EMBRYOGENESIS FROM ISOLATED MICROSPORES OF TULIP; TOWARDS DEVELOPING F_1 HYBRID VARIETIES

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Abstract

This report describes advances in the technique of embryogenesis from tulip microspores in culture. High temperature pretreatment (32°C) of bulbs, which contain fully developed inflorescences, had a positive effect on the production of microspore embryos. The pretreatment shortened the time for embryo formation and stimulated the progression from the multicellular microspore stage into the embryo proper. Early unicellular microspores were found to be better starting material for culture than mid unicellular microspores. The technique of microspore embryogenesis was applicable to various tulip cultivars. The improved protocol now allows the production of large numbers of microspore-derived plants, and the further evaluation of doubled haploid plant technology for tulip.

1. Introduction

Cultivated tulip (*Tulipa gesneriana* L.) is a vegetatively propagated, heterozygous plant species with a very long juvenile phase of 4-5 years. These factors are hampering a fast and efficient genetic improvement of the crop by crossings. Due to the high degree of heterozygosity the understanding of the genetic basis of important traits in tulip is very limited. We know almost nothing about what type of genes, dominant or recessive ones, are controlling important traits such as forcing ability, flower quality or resistances against diseases and pests. We even do not have an estimate for how many loci are involved in these traits. As a consequence, breeding by hybridization in tulip is a relatively random process, of which the outcome is only partly predictable.

For a number of crops, regeneration of plants from gametophytic cells was shown to be of great value for breeding and the study of genetics (Morrison *et al.*, 1991; Ferrie *et al.*, 1995). The plants obtained are derived directly from a segregating population of haploid gametes, in which each gene is represented once, and as a consequence a large enough group of such plants will exhibit the entire genetic variation of the donor plant. Recessive genes are no longer masked by dominant ones. Duplication of the single set of chromosomes will result in doubled haploid plants, which are completely homozygous

and thus equivalent to stable inbred lines. Doubled haploid plants are ideal parents for the production of F_1 hybrid varieties.

The ability to obtain doubled haploid plants in a single generation would be of great benefit to tulip breeding. It would enable fast and efficient genetic analysis of important traits in tulip and might lead to an alternative way of new variety production, *viz.* establishment of F_1 hybrid varieties. Therefore, we started a programme in tulip aimed at the development of doubled haploid plants from isolated microspores in culture, in analogy to successful microspore cultures established for many other crops (Ferrie *et al.*, 1995). First results, including a protocol for the induction of embryogenesis in tulip microspores, have been published previously (Van den Bulk *et al.*, 1994). In the meantime, a batch of microspore-derived plantlets from cv. Rosario have been successfully transferred into soil (unpublished results). This report focusses on improvements of the published protocol for tulip microspore culture (Van den Bulk *et al.*, 1994).

2. Materials and methods

2.1 Plant material

The experiments were carried out with *Tulipa gesneriana* L. cultivars 'Aladdin', 'Bartigon', 'Cassini', 'Gander', 'Leen van der Mark', 'Lucky Strike', 'Lustige Witwe', 'Monte Carlo', 'Rosario', and *T. fosteriana* Hoog cultivars 'Madame Lefeber' and 'Princeps'. Bulbs which had completed inflorescence development, were obtained from commercial growers in October and stored dry at 17°C until the start of the experiments in November and December.

2.2 Isolation and culture of the microspores

Isolation of the microspores from the anthers and their culture *in vitro* were performed as described earlier (Van den Bulk *et al.*, 1994), with some minor modifications: (i) the microspores were released from anther fragments in liquid medium by gently pressing with the back side of a scalpel instead of using a 'flask roller', (ii) embryos and embryo-like structures were transferred to solid medium when they had a size of 0.3-0.5 mm, and (iii) these embryos were put directly on top of the solid medium without a layer of filter paper in between.

2.3 Experiments

The following experiments were carried out:

(i) High temperature (32°C) pretreatment was applied to bulbs of 'Leen van der Mark' and 'Rosario' for various periods of time (2-15 days) in the beginning of December. Upon isolation of the microspores, their stage of development was analysed by staining with the DNA-specific fluorochrome DAPI (4',6-diamidino-2-phenylindole 1.25 µg/ml, according to Custers *et al.* (1994)). The fluorescence intensity of the nucleus and the degree of condensation of the DNA were determined, and cells without visible nucleus or with strongly decondensed DNA were judged as dead. Microspore cultures were carried

out after the various high temperature stress treatments, according to the standard protocol. Per treatment separate microspore populations from 2-4 bulbs were studied.

(ii) Early unicellular and mid unicellular microspores from 'Rosario' bulbs were compared for their ability to regenerate embryos in culture. The early unicellular microspores were isolated mid November and the mid unicellular ones in the beginning of December.

(iii) Nine cultivars were studied for their capacity of microspore embryogenesis. The microspores were isolated in the middle of December, at least 2 microspore populations (i.e. bulbs) were tested per cultivar.

3. Results

Per bulb approximately 800,000 microspores were isolated. The microspores were in the early to mid unicellular stage of development and were 35-50 μm in size (Fig. 1A). After two weeks in culture, several microspores (1-6% depending on the cultivar) were visible with more than two nuclei, which indicated that the normal pathway of gametophytic development had been changed in a sporophytic pathway of development. Continued divisions led to multicellular microspores (±100 μm, with 10-30 nuclei; Fig. 1B) and subsequently globular multicellular structures (±150 μm, with 50-100 nuclei). From week 4 in culture onwards, the globular multicellular structures developed into embryos and embryo-like structures of 0.3-1 mm in size (Fig. 1C). Also callus-like tissues were formed, some of them being very compact and with capacity to regenerate clusters of embryos at a later time in culture (Fig. 1D).

3.1 High temperature pretreatment of bulbs

The high temperature pretreatment of the bulbs strongly affected the vitality of the microspores (Table 1). The frequency of normal looking microspores with a condensed and brightly staining nucleus in the DAPI test, strongly decreased in time. Instead, shrivelled microspores that did not longer show a clear nucleus were found. 'Rosario' appeared more sensitive than 'Leen van der Mark' for damage of the microspores. The second effect of high temperature was on the formation of embryos and embryo-like structures (Table 1). Four weeks after isolation, embryos were produced in the cultures from the 4 and 7 days 32°C pretreated 'Leen van der Mark' bulbs and the 7 days 32°C pretreated 'Rosario' bulbs. Shorter and longer periods of high temperature did not yield embryos. A higher percentage of 'Leen van der Mark' bulbs was responsive than of 'Rosario' bulbs. Following the formation of embryos after four weeks of culture, a second flight of embryos appeared after nine to fourteen weeks of culture (Table 1). With 'Rosario', large numbers of embryos were found in the control and in the 2, 4 and 7 days 32°C pretreatments. No influence of the temperature pretreatment was detectable in this second flight of embryos. 'Leen van der Mark' yielded almost no embryos in the second flight. Notably, the control treatment of this cultivar showed the presence of high numbers of multicellular microspores (50-100 per bulb), which apparently failed to develop into embryos.

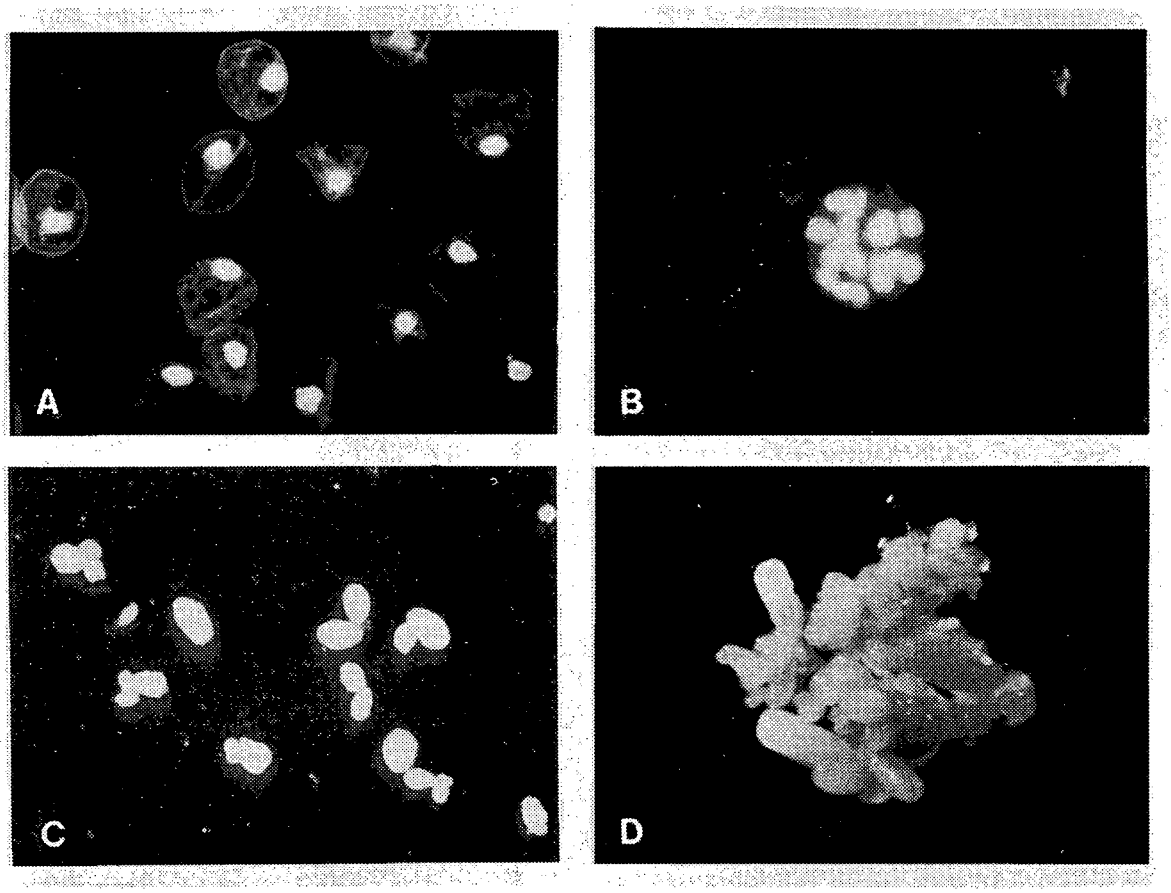

Fig. 1. Stages of development during tulip microspore embryogenesis. In A and B, nuclei have been stained with the DNA stain DAPI. (A) Freshly isolated microspores in the mid unicellular stage (diameter 40-50 μm). (B) Multicellular microspore with 18 nuclei after three weeks of culture (diameter 100 μm). (C) Embryos and embryo-like structures of 0.3-1 mm in size after six weeks of culture. (D) Cluster of embryos, 0.3-2.3 mm long, developed from an embryogenic callus.

3.2 Early unicellular and mid unicellular microspores

In the cultures of 'Rosario' that had been started with early unicellular microspores, a first flight of embryos and embryo-like structures was obtained after four weeks of culture. This early embryo formation was not observed in the cultures starting with mid unicellular microspores (Table 2). The second flight of embryo formation, after nine to fourteen weeks of culture, was much larger when starting with the early unicellular microspores than with the mid unicellular ones (Table 2).

Table 1: Effect of 32°C pretreatment of the bulbs on formation of embryos (ES) and embryo-like structures (ELS) in isolated microspore cultures of 'Leen van der Mark' and 'Rosario'

Cultivar	32°C pre-treatment (days)	Number of bulbs	Percentage viable microspores (%)[a]	Numbers of ES and ELS formed per bulb (percentage responsive bulbs)	
				after 4 weeks	after 9-14 weeks
Leen van der Mark	0	2	85	0	0[b]
	4	1	60	8 (100)	2 (100)
	7	2	45	56 (100)	0
	15	2	5	0	0
Rosario	0	2	75	0	123 (100)
	2	4	75	0	102 (100)
	4	2	35	0	66 (100)
	7	4	25	4 (25)	82 (100)
	15	2	0	0	0

[a] Microspores with a clearly visible, condensed nucleus. ; [b] Numerous (50-100) multicellular microspores, 80-100 µm, were present in culture.

Table 2: Embryos (ES) and embryo-like structures (ELS) produced in 'Rosario' microspore cultures that started with early or mid unicellular microspores

Stage of development of the microspores	Number of bulbs	Percentage viable microspores (%)[a]	Numbers of ES and ELS formed per bulb (percentage responsive bulbs)	
			after 4 weeks	after 9-14 weeks
Early unicellular	5	75	3 (40)	214 (100)
Mid unicellular	2	75	0	123 (100)

[a] Microspores with a clearly visible, condensed nucleus.

3.3 Cultivar specificity

Apart from 'Leen van der Mark' and 'Rosario', nine other cultivars were tested for microspore embryogenesis ability (Table 3). Upon isolation, the microspores were in the mid unicellular stage of development, except for 'Lucky Strike' and 'Princeps' with early unicellular microspores, and 'Gander' with microspores that showed already transition to the late unicellular stage. Embryos and embryo-like structures ≥0.3 mm appeared from week eight of culture onwards in four cultivars, 'Aladdin', 'Bartigon', 'Lucky Strike', and 'Monte Carlo', with on average 8-62 embryos or embryo-like structures per bulb. This development was similar to that in 'Rosario' (Table 1), but the mean embryo yield was lower. 'Cassini', 'Lustige Witwe', 'Madame Lefeber', and 'Princeps' had started sporophytic development, clearly shown by the presence of high numbers (50-500) of multicellular microspores, but these did not show progression to real embryo development (Table 3). This was a similar reaction as with microspores from 'Leen van der Mark' bulbs that were not pretreated with high temperature (Table 1). The cultivar 'Gander' did not show sporophytic development of the microspores.

Table 3: Embryogenic response of various tulip cultivars in microspore culture: +, development of embryos and embryo-like structures; ±, sporophytic development upto the multicellular microspore stage; -, absence of sporophytic development. Data of 'Leen van der Mark' and 'Rosario', from an earlier experimemt (Table 1), are added for comparison

Cultivar	Ability of embryogenesis
Aladdin	+
Bartigon	+
Cassini	±
Gander	-
Lucky Strike	+
Lustige Witwe	±
Madame Lefeber	±
Monte Carlo	+
Princeps	±
Leen van der Mark	±
Rosario	+

4. Discussion

The results of this study show that our earlier protocol for tulip microspore embryogenesis (Van den Bulk *et al.*, 1994) is fully reproducible, and that it is applicable to various cultivars of tulip. It is also demonstrated that the protocol may be further improved. The majority of the 11 cultivars studied showed sporophytic development up to the multicellular microspore stage. In more than 50% of the cultivars embryos and embryo-like structures were obtained. Important new findings were, that high temperature pretreatment of the bulbs, as well as use of early unicellular microspores as starting material had a positive effect on the embryo production.

High temperature treatment is generally accepted as a stress that might induce embryogenesis in microspores (Chuong and Beversdorf, 1985; Custers *et al.*, 1994; Heberle-Bors *et al.*, 1996), and strong correlative evidence suggests that the heat shock response and specific heat shock proteins (HSPs) may be involved in controlling the switch from gametophytic into sporophytic development of microspores (Zarsky *et al.*, 1995; Cordewener *et al.*, 1995). For tulip microspore culture, we postulated previously that the 25°C culture temperature, accompanied by the *in vitro* culture conditions, would be a form of stress that triggers the initiation of sporophytic divisions in the microspores (Van den Bulk *et al.*, 1994). In the present research, however, it was found that high temperature (32°C) pretreatment of the bulbs improved the results of microspore embryogenesis in tulip. Whether this high temperature treatment is optimal for embryogenesis induction in tulip has not been determined, and requires further experiments.

The high temperature pretreatment (32°) had a dual effect, (i) it reduced the time for development of embryos large enough for subculturing from 8-12 weeks of culture, as earlier reported (Van den Bulk *et al.*, 1994), to four weeks of culture, and (ii) it induced

the formation of embryos and embryo-like structures in 'Leen van der Mark' in starting material that was otherwise unresponsive. Independent from the heat pretreatment a second flight of embryos occurred in 'Rosario'. 'Leen van der Mark' microspores from control bulbs, that had not received the high temperature treatment, developed in culture sporophytically upto the multicellular microspore stage, but then failed to proceed into embryo formation. Apparently, microspore embryogenesis entails two critical developmental steps: (i) the initial switch from gametophytic into sporophytic development, and (ii) the transition from multicellular microspore to embryo proper. It therefore seems that the high temperature triggers in the 'Leen van der Mark' microspores mainly the second step, i.e., progression to the embryo proper. In our earlier work (Van den Bulk *et al.*, 1994), where we focussed on the initiation of microspore embryogenesis, we also used 'Leen van der Mark'. There too, high frequencies of multicellular structures were encountered, which produced only a few embryos. The experiment performed here used only two bulbs in the control, which is too low a number to expect embryos.

The developmental stage of the microspores at the start of culture has a strong impact on the success of induction of embryogenesis (Ferrie *et al.*, 1995). The most responsive stage varies between species, ranging from the mid-late unicellular microspore stage in i.e., barley (Hoekstra *et al.*, 1992), to the early-mid bicellular pollen stage, in tobacco (Kyo and Harada, 1986). We found for tulip that the early unicellular microspore stage is the most responsive for induction of embryogenesis. Compared to other plant species, this is an exceptionally young stage. It is quite similar, however, with our earlier finding with microspores from -2°C stored bulbs, that the early-mid unicellular microspore stage was the best starting material (Van den Bulk *et al.*, 1994).

In the cultivar experiment, 4 cultivars behaved similarly as 'Rosario' and embryos were produced from the microspores in culture. Another group of four cultivars ceased sporophytic development after having reached the multicellular microspore stage. This is a similar reaction as with microspores from 'Leen van der Mark' bulbs that were not pretreated with high temperature. Based on this similarity with 'Leen van der Mark', we might expect that high temperature pretreatment might also trigger progression to real embryo development in 'Cassini', 'Lustige Witwe', 'Madame Lefeber', and 'Princeps'. 'Gander' was the only cultivar which was not responsive at all. Because mid-late unicellular stage microspores were used as starting material, younger starting material may improve the result of 'Gander' microspore culture.

This report describes important advances in establishing doubled haploid plant technology for tulip. The technique appears to be applicable to a wide range of tulip cultivars, and we can now produce ample material from various genotypes and accessions. In the near-future (5-15 years), this will allow a thorough genetic analysis of important multiple (recessive) genetic traits in tulip. For the more distant future (20-30 years), it might be possible to hybridize selected homozygous lines, each with genetically well defined characteristics, and to produce sophisticated F_1 hybrid varieties. A prerequisite for this alternative way of production of new cultivars is that the doubled haploid plants do not suffer too much from sterility as caused by inbreeding depression.

All our efforts are now directed to producing high numbers of microspore-derived plants, in order to maximize our chances of finding fertile doubled haploids.

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