal temperature, while in the second, production at 15 °C was divided by production at the optimal temperature.

Pro-embryos (embryos with a few divisions) were determined microscopically in anther cultures of clone 73 incubated at 12 ° for 60 days or at 22 °C for 90 days. Two-hundred pollen grains in each of 20 buds were analysed from each temperature.

Determination of the low-temperature tolerance in anther donor clones

The degree of low-temperature tolerance of a clone was determined from the decrease of chlorophyll fluorescence caused by cellular injury in temperature-stressed leaves. Tubers (4 tubers/clone, 1 tuber/20 cm ϕ pot) were planted in the greenhouse with additional illumination from High Pressure Sodium Lamps, 400 W (General Electric Company, USA) and Metallic Halogen lamps HPI-T, 400 W (Phillips, Sweden). The soil used was a fully fertilized standard mix (W. Weibulls AB, Sweden). Newly expanded and equally sized subterminal leaflets located on the main branches of the plants were detached prior to flowering. In the analysis of the tetraploid clones eight leaflets/clones were used, while in the analysis of dihaploid clones produced from 199.13 ten leaflets/clone were used. The experimental set-up was modified after Smillie et al. (1983, 1987) and Kristjansdottir and Merker (1993). Leaflets were immediately placed, abaxial side up, on wet filter paper on eight (ten) identical aluminium plates, which were then covered with polyethylene film and placed inside a plastic bag. The aluminum plates were thereafter placed in a growth chamber at 10 °C night/20 °C day with an 18-h day/6-h night regime. The average light intensity was 325 $\mu mol\ m^{-2}s^{-1}$ provided by Osram mentallic halogen lamps (HQI-E 250W/D) with a spectral area of 400–700 nm.

The effect of cold treatment was measured by variable chlorophyll fluorescence (Fv) (Walker et al. 1990). Fv is the difference between the maximum fluorescence (Fm) and he initial fluorescence (F0) (Smillie and Gibbons 1981). Prior to measuring, the leaves were dark-adapted for at least 15 min. After 24 h at 10/20 °C the photosynthetic activity of the leaves were considered stable and the chlorophyll fluorescence was measured, which resulted in a control value (Fv0). The aluminum plates were thereafter moved to a growth chamber with similar conditions to those given above except for the temperature, which in this case was 1 °C (day and night). The temperature on the upper surface of the leaves was 1.7 °C during the day and 1 °C during the night. The chlorophyll fluorescence was measured after 48 h of cold treatment and referred to as Fv2, while the degree of low-temperature tolerance was expressed as Fv2/Fv0. The chlorophyll fluorescence was measured on an arbitrary scale with a chlorophyll fluorescence-measuring apparatus (Hansatech instruments, Norfolk, England: a PLS1 programmable light source; an A8 Fiber-optic cable and a MFMS/2S Modulated Fluorescence measurement system). The light output from the LED probe fitted with the standard filter was 4.8 kHz

square wave, and the intensity of the measuring light at the switch position used was 2.2 μmol/m^2 per s.

Statistical calculations

Statistical calculations were performed in the JMP^{TM} (SAS Institute Inc., Cary, N. C., USA) programme for Apple Macintosh computers. The homogenity of means was tested with an analysis of variance. Comparison of individual means were performed with the method of Least Significant Difference, while the correlation studies were performed by regression analysis.

Results

Embryo and callus production of tetraploid clones at different temperatures

The embryo and callus productions at 10, 15, 20, 25, and 30 °C were recorded for six tetraploid clones (Table 1). Significant variations between the production means at the different incubation temperatures for all clones were found, where the level of significance for clones 199.13, 201.12, and Elin were ($P < 0.001$–$P < 0.01$). As shown in Table 1 clones 199.13, S12, and Elin produced the highest number of embryos and calli at 30 °C while 201.12 performed best at 25 °C. Clones 201.5 and 73 were most productive at 20 °C. Using this information, two quotients of the embryo and callus production at 10 and 15 °C divided by the production at the optimal temperature were established. For the quotient 10 °C/optimal temperature, clone 201.5 showed the highest value followed in order by clones 199.13, 201.12, 73, S12 and Elin, while for the quotient 15 °C/optimal temperature the highest value was found for clone 73 followed in decending order by 201.5, 199.13, 201.12, S12 and Elin.

Embryo and callus production of derived dihaploid clones produced in anther culture of clone 199.13

The embryo and callus production for eight dihaploids derived from clone 199.13 was recorded and a significant variation between the embryo and callus production at the different incubation temperatures was found for all clones except HA 13.6 and HA 13.18 (Table 2). For clones HA 13.1, HA 13.7, HA 13.9, and 13.11 the level of significance was $P < 0.001$, while for clone HA 13.2 the level was $P < 0.01$, and finally for clone HA 13.4 a level of $P < 0.05$ was recorded. The highest production of embryos and calli was found at 30 °C for HA 13.1, HA

Table 1 Embryo and callus production (embryos and calli/100 anthers) at different incubation temperatures and the temperature-related embryo and callus production for six tetraploids. Letters (horizontally) indicate means that differ significantly ($P < 0.05$) according to the method of Least Significant Difference

Clone	Embryos and calli/100 anthers (#anthers)					Temperature-related embryo and callus prod.	
	Incubation temp. (°C)					10 °C/optimal temperature	15 °C/optimal temperature
	10	15	20	25	30		
199.13	2.61 (690) ab	11.04 (670) ab	20.0 (570) bc	32.41 (580) c	87.22 (540) d	0.08	0.34
201.5	0.85 (590) a	2.71 (590) ab	7.83 (460) c	3.18 (440) ab	5.78 (450) bc	0.11	0.35
201.12	1.36 (660) a	10.76 (790) a	29.23 (650) b	47.07 (610) c	25.62 (640) b	0.03	0.23
73	1.25 (240) a	34.87 (390) b	49.47 (190) b	40.57 (350) b	5.24 (210) a	0.02	0.70
S12	0 (180) a	0.48 (210) a	0 (190) a	3.64 (220) b	5.71 (140) b	0	0.13
Elin	0 (330) a	0 (470) a	0 (440) a	2.74 (510) a	15.58 (430) b	0	0

Table 2 Embryo and callus production (embryos and calli/100 anthers) at different incubation temperatures and the temperature-related embryo and callus production for eight dihaploids derived from anther culture of clone 199.13. Letters (horizontally) indicate means that differ significantly ($P < 0.05$) according to the method of Least Significant Difference

Clone	Embryos and calli/100 anthers (# anthers)					Temperature related embryo and callus prod.	
	Incubation temp. (°C)					10 °C/optimal temperature	15 °C/optimal temperature
	10	15	20	25	30		
HA 13.1	6.62 (800) a	6.25 (800) a	8.31 (650) a	36.36 (660) b	93.35 (630) c	0.182	0.172
HA 13.2	1.22 (740) a	3.66 (820) a	7.42 (660) b	4.22 (710) ab	0.93 (640) a	0.164	0.493
HA 13.4	3.48 (230) ab	1.92 (260) a	6.09 (230) ab	7.59 (290) b	0.45 (220) a	0.458	0.253
HA 13.6	4.38 (160) a	14.58 (240) a	13.89 (180) a	23.18 (220) a	26.25 (160) a	0.189	0.629
HA 13.7	0 (210) a	0 (250) a	1.85 (270) a	1.72 (290) a	12.50 (240) b	0	0
HA 13.9	6.91 (810) a	22.47 (810) b	14.19 (620) ab	22.17 (600) b	49.68 (630) c	0.312	1.012
HA 13.11	9.72 (360) a	7.63 (380) a	21.25 (320) a	46.90 (290) b	94.61 (260) c	0.207	0.163
HA 13.18	4.44 (90) a	3.33 (60) a	1.25 (80) a	5.56 (90) a	5.50 (80) a	0.799	0.599

13.6, HA 13.9 and HA 13.11, while HA 13.4 and HA 13.18 produced more embryos and calli at 25 °C. HA 13.2 showed the highest production at 20 °C.

Low-temperature tolerance of anther donor clones

The degree of tolerance of low temperatures, expressed as Fv2/Fv0, was analysed for the tetraploid group and the group of anther-derived dihaploids originating from clone 199.13 (Table 3). A significant variation was found between the means within each ploidy group. The level of significance was $P < 0.001$ for the tetraploids, while the level of significance for the dihaploids was ($P < 0.05$).

Correlations between temperature-related embryo and callus production and low-temperature tolerance

A significant positive correlation ($P < 0.05$) was found between the temperature-related embryo and callus production of the tetraploids (10 °C/optimal temperature) and the low-temperature tolerance (Fig. 1 a), while no significant correlation was found between 15 °C/optimal temperature and the chilling tolerance (Fig. 1 b). In the latter regression analysis, clone 73 diverged from the group, due to the unexpectedly high temperature-related embryo and callus production quotient.

Significant positive correlations ($P < 0.05$) were found between the low-temperature tolerance of the dihaploids derived from clone 199.13 and both temperature-related embryo and callus production quotients (Fig. 2).

Percentage of pro-embryos in anther culture of clone 73

At 12 °C, 0.07% of the microspores were classified as pro-embryos, while at 22 °C the percentage of pro-embryos was 1.48%. These values were found to be significantly different ($P < 0.05$).

Table 3 Low-temperature tolerance (Fv2/Fv0) of six tetraploids (4X) and 8 dihaploids (HA) derived from anther culture of clone 199.13. The means of each group (4X and HA) were statistically treated with analysis of variance

Clone (4X)	Fv2/Fv0 (4X) (std)	Clone (HA)	Fv2/Fv0 (HA) (std)
199.13	0.376 (0.078)	13.1	0.281 (0.065)
201.5	0.474 (0.167)	13.2	0.303 (0.074)
201.12	0.273 (0.057)	13.4	0.301 (0.071)
73	0.323 (0.035)	13.6	0.322 (0.070)
S12	0.283 (0.056)	13.7	0.227 (0.046)
Elin	0.096 (0.068)	13.9	0.322 (0.106)
		13.11	0.276 (0.039)
		13.18	0.350 (0.091)
	$P < 0.001$ $R^2 = 0.66$		$P < 0.05$ $R^2 = 0.20$

Discussion

The degree of tolerance to low temperatures of the tetraploid clones 201.5, 201.12, S12 and 73 have been recorded in biomass production studies by Kristjansdottir (1991 a, b), while 199.13, 201.5, 201.12, 73 and S12 were included in a two-group study (Andean and European materials), where the low-temperature tolerance of each group was established using chorophyll fluorescence (Kristjansdottir and Merker 1993). In the latter study the Andean group was found to have a significantly higher level of low-temperature tolerance than the European group. In the present study the degree of low-temperature tolerance of the clones 199.13, 201.5, 201.12, 73, S12 and Elin were established individ-

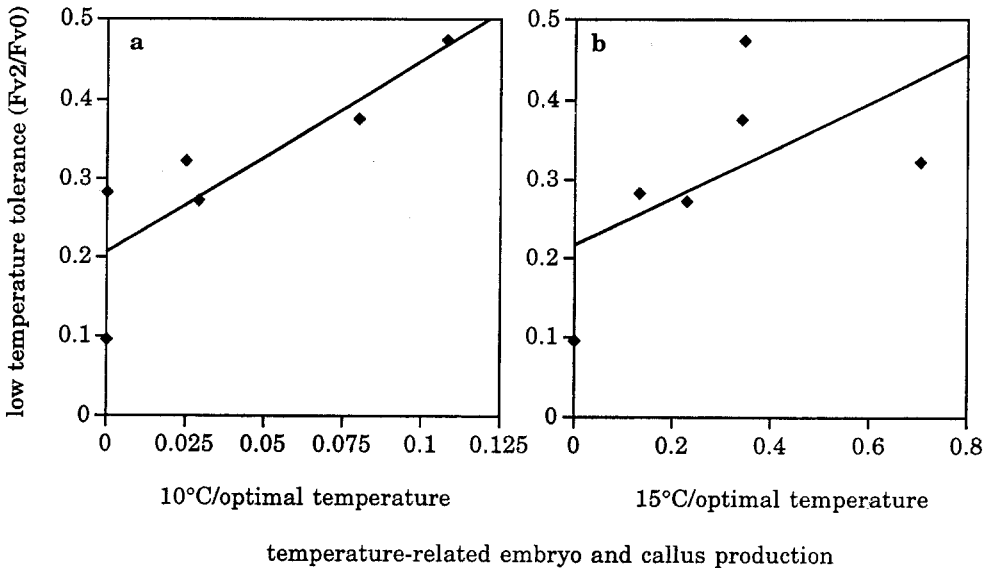

Fig. 1 Correlations according to regression analysis between temperature-related embryo and callus production and low-temperature tolerance (Fv2/Fv0) of six tetraploids. 10 °C/optimal temperature: $P < 0.05$, $R^2 = 0.72$; 15 °C/optimal temperature: $P < 0.24$, $R^2 = 0.33$

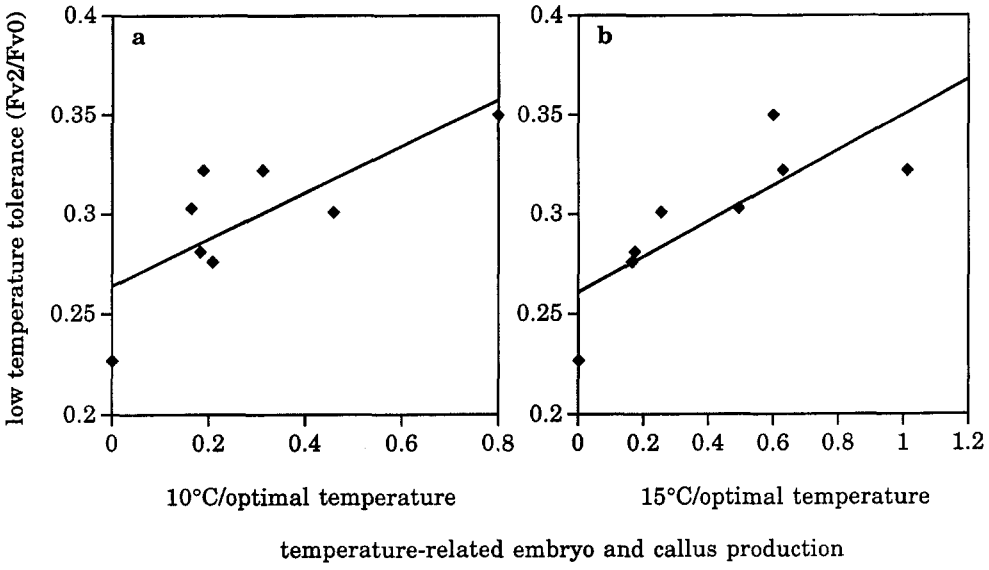

Fig. 2 Correlations according to regression analysis between temperature-related embryo and callus production and low-temperature tolerance (Fv2/Fv0) of eight anther-derived dihaploids from clone 199.13. 10 °C/optimal temperature: $P < 0.05$, $R^2 = 0.59$; 15 °C/optimal temperature: $P < 0.05$, $R^2 = 0.62$

ually with chlorophyll fluorescence and a significant variation between the clones was found, which largely coincided with the observations of Kristjansdottir (1991 a, b) and Kristjansdottir and Merker (1993). The low-temperature tolerance of the dihaploid clones, which was similarly investigated, also revealed a significant variation. A wider variation was found within the tetraploid group compared with the dihaploid, which can be explained by the common origin of the latter group. Generally, the level of tolerance to low temperatures was higher in the tetraploids compared with the dihaploids (Calleberg and Libert, in preparation).

Calleberg and Johansson (1993) demonstrated that different anther donor clones responded differently with respect to embryo and callus production at different incubation temperatures. In the present study additional material, as well as analyses of the correlation between the "temperature-related embryo and callus production" and the low-temperature tolerance of the donor clone, were included. These analyses revealed significant positive correlations for the tetraploids as well as for the dihaploid group. This implies that the embryo and callus production of microspores from clones highly tolerant to low temperatures are, on average, higher at the lower incubation temperatures (10 and 15 °C) compared with clones less tolerant to low temperatures. These results confirm the findings of previous investigations where a correlation between low-temperature tolerance in the sporophyte and in normally developed pollen was shown (Zamir et al. 1981, 1982; Zamir and Vallejos 1983; Kristjansdottir 1990).

Several investigations have demonstrated an extensive overlap of gametophytic and sporophytic gene expression (Tanksley et al. 1981; Rajora and Zsuffa 1986; Sari-Gorla et al. 1986; Pedersen et al. 1987). It is likely that particular characters closely connected with the general metabolism of the tissue, such as tolerance to low temperatures, may be regulated by the same genes in both gametophyte and sporophyte, thus making selection in anther culture feasible. It is, however, possible that the donor or mother plant induces a tolerance to low temperatures in the gametophyte that is not due to the nuclear or cytoplasmic genes of the latter. The main argument against this hypothesis is that cell division and development in the gametophyte is most likely governed by its own gene expression, since an extensive expression is in fact present in the pollen (for a review see Mascarenhas 1989).

The only way to prove that selection at the haploid (or for the potato at the dihaploid) level has genetic effects is to study the progeny obtained. Using this approach Zamir and Gadish (1987), Kovacs and Barnabas (1992) and Lyakh and Soroka (1993) all demonstrated that in vivo pollen selection for low-temperature tolerance was possibe.

The temperature-related embryo and callus production (15 °C/optimal temperature) for clone 73 deviated significantly from the regression line in the correlation analysis (Fig. 1 b). Similarly, Kristjansdottir (1991) noted an unexpectedly high germination rate of pollen from clone 73 at the lower temperature, also contradicting the degree of low-temperature tolerance of clone 73. These results imply that it is not only the expression of tolerance to low temperatures that effects the growth and survival of the pollen at lower temperatures. Other characters, such as vigour, possibly in combination with tolerance to low temperatures, in the microspores as well as the normal pollen, may promote the growth and survival at low temperatures. This has be taken into consideration when designing experiments for in vitro selection.

In the present study the cold stress was applied immediately after the inoculation of anthers containing microspores at the uninuclear stage. The frequencies of pro-embryos (clone 73), as well as of embryos and

calli, decreased with temperatures, which indicates that the sporophytic growth is in fact hampered from the very beginning of the culture period at the lower incubation temperatures. An important factor to consider is that the switch towards a sporophytic developmental pathway has been suggested to be initiated or prohibited by various types of stress (for a review see Powell 1990). According to Custers et al. (1994) the incubation of *Brassica napus* microspore cultures at 17.5 °C instead of 32.5 °C induced a gametophytic rather than a sporophytic development. If this were to be the case also in the present study, then the low embryo and callus production at the lower temperature would be due to a larger number of normally developed pollen grains rather than a decreased sporophytic growth caused by a lower tolerance to low temperatures in the immature microspores. To exclude such factors, microspores from clone 73, incubated at 12 °C for 90 days and at 22 °C for 60 days, were screened for the presence of starch-accumulating pollen and pollen tubes. No differences were found between the two (data not shown).

In this study the optimal temperatures for embryo and callus production for various clones of potato were defined to be either 20 or 25 °C. Even though 30 °C in several cases was superior for embryo and callus production, Calleberg and Johansson (1993) showed that embryos and calli produced at this temperature could not regenerate directly from embryos. It was suggested that this was caused either by a pre-ageing of the embryos or by the embryos originating from secondary embryogenesis. Consequently, when defining the optimal temperature, the temperatures producing the highest frequencies of regenerable embryos and calli were chosen. Presuming that the vast amount of embryos produced in anther culture, by some clones, at 30 °C is not caused by secondary embryogenesis, it would be interesting to investigate whether correlations, similar to this study, could be found for heat tolerance.

In conclusion, this study provides strong indications that there is a correlation between the gene expression for low-temperature tolerance of the sporophyte (anther donor) and the gene expression for tolerance to low temperatures in the immature microspore developing sporophytically via anther culture. It is suggested that the embryos and calli produced at a low temperature are the result of the embryogenesis of immature microspores expressing genes for low-temperature tolerance, which would make in vitro selection for increased tolerance to low temperatures through anther culture of potato possible.

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