Cyto-histological Studies on Callus Formation and Its Regeneration in Anther Culture of *Oryza sativa* L.*

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Abstract : Histological characteristics of globular shaped pollen callus (globules) developed from multicellular pollen grains were examined in rice anther culture (*Oryza sativa* L.). The ultrastructure of pollen grains, anther wall tissues and the globules *in vitro* were also clarified. It was speculated that the microbodies observed in the viable pollen grains and anther wall tissues were associated with the lipid metabolism during the formation of the multicellular pollen grains. Before de-differentiating cells emerged from the pollen wall (exine), mucilage was secreted on the outer-surface of the exine. The presence of multivesicular bodies in the multicellular pollen grains was analyzed in relation to their function in mucilage secretion. Lipid droplets and starch grains were formed in the globules after the emergence. Thereafter, a shoot-root axis appeared in the globules in the anther culture medium. When the meristem of the future root apex appeared at the root pole of the globules, the starch grains and lipid droplets decreased in the meristem. These results suggest that the formation of the globules in the anther culture and plant regeneration *in vitro* were accompanied with dynamic changes in the carbohydrate and lipid metabolism.

Key words : Anther culture, Callus, *Oryza sativa*, Plant regeneration, Rice, Ultrastructure.

イネ葯培養におけるカルス形成と植物体再生に関する細胞・組織学的研究：中野 寛・前田英三（名古屋大学農学部）

要 旨：葯培養条件下の花粉細胞や葯壁組織、および花粉細胞起源カルスの組織構造・細胞内微細構造を調査し、イネ花粉細胞の脱分化に伴う若干の微細構造的特徴を認めた。その一つは、培養条件下で生存している花粉細胞や葯壁組織に認められたマイクロボディである。このマイクロボディは、イネ葯培養での花粉細胞の脱分化過程に伴う脂質代謝に機能している可能性があると考えられた。また、花粉由来の脱分化細胞が花粉外殻を破り外に現われる際、粘液性物質が外殻の表面に分泌された。この粘液性物質の生理的機能は不明であるが、多細胞花粉の中に認められたマルチベシキュラーボディが、この分泌に機能しているものと推察された。

多細胞花粉は増殖を続け球状カルス塊になり、さらにそれはイネカルスからの不定根分化と不定芽分化の両初期過程に現われる2種の組織へと分化し、そして最後には不定根原基も形成された。花粉起源球状カルス塊の形成時に現われたデンプン粒や脂質顆粒は、不定根原基の分化に伴って細胞内から消失することが示された。これらの結果は、葯培養条件下でのイネの花粉細胞の脱分化と植物体再分化の過程において、炭水化物代謝や脂質代謝の変動が関与していることを示唆していると考えられた。

キーワード：イネ、カルス、植物体再分化、微細構造、葯培養。

Several ultrastructural studies have already been carried out on the initial cell divisions of embryogenic pollen grains in the anther culture of *Nicotiana tabacum*^{4,5,6,16,19,25,26}, *Datura innoxia*^{7,8,9}, *Hordeum vulgare*² and *Hyoscyamus niger*^{17,18}. However, very few ultrastructural observations on multicellular pollen grains and somatic embryos during or just after the emergence from the exine in the anther culture of these plants and in other plant species have been reported¹⁹. In our previous study, we

described the ultrastructure of the rice pollen cells at the time of inoculation into the anther culture medium¹⁵. The current study presents the ultrastructure of multicellular pollen grains before the emergence of de-differentiating cells through the exine and globular shaped pollen callus (globules) after the emergence. This paper also describes the histological features of the globules.

Materials and Methods

Anthers of rice plant (*Oryza sativa* L. cultivar IARI 5995) were cultured as previously described¹⁵. Cultured anthers were sampled at 23 days after inoculation for SEM observation and they were harvested from 2 to 4

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weeks after inoculation for histological and TEM observations and for subculture into the plant-regenerating medium. The plant-regenerating medium was the same as the anther culture medium except that auxins were omitted. On this medium, the globules, whose diameter was less than about 0.5 mm, were transplanted and maintained at 25°C under a continuous illumination of about 3,000 lux.

For SEM observation, the sampled anthers were fixed in 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 at 4°C overnight. After dehydration through an ethanol series and isoamyl acetate, they were dried in a critical point dryer Hitachi HCP-1. Dried materials were coated with a thin layer of gold by the ion coater Eiko IB-3 and examined in a scanning electron microscope Hitachi SSM-2.

For histological observation, the harvested anthers were embedded in 2% agar in order to avoid the loss of pollen grains and the globules during their passage through the dehydration series. Then, they were fixed in FAA and embedded in paraplast through a tertiary butanol series. Sections were stained with Delafield's haematoxylin.

For TEM preparation, anthers were preliminarily fixed in 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 at 4°C for about half an hour. They were also embedded in 2% agar and further fixed in the same glutaraldehyde solution for 4 hours. After prefixation with glutaraldehyde, the samples were processed and examined, as previously described¹⁵⁾.

Results

Ultrastructure of the surface of pollen grains in vitro

At inoculation, microspores at the mid-uninucleate and late-uninucleate stages were compactly packed and aligned against the tapetum in the locule of the anther (Fig. 1A). Their surface ornamentation was granular as that of mature pollen grains. Many Ubisch bodies had also been formed on the surface of the tapetal cells.

Globules (G) had begun to appear in the cultured anthers 2 weeks after inoculation (Fig. 1B). Exine of some of the pollen grains was occasionally covered with a secreted material in some areas (Fig. 1C). It was often cracked by the irradiation of the electron beam for a few minutes. In some of the pollen grains, the exine was torn and a mass of de-differentiating cells emerged through the broken exine (Fig. 1D). The exine of these pollen grains was also covered with the secreted material in scattered areas. There were pollen grains on the surface of the exine where this material accumulated (Fig. 1E). This material may correspond to a mucilaginous secretion. Multicellular pollen grains adhering to dead pollen grains by the mucilage were frequently observed by light microscopy (Fig. 2A). On the other hand, the exine of the necrotic pollen grains on browning anthers underwent degenerative changes (Fig. 1F).

Histology of the globules

When anthers were left on the original anther culture medium, small globules grew into a callus mass. However, when the anthers were subcultured to the plant-regenerating medium, frequently the globule developed into an adventitious shoot and a plantlet with a coleoptile-like leaf (C) (Fig. 2B).

Small globules on the anther culture medium were compared histologically with zygotic embryos of the rice plant whose embryogenesis had been studied in detail²¹⁾. Their histology was different from that of the zygotic embryo. They were composed of randomly arranged meristematic cells in the early period after their emergence from the pollen wall (Fig. 2C, two small globules). However, as they grew, they gradually differentiated into tissues with two types of organization. For example, as the peripheral meristem (PM) shown in Fig. 2C, meristematic cells were randomly arranged in the lower half of the largest globule, which faced the anther wall. This tissue corresponded to that which had been observed in our previous histological studies on rice seed callus. It appeared at the early stage of callus development in the course

Abbreviations for Fig. 1 to 4 : C ; Coleoptile-like leaf, E ; Exine, G ; Globular pollen callus (globule), L ; Lipid droplet, Mb ; Microbody, Mt ; Mitochondrion, MVB ; Multivesicular body, N ; Nucleus, P ; plastid, Pb ; Protein body, PM ; Peripheral meristem, PP ; Peripheral parenchyma, R ; Root, RT ; Radial tissue, S ; Starch grain, SSM ; Sub-surface meristem, V ; Vacuole.

Fig. 1. SEM micrographs of pollen grains at the time of inoculation and *in vitro* on the anther culture medium. A : Mid-uninucleate microspores at inoculation. $\times 690$, B : Globules and pollen grains (arrow) on an anther cultured *in vitro* which underwent dehiscence. The globules adhere to each other. $\times 70$, C : A pollen grain covered with some patches of a secreted material (arrow). $\times 1,300$, D : Globule with de-differentiating cells emerging through the broken exine. Arrow indicates a patch of the secreted material. $\times 780$, E : Pollen grains heavily covered with the secreted material. Its exine is also torn by the proliferation of de-differentiating cells (arrow). $\times 740$, F : Degeneration of surface granular ornamentation of the exine into the smooth membranous structure in a necrotic pollen grain. $\times 1,300$.

Fig. 2. LM micrographs of multicellular pollen grain, globules and a plantlet regenerated from the globule. The horizontal bars represent $100\ \mu\text{m}$ in Fig. 2A and $0.1\ \text{mm}$ in Fig. 2C and 2D. A : A multicellular pollen grain with its mucilaginous secretion adhering to shrivelled dead pollen grains. $\times 790$, B : Plantlets with a coleoptile-like leaf formed directly from the globule without callus proliferation on the plant-regenerating medium, C : Two small globules on the anther culture medium are composed of randomly arranged meristematic cells. The upper half of the largest globule consists of the radial tissue (RT) in which cells are radially arranged from the

of the root formation on the subculture medium. It had been called the peripheral meristem¹³⁾. In the lower half of the globule as shown in Fig. 2D and 2E, parenchymatous tissue in the peripheral zone and meristematic tissue in the inner zone were observed. The former had been called the peripheral parenchyma (PP) and the latter the sub-surface meristem (SSM) in the previous paper. The latter changed into root apical meristem at the later stage of callus development on the above-mentioned subculture medium. Histological organization of the upper half of the largest globule in Fig. 2C was the same as that which appeared in the rice seed callus transplanted into the shoot-forming medium¹⁴⁾. The earliest histological change on this medium was characterized by the appearance of callus tissue in which cells were arranged in radial arrays from the inner part to the surface of the callus mass. This tissue had been called the radial tissue. The radial tissue (RT) was observed also in the upper half of the globule as shown in Fig. 2D and 2F. These histological characteristics indicated that a shoot-root axis appeared in the globules on the anther culture medium.

Ultrastructure of the pollen grains and globules

In the cultured anthers where the globules occurred, most pollen grains were shrivelled and lost their cytoplasmic contents. However, some pollen grains remained spherical and their cytoplasmic contents were retained. Microbodies (Mb) containing a crystalline material were observed in such viable pollen grains (Fig. 3A and 3B). On the other hand, many small microbodies with a granular matrix were observed in the multicellular pollen grains (Fig. 3C). These pollen grains showed an exine and inter-cellular space where electron-dense material accumulated (Fig. 3D, arrow). This electron-dense material may correspond to the mucilaginous substances which were secreted around the multicellular pollen grains. Furthermore, these multicellular pollen grains contained a large

number of multivesicular bodies (MVB) (Fig. 3D).

The anther wall, on which the globules occurred, consisted of endothecium and epidermis. Its tapetal cells had already disappeared. Microbodies containing a crystalline material and large protein bodies (Pb) were observed in the endothecium and epidermis (Fig. 3E). Numerous mitochondria (Mt) with well-developed cristae were sometimes observed in the endothecium (Fig. 3F). Ubisch bodies were still present at the surface of the endothecium.

The small globules were composed of randomly arranged meristematic cells before the acquisition of a shoot-root axis, although they were occasionally composed of senescent large vacuolated cells. These meristematic cells were rich in cytoplasm (Fig. 4A and 4B). Their nucleus (N) was large and two nucleoli were rarely present. Many mitochondria and only a few microbodies were observed. The density of the rough endoplasmic reticulum was moderate. Each globule contained only one type of plastid (P) among the three types, that is, proplastid, proplastid with prolamellar body and plastid containing starch grains. Occasionally large lipid droplets (L) were also observed (Fig. 4A).

During the differentiation of the shoot-root axis, the ultrastructure of the meristematic cells did not exhibit appreciable changes in both the root pole and shoot pole, that is, the peripheral meristem (Fig. 4C) and the radial tissue (Fig. 4D), except for the enlargement of vacuoles and the reduction in the number of mitochondria. Thus there were no differences in the ultrastructure between the peripheral meristem and the radial tissue. However, when the sub-surface meristem appeared in the inner part of the root pole, the ultrastructure of the cells in this tissue became different from that in the peripheral meristem. The shape of the cells was rectangular and the cell wall was thin, resulting from rapid cell divisions in a unilateral direction. The number

inner part to the surface. In the lower half, cells are randomly arranged into the peripheral meristem (PM). $\times 140$, D: A globule on the anther culture medium. It is divided into two histologically different parts, that is, the lower half which faces the anther wall and the upper half. $\times 84$, E: Enlarged view of the peripheral parenchyma (PP) and the sub-surface meristem (SSM) in the lower half of the globule in Fig. 2D. $\times 200$, F: Enlarged view of the radial tissue in the upper half of the globule in Fig. 2D. $\times 200$.

of mitochondria increased again and the cells contained a larger amount of cytoplasmic matrix (Fig. 4E). In the peripheral parenchyma, the cytoplasm was pushed to the cell wall by a large central vacuole (V) (Fig. 4F). The cells contained a lenticular nucleus in the thin cytoplasm layer. Ribosomes and endoplasmic reticulum were sparse in the cytoplasm and there were plastids containing starch grains and few lipid droplets. Root-hair like cells, which appeared on the peripheral parenchyma, contained numerous Golgi bodies and a well-developed endoplasmic reticulum.

Discussion

Plant regeneration via somatic embryogenesis has been documented for a number of graminaceous species²⁴. Also in the anther culture of the rice plant, the occurrence of an embryoid (androgenic embryo) has been reported in several studies^{1,10}. In dicotyledonous plants, it is very common that the morphogenesis of the embryoid *in vitro* proceeds through a globular, heart-shaped and torpedo-shaped stage which has been described in the zygotic embryo in a seed. In the Gramineae, however, there are no such distinct gross-morphological characteristics in zygotic embryogenesis. Therefore, histological examination is essential in order to identify the embryoid in tissue cultures.

In this study, in order to obtain androgenic embryos we cultured anthers according to the method of Guha-Mukherjee¹⁰ and we obtained globular structures (globules) in the cultured anthers. After transplantation into the plant regenerating medium the globules developed into a plantlet with a coleoptile-like leaf as well as a germinated seed embryo. However, the globules were histologically different from a zygotic embryo, suggesting that they did not follow the morphogenesis and histogenesis of a zygotic embryo in the plant regenerating medium.

However, this study showed that the globules differentiated subsequently into tissues

with two different types of organization which had been already recognized in rice seed callus. The root-like organization could be observed on the subculture medium containing 10^{-5} M 2, 4-D¹³. On the other hand, the shoot-like organization was observed in the rice seed callus which was transplanted on the shoot-forming medium containing 10^{-7} M 2, 4-D and 5×10^{-5} M kinetin¹⁴. In the present investigation, the anther culture medium contained 0.9×10^{-5} M 2, 4-D, 1.1×10^{-5} M IAA and 0.47×10^{-5} M kinetin. The ratio of auxin to cytokinin in this medium was intermediate between that of the subculture medium and the shoot-forming medium. These findings suggested that the ratio of auxin to cytokinin regulated the organization of the callus tissues as well as organ differentiation in the rice plant and other species.

At the time of inoculation, rice microspores on the mid-uninucleate and late-uninucleate stages were vacuolated¹⁵. In this study, the increase in the amount of cytoplasmic matrix and organelles was recognized during the development from the microspores to the globules *in vitro*. It is assumed that such cell activation is associated with the presence of systems supplying energy in the cells. Actually the accumulation of starch grains and lipid droplets were found to be associated with the formation of the globules and the decrease with the subsequent histogenesis of root. Such a phenomenon had been reported in adventitious shoot organogenesis²², somatic embryogenesis^{6,20} and callus formation²⁷. Increase of the number of mitochondria, which are associated with the respiratory system, was also encountered during the histogenesis of root in the lower half of the globules. Although Tsay et al.²³ detected cytochemically a large amount of lipid in the multicellular pollen grains in the rice plant, we did not observe a large amount of lipid droplets. However, large microbodies were found in viable pollen grains and small microbodies in the multicellular pollen grains. It can be assumed that these

Fig. 3. TEM micrographs of pollen grains and anther wall tissues *in vitro* on the anther culture medium. A: A viable pollen grain containing many large microbodies. Electron-dense mucilaginous material accumulates in the exine. $\times 7,900$, B: Enlarged view of crystalline material in microbodies in Fig. 3A. $\times 39,000$, C: Many small microbodies with a granular matrix in a multicellular pollen grain whose exine had already ruptured. $\times 8,000$, D: A

multicellular pollen grain in which de-differentiating cells emerged through the ruptured exine. Many multivesicular bodies are seen in the cytoplasm. The electron-dense mucilage accumulates in the inter-cellular space (arrow), in the exine and on the surface of the pollen grain. $\times 5,000$, E: Nucleus, protein bodies and microbodies with crystalline material in an epidermal cell of the anther wall. $\times 4,000$, F: Numerous mitochondria in a cell of the endothecium of the anther wall. $\times 6,000$.

Fig. 4. TEM micrographs of the globules on the anther culture medium. A and B : Globules with randomly arranged meristematic cells. Many mitochondria are seen. Lipid droplets and proplastid are present in a globule in Fig. 4A. Plastids containing starch grains are present in a globule in Fig. 4B. $\times 6,400$, C : Cells of the peripheral meristem after the acquisition of shoot-root axis. They contain fewer mitochondria than the cells in Fig. 4A and 4B. $\times 5,900$, D : Cells of the radial tissue which are considerably vacuolated. It also contain few mitochondria. Plastids containing starch grains are present. $\times 7,900$, E : Cells of the sub-surface meristem. They do not contain lipid droplets and plastids containing starch grains but contain many mitochondria. $\times 4,800$, F : A vacuolated outermost cell in the peripheral parenchyma. $\times 6,500$.

microbodies had functioned as in the case of glyoxysomes in lipid catabolism.

In the rice plant, Tsay et al.²³⁾ noted that the epidermis and endothecium of cultured anthers accumulated lipid droplets and starch grains and that they transported the nutrients into pollen grains. We observed that these tissues included protein bodies, microbodies containing a crystalline material and many mitochondria. This observation may support the hypothesis that these tissues were a source of nutrients for the pollen grains *in vitro*.

In the anther culture of *D. innoxia* the exine of embryogenic pollen grains disappeared¹⁹⁾. In this study, although the exine degenerated in necrotic pollen grains, it was mechanically broken by cells undergoing de-differentiation which had been confined inside the multicellular pollen grains. Before the emergence of de-differentiating cells from the exine, the mucilage substance had been observed around the multicellular pollen grains. Furthermore, multivesicular bodies were also observed in the multicellular pollen grains. It has been considered that they participated in the intracellular transport system of endocytosis and exocytosis^{11,12)}. It is assumed that these bodies were involved in the mucilage secretion in the rice anther culture. Mucilage secretion was reported also in protoplast culture³⁾ and suspension culture²⁴⁾. Cocking³⁾ speculated that the aggregation of protoplasts by mucilage enhanced their cell-division potential.

In this study, we described the ultrastructure of pollen grains, anther wall tissue and globules *in vitro*. Further studies should be carried out in order to relate the morphological characteristics with physiological functions.

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