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Light Microscopic Observations on Pollen and Anther in Rice (*Oryza sativa* L.)*

II. Stages from early microspore to mature pollen

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Abstracts : Development of pollen cells as well as the fate of tapetal cells were studied by the glycol methacrylate semithin method for light microscopy. Sections including artificially produced images of pollen cells were eliminated and some probale backgrounds of these images were discussed. The spatial close interrelationship between developing pollen and tapetal cells was found to be maintained up to mature pollen. Germ pore was observed mostly pointing toward the tapetal side instead of being constantly appressed tapetum. Degeneration of the tapetum seemed to be related to the development of microspore. Storage process of engorged pollen and easy release of mature pollen were seen to be accompanied by probable water decrease in the anther loculus.
Key words : Development, Germ pore, Light microscopy, Microspore, Mitosis, Pollen, Rice, Tapetum.

イネの花粉と葯の発達に関する光学顕微鏡観察　第2報　小胞子初期から花粉成熟期まで：和田富吉・伊藤辰也・伊藤雅章・武岡洋治（名古屋大学農学部）

要　旨：イネの花粉細胞の発達とタペート細胞の推移をグリコールメタクリル樹脂準超薄切片法により観察した．調整により変形を生じた花粉像を含む切片を除き，このアーティファクト像が生成する背景を考察した．発達しつつある花粉細胞とタペート細胞の間に密接な位置的関係があり，この関係が成熟花粉に至るまで維持されることを認めた．発芽孔はタペート組織に常に押し付けられているのではなく，その多くが，この組織の方向を向いているように観察された．タペート組織の退化には小胞子の発達が関係していると考えた．成熟花粉内への物質の蓄積ならびに花粉が最終的にタペート組織の内壁から離れやすくなることは，葯内の水分の減少に伴って生ずるものと推察した．
キーワード：イネ，花粉，光学顕微鏡，小胞子，タペート組織，発育，発芽孔，有糸分裂．

Pollen and anther development in rice plant have been investigated rather intensively[6,12,14,15,21] because they are important in understanding the process for mature pollen production. Developing pollen cells were damaged by cool temperature[10,12,15], other environmental factors[10,16] and genetic backgrounds[1]. To understand the mechanism more precisely, further information about developing pollen and tapetal cells are thought to be essential[16]. However prior to that, it would be important to revise the normal course of development because there has been still little information in some stages and some discrepancies appeared in the recognition of the structure of pollen cells[7-12,14-16,21].

Development of pollen mother cell and anther wall was reported in the previous paper[19], and in the present paper, development of pollen and tapetum during the stages from microspore to mature pollen is described.

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Materials and Methods

Two cultivars of paddy rice (*Oryza sativa* L. cv. Kinmaze and Somewake) were cultured in Wagner pots under natural conditions and young panicles containing developing spikelets in various growth stages were used and prepared as described in the previous paper[19]. Sections obtained were stained with basic fuchsin, thionine, amide black and periodic acid Schiff's (PAS) reaction. Observations and photographs were made with an Vanox microscope (Olympus Co. Ltd.). Some unfixed or fixed anthers were initially observed to check the structure of developing pollen cells.

Results

Classification of the developmental stages by Satake[15] was adopted to our observations. Profiles of contracted pollen cells as well as their cytoplasm showing plasmolysis were obtained from some sections of materials through preparation procedures, which could not be found from unfixed pollen in the

anther loculus. Therefore these were regarded as artifacts produced during the preparation, and they were eliminated, although these sections could be decreased by the improved preparation in combination with checking the pollen profile during the preparation procedure.

1. Early microspore stage

About 11 days before heading, microspore cells were formed from the daughter cells of the tetrad. The intersection zone of callose wall among four daughter cells was at first marked to be dissolved (Fig. 1), and then callose wall in the boundary region of four daughter cells became disorganized. Each cell released from neighboring cells appeared as an isolated state in line with the tapetal inner surface (Fig. 2). The non-bordered region in the wall of daughter cells just before release showed uneven or a finely undulated figure. Immediately after the release, microspore cells appeared in somewhat irregular shape with thin walls and soon turned into the spherical profile. A similar process was described by Ranghavan[14]. although the wall undulation might possibly be produced during preparation, it was characteristic for this process.

The thin cell wall of an isolated microspore, which was identified by slightly PAS positive reaction, soon became thickened within a few days (Figs. 2, 3). Initiation of germ pore formation was noticed by the occurrence of annulus (Fig. 3), and thereafter an operculum appeared as a spot in the ring. Germ pore was formed on the region of the cell surface where daughter cells of the tetrad were not bordered in the former phase, because isolated microspores were for a while seen to leave the spatial disposition of four daughter cells of one tetrad. The four germ pores appeared distal to the older boundary region (Fig. 4), as previously illustrated[7,8]. These microspores maintained close contact with the tapetal inner surface. Most of the pores pointed toward the tapetal inner surface without touching it directly. The disposition was seen to be changed during the following development.

In the late early microspore stage, the nucleus and nucleolus of microspore cells changed to spherical figures and the nucleolus dyed more deeply in color with thionine and basic fuchsin (Figs. 4, 5).

2. Middle microspore stage

Microspore cells became enlarged containing a number of small vacuoles, and cytoplasmic strands appeared among the vacuoles, which were seen to spread out readily from the nucleus when located at the central part of the cells (Fig. 6).

The microspore cell wall thickened to form exine. The exine wall was discriminated as three layers, tectum, cavea and nexine in other plants[5]. The outer and innermost layers showed metachromatic light blue color with thionine, although the middle layer was not stained. Presence of Zwischenkörper[5] beneath the germ pore was confirmed by its dark blue color with thionine and by its dark red color with basic fuchsin and PAS reaction.

3. Late microspore stage

Microspore cell became more enlarged containing a large central vacuole. It could be formed by fusing small vacuoles. Nucleus and nucleolus further enlarged and nucleolar vacuoles appeared in the nucleolus (Fig. 7). Microspore nucleus migrated to the periphery of the cell, and then localized at the opposite site against the germ pore, while a large vacuole occupied the region in the nearby germ pore. As the microspore enlarged, the cells were seen to press each other at the boundaries and also to push the tapetum aside.

4. Vacuolated pollen stage

About 7 days before heading, a vacuolated pollen, which constituted vegetative and generative cells, was developed from a microspore (Figs. 8—11). Microspore nucleus divided into two nuclei and by the successive cytokinesis, these nuclei were separated from one another into different cells. The mitotic division was not completely synchronous and mitotic figures on different phases as well as figures on pre or post mitotic stages were seen in the same loculus. Profile of both nuclei just after mitosis was somewhat ellipsoidal, and their sizes were almost similar, although apparently smaller than the mother microspore nucleus (Fig. 9). The cytoplasmic region was divided unevenly, and the smaller cell located distal to the germ pore was developing to the generative cell, while the larger one, including a large vacuole and positioned at the pore side, was forming the vegetative cell (Fig. 9). The generative cell was still positioned distal to

the pore, but changed to an arched figure (Fig. 10). The cell was mainly filled with nucleus by decreasing the volume of the cytoplasm. The generative cell was recognized by deeply staining in color with basic fuchsin and thionine. Vegetative nucleus as well as some cytoplasm of the vegetative cell migrated to the periphery apart from the generative nucleus and then located in the region of the germ pore side of the vegetative cell (Fig. 11). Consequently a large vacuole in the vegetatve cell dispositioned distal to the germ pore. Vegetative nucleus somewhat enlarged, but nucleoplasm stained relatively pale in color with dyes.

After migration of the vegetative nucleus, the generative cell detached from the portion distal to the pore. It might be preceded by dissolution of the cell wall that fixed the generative cell at the distal region. Thereafter the generative cell migrated into the cytoplasm of vegetative cell to be positioned nearby the vegetative nucleus (Fig. 12).

5. Engorged pollen stage

About 5 days before heading, starch grains were remarkably increased in the cytoplasmic region of vegetative cells (Figs. 12—14), although tiny starch grains identified by PAS positive particulated reaction were observed in the previous stages. During the initiation of starch accumulation, a large number of grains appeared at the region nearby germ pore, and the long axis of starch grains aligned almost radially from the pore (Fig. 13), as was pointed by Kihara and Hirayoshi[8], but this alignment soon became obscured. Within a few days, starch grains increased and spreaded over the cytoplasmic region. As the pollen grain enlarged, starch grains further increased, while the volume of the large vacuole decreased (Figs. 15, 16). Some proteinaceous substances identified by basic fuchsin and amide black positive reactions were deposited in the vacuole (Fig. 12), and finally filled the vacuole after decrease of its watery region. These results suggest the engorged pollen development might be accompanied by water loss of the pollen cell. Polysaccharide particles, estimated by a slightly PAS positive reaction, appeared in the cytoplasmic region (Fig. 15). The intine, with pale PAS positive reaction, distinctly developed between the exine and the cytoplasm of the vegetative cell. The intine in the germ pore side was more thickened than at the opposite side.

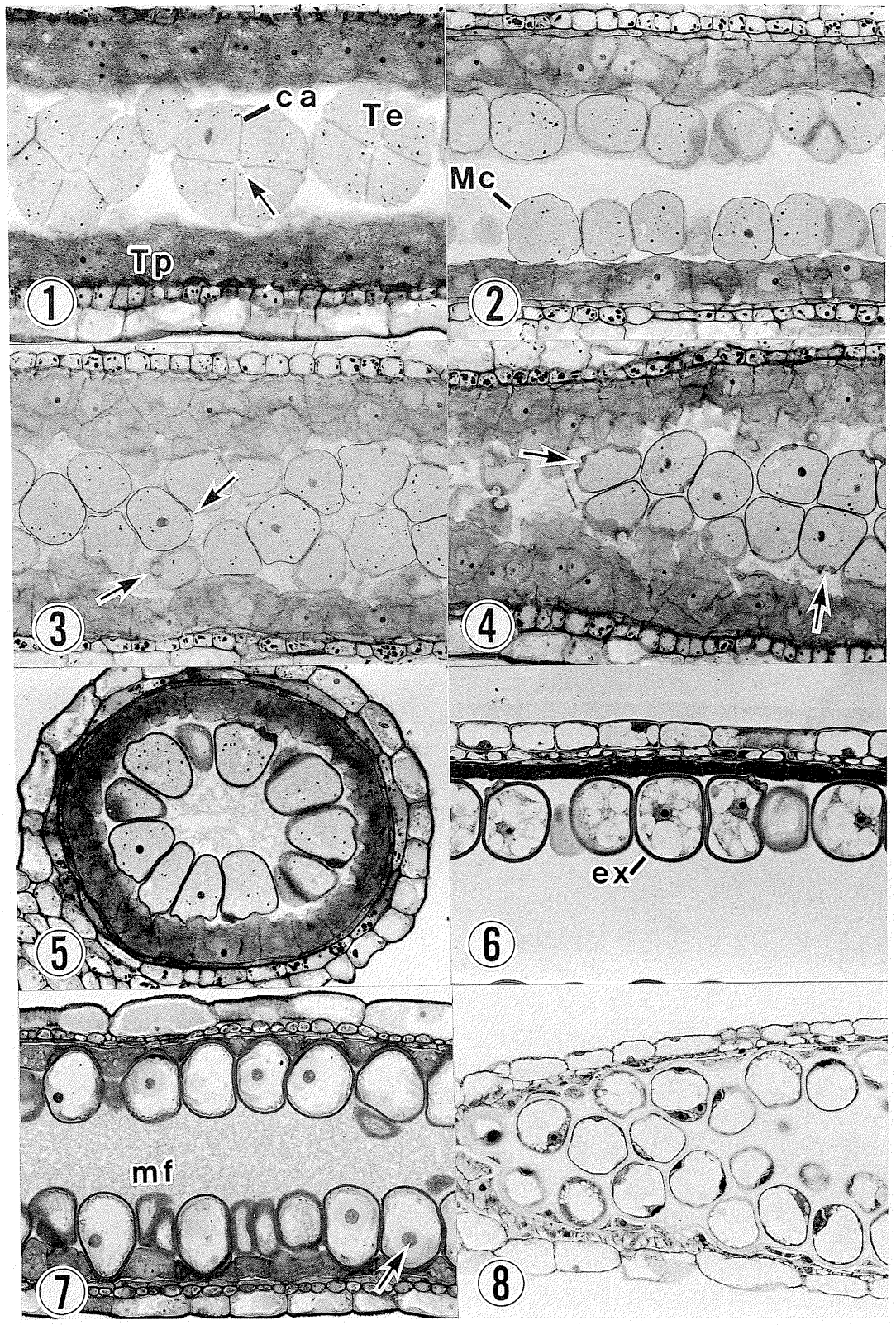
In the early engorged stage, generative cell divided at the nearby vegetative nucleus to form a couple of sperm cells (Figs. 12, 14). The initiation of the division was marked by change in the figure of the generative nucleus to spindle shape (Fig. 14, arrow). Sperm cells developed a chalk-like outline enclosed by

Abbreviations;

Gc : generative cell, **Mc** : microspore cell, **Sp** : sperm cell, **Te** : tetrad, **Tp** : tapetum, **Vn** : vegetative nucleus, **ca** : callose wall, **ex** : exine, **mf** : microfibrillar substance, **po** : polysaccharide particle, **ps** : proteinaceous substance, **st** : starch grain.

Explanations of figures (Figs. 1—11)

Figs. 1—4. Longitudinal sections of young anther locules during early microspore stage. Fig. 2. Median and Figs. 1, 3, 4. non-median sections. Callose wall of tetrad is degenerating particularly at the intersection zone (Fig. 1, arrow), and microspore cells were developing from daughter cells of the tetrad during the first phase. Cell wall of the microspore is thickening, and the germ pore is forming during the late early phase (Figs. 3, 4, arrows). Fig. 5. A cross section during the late early phase, showing a spatial close relationship between microspore and tapetum. Nucleolus is seen to be spherically shaped. Fig. 6. Middle microspore stage. Microspore cell is vacuolated and the tapetal layer becomes thinner as compared with that in the former stage. Fig. 7. Late microspore stage. Microspore cell became enlarged, including a large vacuole. Nucleus and nucleolus are apparently enlarged and nucleolar vacuoles are observed (Arrow). Figs. 8—11. Vacuolated pollen stage. Microspore cell is divided to form generative and vegetative cells (Fig. 8). The two cells are divided distal to the germ pore and just after mitosis, the size of both nuclei is almost similar (Fig. 9, arrow). Vegetative nucleus soon begins to migrate from this position (Fig. 10), and finally localized in the region of germ pore side (Fig. 11).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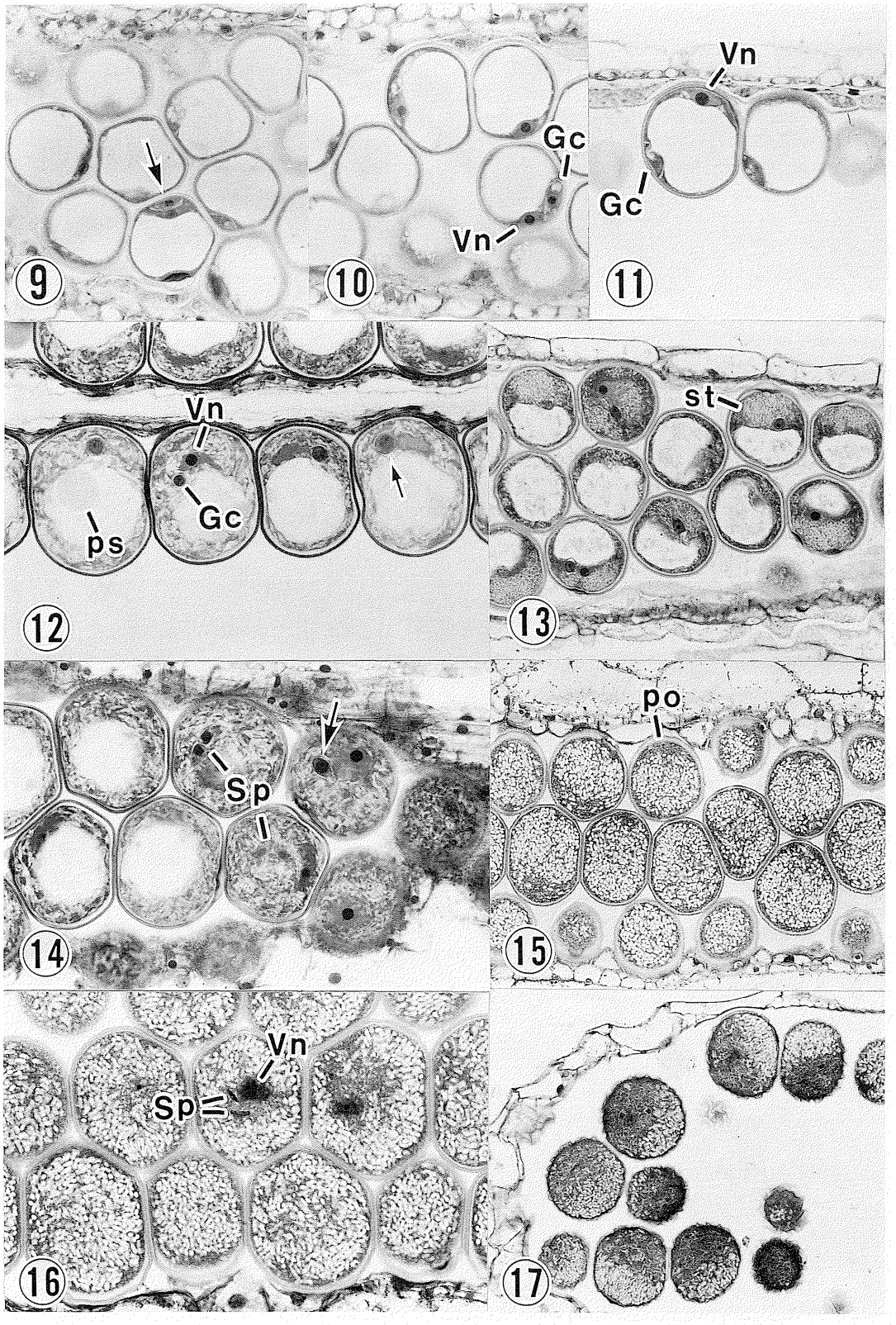
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membranous structure, as Yamada reported[21], although the envelope soon became indistinct. The phenomena were accompanied by decrease in the sperm cell size and the cell was seen to be occupied mainly by the nucleus. Sperm cells changed into the wedge shape and were apart from, one another (Fig. 16).

On the heading day, a definite polar distribution of storage particles and other constituents were observed (Fig. 17). Starch grains mainly localized in the region nearby the germ pore of vegetative cell, while other components including most of the polysaccharide particles, protein deposit, vegetative nucleus and sperm cells positioned at the region distal to the pore. This state of mature pollen reported by some authors[5,6,9,20], and named as a sugary type pollen[20], was rather easily released from the inner surface of reduced tapetum.

6. Tapetum

During the early microspore stage, the tapetal cell enlarged, and the thickness of tapetal layer increased (Figs. 1, 2). The cell contained dense cytoplasm, many small vesicles and fine mebranous structure, but not contain a large vacuole. During the later stage, the tapetal wall became obscured, and fine undulation appeared on its inner surface (Figs. 3, 4). During middle microspore stage when the microspore cell began to enlarge, thickness of tapetal layer began to decrease and the inner surface became smooth, while the cell still contained dense cytoplasm and small vesicles (Fig. 6). During the late microspore stage, when microspore cell further enlarged, the tapetal cell was seen to be degenerated by decreasing in thickness, and looked concave or wavy in appearance probably due to the pressure of enlarged microspores (Fig. 7). A large number of orbicules[4,5] which were identified by the same bright blue metachromatic color with thionine as exine appeared on the inner surface of the tapetum. Until the late microspore stage, anther loculus was filled with a fibrillar substance regarded as polysaccharides by PAS positive reaction, but it disappeared in this stage. During the stages of vacuolated and engorged pollen, most of the tapetal cells became thoroughly degenerated to leave a thin film of wall, although very few tapetal cells containing a small volume of cytoplasm and nucleus were seen between a line of developing pollens and anther wall tissue (Figs. 8—11).

Discussion

Deformation on the structure of the developing pollen cells using conventional preparation methods should be discussed, because preserving the structure without artifacts was found to be difficult in the case of rice, as well as sorghum as pointed out by Christensen and Horner[3,4]. The first and second contractions of microspore cells were produced artificially by conventional fixatives such as formalin acetic acid alcohol as indicated by Satake[15]. He indicated that the preservation of microspore was improved using glutaraldehyde, while pointed out that there is the occurrence of some shrinkage even when this fixative was used. And a revised classification of the developmental stage was proposed by Satake[15], because an artificially deformed microspores should be distinguished from

Explanations of figures (Figs. 12—17)

Figs. 12—14. Longitudinal sections of developing anther locules during early engorged pollen stage. After the generative cell has migrated at the nearby vegetative nucleus (Fig. 12), the cell begins to divide (Figs. 12, 14, arrows), and then forming a pair of sperm cells (Fig. 14). Starch grains are increasing in the cytoplasmic region and proteinaceous substance appeared in the vacuole. Figs 15—17. Engorged pollen stage. Starch grains and polysaccharide particles had accumulated, while the watery region of vacuole has almost disappeared, and the sperm cells assume the wedge-shaped outline (Fig. 16). During the late engorged pollen phase, sugary type of pollen is forming, being most of starch grains oriented to the germ pore side, and other components located on the opposite side (Fig.17).

Figs. 1—6, 7—17 were fixed with aldehydes, and Fig. 6 was prefixed with aldehydes and postfixed with osmium tetroxide (Figs. 1—4, 6, 7, 9—12, 14, 16 : × 450, and Figs. 5, 8, 13, 15, 17 : × 300).

microspore damaged by cool temperature[16]. Our results support this recognition and the revised classification was adopted to our study.

However, such stage specific occurrence of contractions could probably be derived from the nature of microspore on the development. The first contraction of microspore cell occurred after the microspore being released from the mother tetrad, and the second appeared after enlargement. Therefore these microspores might not have enough hardness to preserve the cell structure during preparation. Differences in the structural nature of developing microspores between the two contraction phases were discussed by Yamada[21]. Even during the other stages, figures of developing pollen cells, showing a hollowed profile as well as indecating plasmolysis, were found out from our sections and also seen in photographs and illustrations in some reports[8,14,15,21] and a book[7]. These might be produced during preparations, because such images as well as the first and second contractions could not be discovered from unfixed and/or most of the carefully prepared materials. Light microscopic observation using unfixed materials was reported by Nishiyama[12], and this was useful to check the developing pollen structure.

The spatial disposition between developing pollen and tapetum could have been maintained up to the late engorged stage. This recognition is identical to that in *Sorghum* and other gramineous plants[3]. However, in our sections as well as others[12,15], there included figures which showed developing pollen cells detached from tapetum. These might be produced during preparation, because such figures decreased after inprovement of the procedure. The detached figures occurred especially during the early microspore and late engorged stages. The microspore figure could be related to a weak binding between microspore and the tapetal cell wall because even in the case of non-detached figures, the boundary area between the microspore and tapetum was obviously very small in this early microspore phase. The late engorged pollen was seen to be dispersed from the anther loculus during dissection, and so it could be estimated as the result of the decrease in affinity, which was presumably accompanied by water withdrawal from the anther. In the periods between the two phases, the spatial disposition of developing pollen cells were rather stable, and it could be explained by the support with the adhesive nature of exine and orbicules which was suggested by Banerjee and Barghoorn[1]. On the whole, to preserve the structure and disposition of developing pollen of rice and some other gramineous plants using conventional methods would still have some technical difficulties, and several methods should be used to understand the exact structure.

The importance in the functional relationship between the microspore and tapetum could be clarified by the present result. During the early microspore stage, the tapetal cell was seen to be activated and this state maintained up to the vacuolated pollen stage, while the thickness of tapetum decreased. In the vacuolated pollen stage the tapetal cell layer became degenerated to form a thin film. During the microspore stages, microspore cells were seen to be developing step by step. Changes in the fine structure of tapetal cells during microspore stages were described and discussed by Nishiyama[11], and those in the microspore cell depicted by Nakano and Maeda[10]. Our results roughly corresponded with their studies. Their conclusions seem to be acceptable, although both of them did not fully discuss the relationship between the two tissues, tapetum and microspore. The fate of tapetum could be recognized as having in close relation to the development of the microspore. And therefore the tapetal hypertrophy[15,16] induced from cool temperature and other factors should be estimated in this correlation.

Some figures of the developing pollen cells observed in our study could be discussed as follows. During the early microspore stage, germ pore as well as primexine was seen to be forming. Similar information was provided by Kihara and Hirayashi[8]. Germ pore in the later stage would probably influence microspore development by restricting substance flow into the microspore. The disposition of the microspore nucleus distal to the germ pore during the late microspore stage, and the occurrence of starch grains near the germ pore and their radial arrangement at the early engorged pollen stage could be related to this substance flow.

The germ pore of gramineous plants was tightly appressed to the tapetal surface in all the stages during pollen development, according to Christensen and Horner[3]. However, the germ pore of rice was seen to be not directly in contact with the tapetal inner surface, but mostly pointing toward the tapetal side and the disposition changed during the pollen development. Similar observations were presented by Kihara and Hirayoshi[8], and in other grass pollens, according to Banerjee and Barghoorn[1], and by Scole and Evans[17]. Tapetum was thought to play a role as a secreting tissue[11] and germ pore function as a probable flow route as discussed above. But considering from the pore disposition, substances might be transferred from the tapetum to the anther loculus and then through the germ pore into the pollen cytoplasm indirectly.

Microspore cells were seen to be activating gradually from the late early to the late microspore stages. The distinct appearance of the radially arranged cytoplasmic network from the nucleus during vacuolation suggested the presence of cytoskeleton along the strand. As the cytoskeleton could be thought to play a role of intracellular motion[18], the cyclosis along the cytoplasmic strand might probably occur rather actively. In addition, vacuolated microspore cells might have a sink activity such as elongated cells[13]. Microspore cells in the late microspore stage were more activated than those in the early stages as pointed out by Nakano and Maeda[10]. Particularly microspore nucleus vigorously changed during the stages from the middle to the late microspore and in the successive vacuolated pollen stage, microspore division occurred. Therefore, activation and premitotic process could be characteristic for these microspore stages.

During the early engorged microspore stage, the generative cell divided to form two sperm cells. Prior to this mitosis, the duplication of deoxynucleic acid and other preparations for the division must occur. Consequently, the generative cells would be in a rather active state, as Yamada discussed[21]. The developing pollen cells were rather small and the cytoplasm became condensed during later stages. Therefore further detail information should be obtained using electron microscope.

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References

1. Banerjee, U.C. and E.S. Barghoorn 1971. The tapetal membranes in grasses and ubisch body control of mature exine pattern. In Pollen : Development and Physiology. (ed.) J. Heslop-Harrison. Butterworths, London. pp126—127.
2. Cheng, Y.K. and C.S. Hung 1980. Studies on cytoplasmic-genetic male sterility of cultivated rice (*Oryza sativa* L.) II. Morphological-histological investigation on functional male sterility. Jour. Agric. Res. China 29 : 69—80*.
3. Christensen, J.E. and H.T. Horner, Jr. 1974. Pollen pore development and its spatial orientation during microsporogenesis in the grass *Sorghum bicolor*. Am. J. Bot. 61 : 604—623.
4. ————, ———— and N.R. Lersten 1972. Pollen wall and tapetal orbicular wall development in *Sorghum bicolor* (Gramineae). Am. J. Bot. 59 : 43—58.
5. Heslop-Harrison, J. 1979. Aspects of the structure, cytochemistry and germination of the pollen of rye (*Secale cereale* L.). Ann. Bot. 44 (Supl. 1) : 1—47.
6. Hirayoshi, I. 1938. Structure of nucleus in the pollen and its behavior during germination of rice. Proc. Crop Sci. Soc. Japan 10 : 65—70**.
7. Hoshikawa, K. 1975. Development of flower organs. In The Growing Rice Plants. Nobunkyo, Tokyo, 226—227***.
8. Kihara, H. and I. Hirayoshi 1942. Entwicklung des Pollenkorns bei *Oryza sativa* L. Agric. Hortic. 13 : 685—690****.
9. Koike, S. and T. Satake 1987. Sterility caused by cooling treatment at the flowering stage in rice plants II. The abnormal digestion of starch in pollen grain and metabolic chantes in anthers following cooling treatment. Jpn. J. Crop Sci. 56 : 666—672.
10. Nakano, H. and E. Maeda 1989. Ultrastructure of inoculated anthers in relation to the frequency of induction of pollen callus in anther culture of *Oryza sative* L. Jpn. J. Crop Sci. 58 : 204—211.
11. Nishiyama, I. 1970. Male sterility caused by cool treatment at the young microspore stage in rice plants. VI. Electron microscopical observations on normal tapetal cells at the critical stage. Proc.

Crop Sci. Soc. Japan 39 : 474—479.
12. ———— 1981. ————XX. Optical microscopical observations of unfixed, intact anthers. Jpn. J. Crop Sci. 50 : 495—501.
13. Okamoto, A. 1980. Electrophysiological structure of elongation sink in growing zone of hycopotyl. Function of H^+ pump. Seibutsukagaku 32 : 25—35**.
14. Ranghavan, V. 1988. Anther and pollen development in rice (*Oryza sativa*). Am. J. Bot. 75 : 183—196.
15. Satake, T. 1974. Male sterility caused by cool treatment at the young microspore stage in rice plants. IX. Revision of the classification and terminology of pollen developmental stages. Proc. Crop Sci. Soc. Japan 43 : 31—35.
16. ———— 1977. A course of study on the mechanism of sterility caused by cooling treatment at the young microspore stage in rice plant. Chem. Regul. Plants 12 : 29—39***.
17. Scoles, G.J. and L.E. Evans 1979. Pollen development in male-fertile and cytoplasmic male-sterile rye. Can. J. Bot. 57 : 2782—2790.
18. Van Lammeren, A.A.M., C.J. Keijzer, M.T.M. Willemse and H. Hieft 1985. Structure and function of the microtubular cytoskeleton during pollen development in *Gastria verrucosa* (Mill.) H. Duval. Planta 165 : 1—11.
19. Wada, T., K. Ogawa, T. Ito, H. Suzuki and Y. Takeoka 1990. Light microscopic observations on pollen and anther development in rice (*Oryza sativa* L.) I. Stages from pollen mother cells to tetrads. Jpn. J. Crop Sci. 59 : 769—777.
20. Watanabe, K. 1961. Studies on the germination of grass pollen II. Germination capacity of pollen in relation to the maturity of pollen and stigma. Bot. Mag. Tokyo 74 : 131—137.
21. Yamada, N. 1972. Studies on the developmental physiology in rice pollen. I. The metabolic patterns connected with the structural chantes in developing poollen. Proc. Crop Sci. Soc. Japan 41 : 320—334*****.

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