Dedifferentiating initiation and embryogenesis from freshly-isolated microspores of barley*

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Abstract By using DNA-specific fluorescent dye and a confocal laser scanning microscope, the present study was designed to investigate the cytological characteristics of dedifferentiating initiation during pretreatment and embryogenesis during culture in freshly-isolated microspores of barley, and the difference in main developmental pathway between freshly-isolated and cold-treated microspores. The results revealed that (ⅰ) freshly-isolated microspores started the initiation within 12 h of mannitol pretreatment, whose main cytological characteristics were that: cell volume was obviously extended; the volume of nuclei and nucleoli were also greatly increased; nucleoli were extremely clear and highly condensed; N/C ratio was very high; (ⅱ) all the pretreatment methods led to the initiation of the microspores, thus triggering the embryogenic process; (ⅲ) pretreatment methods influenced the main developmental pathway of microspores by changing the pattern of the first mitosis. The cold-treated microspores formed main developmental pathway via A patterns, but freshly-isolated microspores via B pattern.

Keywords: barley, freshly-isolated, microspore culture, dedifferentiating initiation, embryogenesis

In vivo, microspores grow into mature pollen grains, but *in vitro* culture they develop into embryoids. This redirection process, namely dedifferentiating initiation, or the induction of embryogenesis, was said to be the key and prerequisite for microspore embryogenesis. If the microspores were not initiated, embryogenesis would not be produced. Once the initiation is induced, no matter in what phase the microspore differentiation is at this time, the differentiation will be reversed to embryonic state: first developing into embryoids, then redifferentiating into plantlets. Essentially, dedifferentiating initiation is the process of making living clock to reverse[1]. G_1 phase in cell cycle plays a key role in differentiation and dedifferentiation. As this phase, the microspores are most sensitive to external inductive conditions. Therefore, most probably pretreatment induces the initiation of the microspores[2]. The regeneration system of freshly-isolated microspore culture at high induction frequency established in barley provides an ideal experimental system for direct observation and study of embryogenesis, especially dedifferentiating initiation of the microspores free of the interference of anther wall. A confocal laser scanning microscope (confocal microscope) offers greater resolution than a conventional microscope, reduces interference from out-of-focus fluorescence and operates accurate non-invasive optical sectioning; therefore, it is so well suited to the imaging and three-dimensional tomography of stained biological specimens[3].

In this study, by using DNA-specific fluorescent dye PI and a confocal microscope, we report

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the observations on cytological characteristics of dedifferentiating initiation prior to the first mitosis and embryogenesis in freshly-isolated microspore culture, and comparative results of the difference in main developmental pathway between freshly-isolated and cold-treated microspores.

1 Materials and methods

Hordeum vulgare cv Igri grown in open field was as the donor plants. The spikes were selected and harvested when most of microspores were at the mid-late uninucleate stage. The excised spikes were sterilized with 10% sodium hypochlorite (v/v) for 10 min followed by 3—4 times of washing with sterile water. Approximately 10—15 spikes were placed in a microblender containing 20—30 mL cool (refrigerated) 0.3 mol/L mannitol for 30 s at 20 000 rpm. The blended slurry was filtered through a 100-μm mesh, and then centrifuged at 50 g for 5 min. The microspores were adjusted to the density of $(1.0—1.2)\times10^5$ per mL in 0.3 mol/L mannitol. 1 mL microspore suspension was placed in petri dishes (30×15 mm), and incubated in the dark at 25℃ for 3 d. Subsequently, microspore suspension was centrifuged to remove mannitol solution, and cultured in 1 mL of MN_6 medium[4] supplemented with 75 000 mg/L maltose, then incubated in the dark at 25℃. Mannitol solution and medium were filter-sterilized. Some spikes were cold-treated in a refrigerator at 4℃ for 21—24 d. Microspore isolation and culture were done as described above. During pretreatment and culture, the microspores were collected by centrifugation and fixed with carnoy every 12 h, and mixed well with fluorescent dye PI (Propidium iodide) diluted to 1—1.25 μg/mL in PBS. The mixture was stored at room temperature for 15 min, observed and photographed in a BioRad MRC-600 confocal microscope.

2 Results

2.1 Dedifferentiating initiation of microspores during pretreatment

Usually most of the microspores freshly isolated from the spikes were at the mid-late uninucleate stage. The nuclei of the microspores at this time were generally elliptic-shaped and stuck close to cell wall. After 12 h of mannitol pretreatment the nuclei changed obviously: the nuclei were apparently enlarged and gradually moved to the center of cells. The nucleus-cytoplasm ratio (N/C ratio) was increased, and the staining degree of the nuclei was also greatly enhanced, indicating that the nuclei were undergoing intense DNA synthesis. Meanwhile, the chromatin in the nuclei of a great number of microspores was condensed, and the nucleoli were very clear. These phenomena often occurred within 1 or 2 d of pretreatment. On the third day, before the end of pretreatment, the microspore would be transferred to induction medium. The cell volume of quite a part of the microspores was obviously extended, the nuclei and nucleoli were also correspondingly increased, especially nucleoli; the changes were extremely clear. These phenomena showed that these microspores, making up 30%—40% of whole population, had basically completed DNA duplication, and were at S—G_2 phase, near the first mitosis, of which the chromatin of little part of the microspores was condensed, and the chromosomes were formed, showing that these microspores had entered into the prophase. These observations revealed that *in vitro* these microspores had deviated from developmental pattern *in vivo* to embryogenic pattern *in vitro*, in other words, they were initiated.

Similar phenomena were also observed on the microspores in the anthers pretreated in manni-

tol. Whereas on the 2nd day the chromosomes were visible in the nuclei of a few microspores, on the 3rd day a lot of the microspores with 2 cells (nuclei) appeared. These showed that, compared with freshly-isolated microspores, the microspores in the pretreated anthers, initiated more rapidly, and thus their development advanced. We observed that, quite a part of the microspores isolated from the cold-treated spikes had completed the first mitosis, forming one vegetative and one generative cell. Generally the vegetative cell was near or had entered into next division (Plate Ⅰ-1—6). The above-mentioned observations indicated that all the pretreatment methods led to the initiation of the microspores *in vitro*, thus beginning the embryogenic process. From the cell cycle point of view, the dedifferentiating initiation of microspores, had apparently been completed prior to the first division, namely within interphase. It was most likely that the switch point was at G_1 phase.

2.2 Embryogenesis of initiated microspores

After the microspores were transferred to induction medium, they began the embryogenic process rapidly. The majority of initiated microspores divided once or twice within 1—2 d. In the first mitosis, the frequency via equal division was higher. This produced two daughter cells similar in size and appearance. A part of the microspores divided still via gametophytic pattern (unequal division), forming one vegetative and one generative. Following that, vegetative cell repeatedly divided, but generative cell was gradually degenerated; or vegetative cell continued to divide after generative cell divided once and ceased. These microspores, usually with big vegetative cell, thin wall, rich cytoplasm and no vacuole, looked like the meristem cells. In a minority of the microspores, vegetative and generative cell divided independently, forming two quite different types of the cells, but the daughter cells resembled their mother cells. Multicellular (nuclei) pollen grains (MPGs) could be formed via various developmental pathways within a few days. Embryoids varying in size and appearance were produced after the cells in MPGs burst through the exine. We found by a confocal microscope that embryoids were essentially a kind of the aggregate loosely arranged by a lot of various undifferentiated cells. It is likely that embryoids contained both a lot of vacuolizated cells analogous to parenchyma and a high proportion of meristem-like cells. During the embryogenic process, we observed that the nuclei in the microspores unable to be initiated, began to degenerate. Some of them were disintegrated, leaving the empty cells. After dividing once or twice, some initiated microspores ceased and were gradually degenerated. And more of the microspores failed to become the embryoids after they formed into MPGs (Plate Ⅱ-1—10). These results demonstrated that, during the embryogenic process, most of initiated microspores could not sustain from the formation of MPGs to embryoids. Similar results were obtained in maize anther culture by Pace *et al.* (1987)[5], and Pescitelli and Petolino (1988)[6]. They held that low survivability may be a major limitation to overall culture response.

2.3 Influence of pretreatment methods on the developmental pathway of isolated microspores

Our observations revealed that freshly-isolated microspores differ from those isolated from cold-treated spikes in main developmental pathways. Table 1 shows that in freshly-isolated microspore culture, 61.4% of MPGs were developed via B pattern (equal division), and only 38.6% via two types of A patterns (A-V, A-VG). But on the contrary, in cold-treated microspore culture, 63.6% of MPGs were developed via A patterns, and only 36.4% via B pattern. For

both pretreatment methods, the frequencies via A-V were higher in A patterns. These results suggested that during cold pretreatment, the microspores in excised spikes developed still via the pattern *in vivo*. As a result, a great part of the microspores underwent the first division, forming one vegetative and one generative cell. But the vegetative cell at this time was quite different from the counterpart developing *in vivo*: its DNA synthesis in many microspores had been completed, thus forming main developmental pathway via A patterns. But freshly-isolated microspores, without interference of the somatic tissues like anther wall, formed main developmental pathway via abnormal B pattern.

Table 1 Influence of pretreatment on the developmental pathway of isolated microspores

Treatment	No. of microspores observed	Developmental pathways % (No.)		
		A-V	A-VG	B
Cold pretreatment	228	42.1(96)	21.5(49)	36.4(83)
Freshly-isolated	197	22.9(45)	15.7(31)	61.4(121)

3 Discussion

Studies on anther and microspore culture showed that "*in vitro*" was indispensable to the induction of microspore embryogenesis, but only this fact was not enough for it. The microspores *in vitro* had to alter from the gametophytic to sporophytic development, namely through external stimuli, inducing the initiation of the microspores. Without numbers of initiated microspore, it was impossible to undergo embryogenic process. During pretreatment, or the inductive phase of embryogenic process, three important events took place: (ⅰ) pollen development was apparently inhibited; (ⅱ) microspores became committed to the embryogenic process, or initiated; (ⅲ) commitment to the embryogenic process and its sustainment became irreversible and independent of the pretreatment process[7]. Pretreatment has been essentially proved to be of certain stress treatment, but the type of the stress treatment can vary in species, even in the same species. For example, in barley[8] and tobacco[9], it represents the sugar starvation, and in *Brassica*[7,10], it exhibits proper high temperature (32℃). Even though the types of the stress treatment can vary, they all serve as external stimuli to trigger the embryogenic process[11].

The characteristics of initiated microspores in cytology, cytochemistry and ultrastructure have not been concluded yet, but the DNA synthesis undergone in the nuclei is undoubtedly one of the important conditions for the deviation of microspores from gametophytic to sporophytic development[2,12]. After the pretreatment of freshly-isolated microspores of barley, the volume of quite a number of microspores (30%—40%) was obviously extended, and the sizes of the nuclei and nucleoli were also significantly enlarged. N/C ratio was very high. Nucleoli were extremely clear and highly condensed. These seem to be regarded as the cytological characteristics of initiated microspores. Considering that rRNA components are transcribed in the nucleoli, and the size of the nucleoli is also relevant to the intense degree of protein synthesis in the cells, the presence of the nucleoli indicates the start of transcription and synthesis after DNA duplication. More investigations are required to make clear whether it has relevance to new cytoplasmic synthesis associated with sporophytic development after the reorganization of the cytoplasm in initiated microspores.

The pretreatment methods were shown to influence the main developmental pathway of microspores by changing the pattern of the first mitosis. Sunderland[13] held that cold pretreatment was conducive to the developmental change of the pollens, but this change did not mean to lead to equal division. In maize[6] and rice[14], the main developmental pathway via A patterns was observed in cold-treated anther culture. In freshly-isolated microspore culture of Chinese cabbage, high temperature was found to effectively suppress gametophytic pathway and enhance the frequency of symmetric division of uninucleate microspores, reaching 55%[10].

In comparison with conventional microscope, the confocal system that reduces interference of the noise from out-of-focus structures greatly enhances resolution, and offers perfect imaging of observed specimens. Meanwhile, it gives effectively optical section for thick specimens with little degradation in the image quality. Since the images produced by the confocal system are stored in a digital frame, they are extremely convenient for image analysis and processing in a computer. Consequently, the confocal system has been widely used in nuclear structure, *in situ* hybridization, cytoskeleton and three-dimensional tomography[15].

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