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Electron Microscopic Observation of Megasporogenesis of *Tricyrtis hirta*

By

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Abstract. The process of the megaspore formation in *Tricyrtis hirta* (Liliaceae) was examined electron-microscopically. This species produces four megaspores of tetrad and the chalazal-most one of them becomes functional. The megasporocyte and the functional cell of dyad and tetrad have some polarity between the chalazal and the micropylar poles; the organelles enclosed with a double membrane and the concentric ER are not distributed uniformly in the cytoplasm of these cells. In particular, the concentric ER appears in the nonvacuolate cytoplasm of the megasporocyte, two dyad cells and four tetrad cells, but it disappears in the vacuolate cytoplasm of these cells. The nucellus of this species is tenuinucellate; the megasporocyte is situated at the top of nucellar column which is invested with a layer of the epidermal cells. The chalazal part of the megasporocyte, the dyad and the tetrad is invested with the cells of nucellar column, while the micropylar part of them is invested with the cells of nucellar epidermis. Only at the chalazal part, there are plasmodesmata, but at the micropylar part there are not plasmodesmata. It has been discussed that the unequal distribution of the plasmodesmata correlates with the mode of the megaspore formation such as the monosporic, bisporic and tetrasporic type of development (Kapil & Bhatnagar 1981). Besides such a discussion, the correlation between the distribution pattern of plasmodesmata and the kind of the nucellar cells to which the cell or cells at the meiotic stage are contiguous should be discussed as well.

The developmental process of an embryo sac of some species in the genus *Tricyrtis* was examined by Ogura (1964, 1966), using the light microscope. In brief, the process is stated as follows. A megasporocyte differentiates directly below epidermal cells at the top of nucellus. It successively undergoes the heterotypic and the homotypic divisions to become a dyad and a tetrad in sequence. The tetrad invariably is of a T-shaped arrangement. The chalazal-most functional megaspore of the tetrad cells undergoes three nuclear divisions to become an eight nucleate embryo sac. Thus, the embryo sac of the genus *Tricyrtis* is formed according to the monosporic eight nucleate *Polygonum* type. In every organized embryo sac examined by Ogura, the antipodal cells enlarge and become very prominent at the time of fertilization or some later.

Unfortunately, the developmental process of the embryo sac in *Tricyrtis hirta* has not described yet. But Satô, who is the senior author of this paper, has already finished the light microscopic examination of the process, though it is not published yet. He has confirmed that during the embryo-sac formation,

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T. hirta has the same features as the species examined by Ogura (1964, 1966) have. He will report on the light microscopic examination of the embryo-sac formation of *T. hirta* in the near future.

Electron microscopic examination of the haploid generation in Angiosperms has not been carried out enough. In particular, our knowledge on the developmental process of megaspore and megagametophyte (embryo sac) obtained from the electron microscopic examination is very scanty. Perhaps this seems to depend on unsatisfactory penetration of fixatives to a haploid cell through thick nucellar cells and difficulty in cutting the nucellus along the desirable orientation. *T. hirta* forms many ovules in an ovary and they arrange in well-regulated state; the long axis of the ovule invariably meets at right angles to that of the ovary. It is relatively easy to arrange removed ovules in desirable orientation and embed them in resin. That is, ultra-thin sections cut along the desirable orientation are obtained with ease. We have begun to examine the developmental process of the embryo sac of *T. hirta*, using the electron microscope. Our paper presents the knowledge obtained from the electron microscopic observation of the megasporogenesis of this species.

Material and Method

Many young flower buds were collected from individuals of *Tricyrtis hirta* grown on the campus of the Yokohama National University extending from September to October in 1990. The clusters of several ovules were excised from an ovary of buds collected. The clusters were fixed in 2.5% glutaraldehyde buffered with 0.1 M phosphate buffer and post-fixed with 1% osmium tetroxide in 0.1 M phosphate buffer. These fixed materials were dehydrated in a graded ethanol series and embedded in low viscosity epoxy resin (Spurr 1969). Thin sections were stained with 2% uranyl acetate and lead citrate. The stained sections were examined under the JEM-100CX transmission electron microscope.

The ratio of a total area of the organelles enclosed with a double membrane to an entire area of the cell was calculated in photographs which are taken from the sections cut along the long axis of the cell. These areas are measured from these photographs, using the image analysis program, Nikon Cosmozone 1SA.

Observation

1. Cell Wall

A nucellus of *T. hirta* is tenuinucellate (Fig. 1A). A megasporocyte (Fig. 1A), a dyad (Fig. 2A) and a tetrad (Fig. 3A) are directly surrounded with epidermal cells of nucellus and other nucellar cells except them. The nucellar cells except the epidermal cells are contiguous to the chalazal half of the mega-

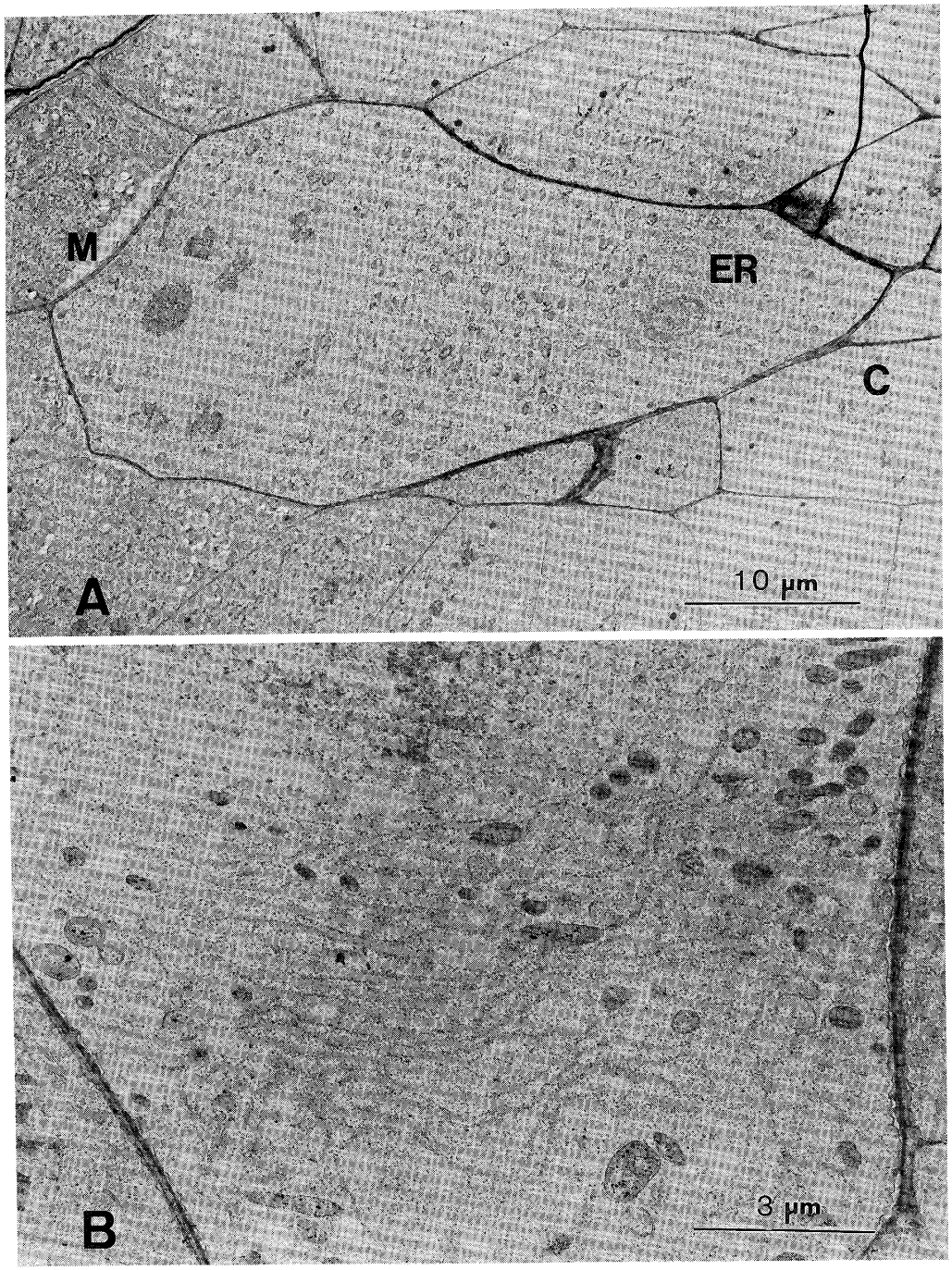

Fig. 1. Megasporocyte (M: micropylar side, C: chalazal side, ER: concentric ER). A: Non-vacuolate megasporocyte. Plenty of organelles enclosed with a double membrane are situated at and near the center of the nonvacuolate megasporocyte. B: Chalazal half of vacuolate megasporocyte. The concentric ER disappears and the stratified ER appears.

sporocyte, to the chalazal half of the chalazal dyad cell and to the chalazal half of the functional megaspore. The micropylar half of the megasporocyte, the micropylar cell of the dyad and three non-functional cells of the tetrad are not

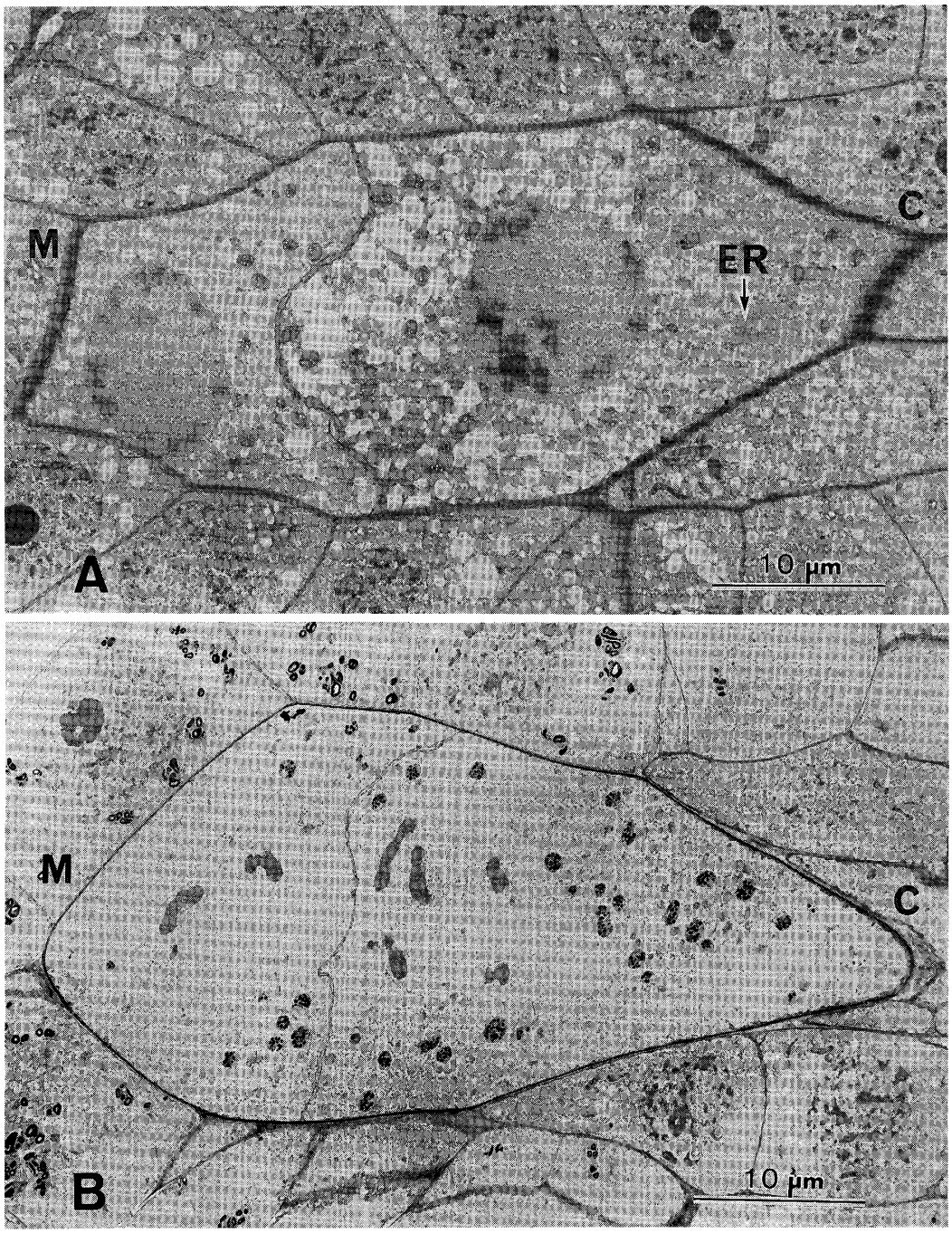

Fig. 2. Dyad (M: micropylar side, C: chalazal side, ER: concentric ER). A: Two dyad cells at intermediate phase. (photograph by Noriyuki Nagai) B: Two dyad cells undergoing the homotypic division.

contiguous to the nucellar cells except the epidermal cells, but they are contiguous to the epidermal cells of nucellus at and near its top. Although the cell wall of the nucellar diploid cells inclusive of the epidermal cells is low in electron density, the wall of the megasporocyte, the wall wholly enclosing a dyad

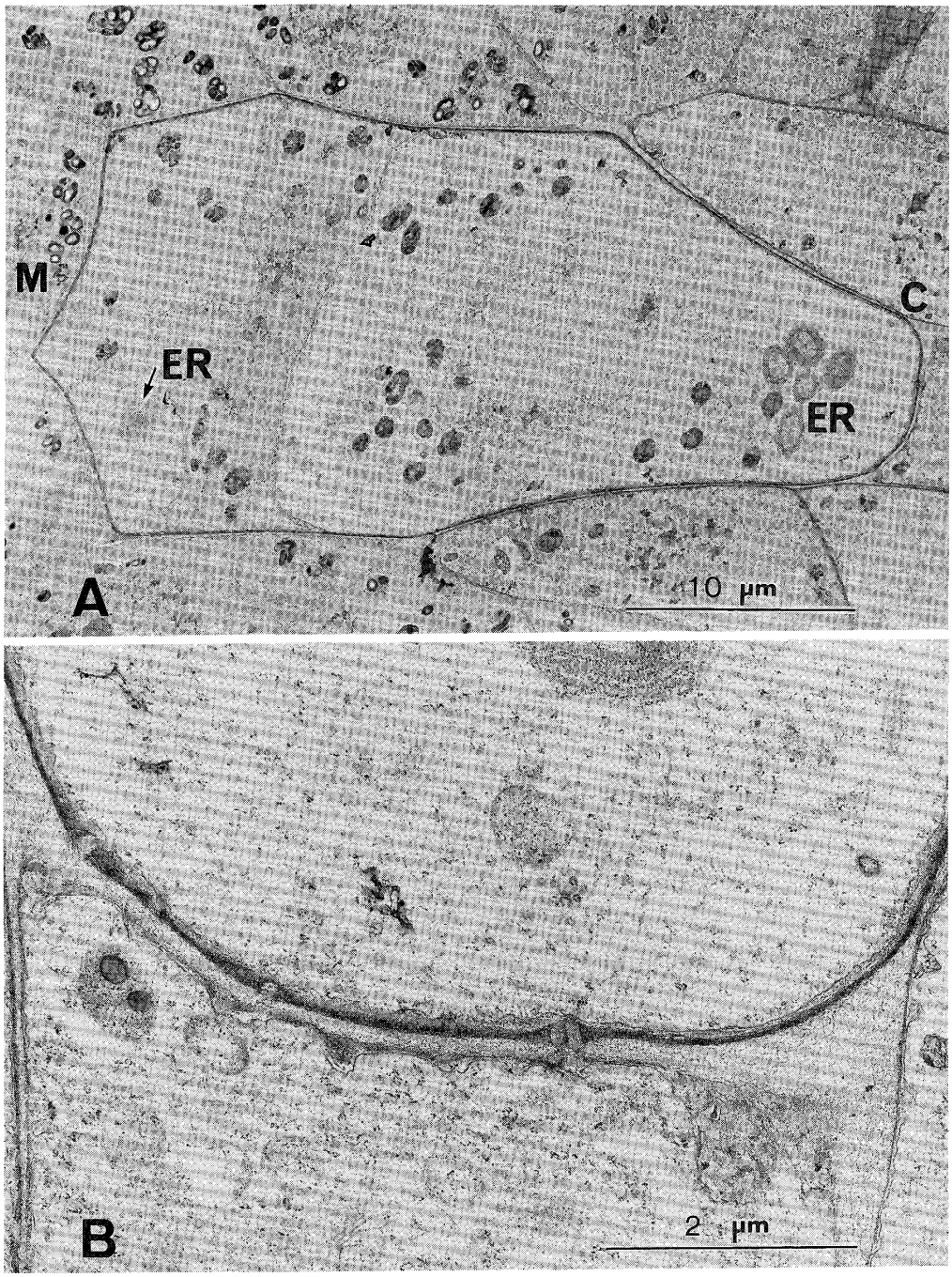

Fig. 3. Tetrad (M: micropylar side, C: chalazal side, ER: concentric ER). A: Four tetrad cells. In the chalazal-most cell, plenty of mitochondria and plastids occur on the micropylar side, and several concentric ERs occur at chalazal end. B: Chalazal end of functional megaspore. Plasmodesmata occur in the wall of the chalazal end.

and the wall wholly enclosing a tetrad have high electron density (Figs. 3B, 4C). Furthermore, fibrillar material permeates wholly and richly in these enclosing walls. The wall separating two dyad cells and three walls separating

four tetrad cells are very low in electron density and fibrillar material is absent or nearly absent in the walls (Fig. 4C). As the wall matures, the electron-translucent wall becomes to be put between two electron-opaque layers with high electron-density (Fig. 4D). The wall wholly enclosing a dyad and the wall wholly enclosing a tetrad are directly derived from the wall of megasporocyte. These enclosing walls seem to be different from the walls of nucellar cells inclusive of the epidermis and the separating walls formed after the heterotypic and homotypic divisions in chemical and morphological properties.

Plasmodesmata do not occur in the wall on the micropylar side of megasporocyte, but they occur in the wall on the chalazal side of megasporocyte. That is, the megasporocyte is not connected with epidermal cells of the nucellus by plasmodesmata, but by plasmodesmata, it is connected with other cells of the nucellus except its epidermis. In a dyad and in a tetrad, similarly, no plasmodesma occurs in the boundary between the cell constructing them and the cells of nucellar epidermis, while it occurs in the boundary between the chalazal cell of the dyad or the functional megaspore of the tetrad (Fig. 3B) and the cells of nucellus except its epidermal cells. There is no plasmodesma in the wall separating two dyad cells and in three walls separating four tetrad cells (Fig. 4D). Whether the plasmodesma is produced in the boundary or whether it is not produced seems to depend on the kind of the cell to which the cell at the meiotic stage is contiguous.

2. Vacuole and Endoplasmic Reticulum

Although a young megasporocyte whose nucleus contains a distinct nucleolus has no vacuole in its cytoplasm (Fig. 1A), an old one which is shortly after the onset of meiotic division has vacuole or vacuoles in its cytoplasm (Fig. 1B). Not only in a young dyad where the heterotypic division has just been over, but also in a young tetrad where the homotypic division has just been over, the cells of them have no vacuole, but vacuole or vacuoles are produced in the cytoplasm as the young dyad and tetrad become mature respectively (Figs. 2A, 3A). That is, although these cells where a division has just been over have no vacuole, the vacuoles are gradually produced as these cells come to maturity.

An endoplasmic reticulum (ER) permeates throughout the cytoplasm of the cell at the meiotic stage. The ER usually is in strata, but in the cells at the meiotic stage of *T. hirta*, it is often viewed as concentric circles (Fig. 4A). The concentric ER occurs in the young nonvacuolate cytoplasm of a megasporocyte (Fig. 1A), two dyad cells (Fig. 2A) and four tetrad cells (Fig. 3A). That is, it is always viewed in the nonvacuolate cell, and even in the vacuolate cell (Fig. 2A), it occurs in the cytoplasm where a vacuole is nearly absent near it yet. The membrane of concentric ER becomes gradually indiscernible in the cytoplasm where small vacuoles are been producing near the ER (Fig. 4B). Perhaps the concentric ER has something to do with the production of vacuole. After

this, the concentric ER disappers and only the stratified ER occurs in these cells (Fig. 1B). The ER seems to be replaced with the stratified ER together with the progression of vacuolization. However unfortunately we could not clarify enough the relation between the stratified ER and the concentric ER. We have not found the concentric ER in any cytoplasm of the nucellar diploid cells yet.

3. Organelle Enclosed with Double Membrane

There are many organelles enclosed with a double membrane in a cytoplasm of the megasporocyte (Fig. 1A). They are ellipsoidal or gourd-shaped, and

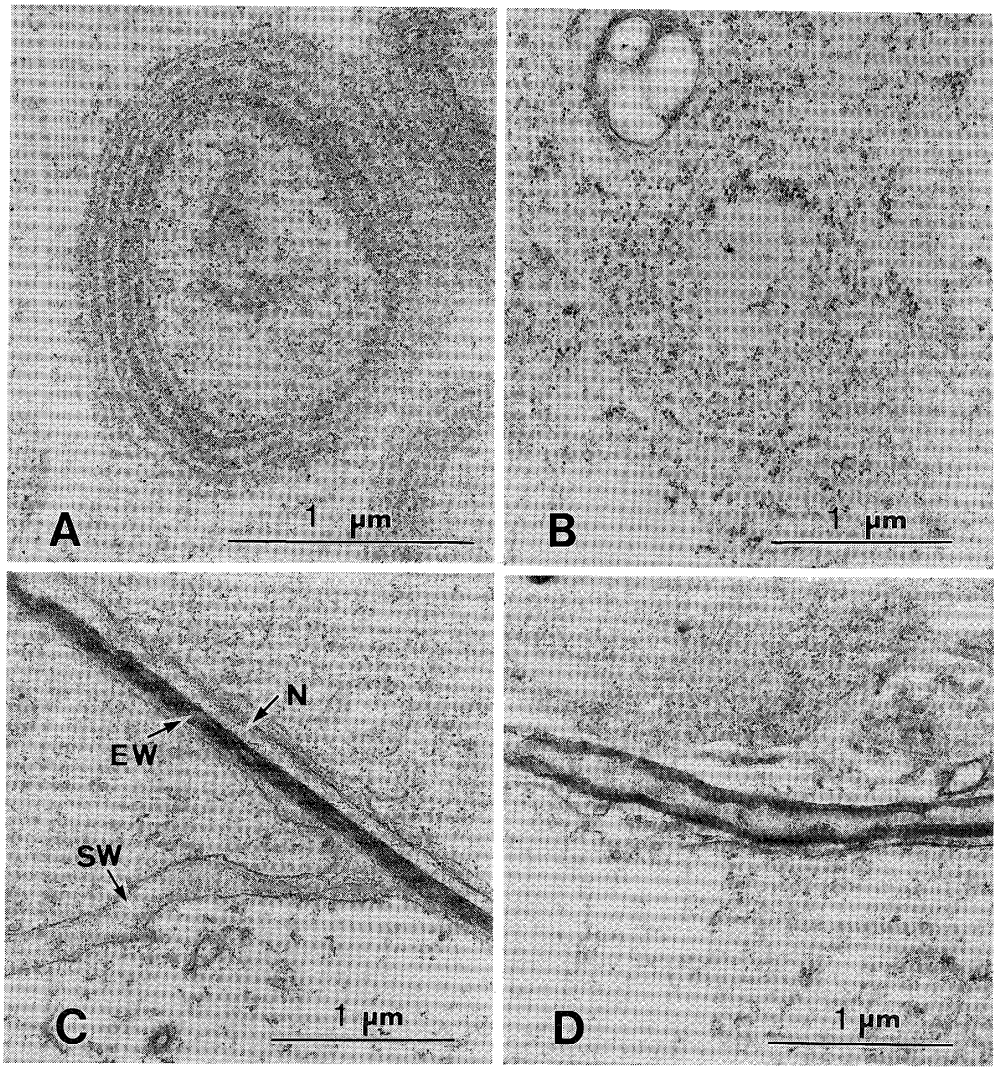

Fig. 4. Concentric ER and cell wall. A: Concentric ER with discernible membrane. There is no vacuole near the ER. B: Concentric ER with indiscernible membrane. There are vacuoles near the ER. C: Cell walls of tetrad. (N: wall of adjacent nucellar cell, EW: Wall wholly enclosing tetrad, SW: Wall formed after heterotypic division) D: One of three walls separating four tetrad cells, formed after heterotypic division.

inner membrane systems of them such as thylakoids or cisternae are of a very immature state. Many of these organelles are too immature for us to identify as young mitochondria or as pro-plastids. But there are a few organelles identified either as young mitochondria or as pro-plastids, too. The membrane enclosing the mitochondrion is more prominent than that of the pro-plastid. The matrix of mitochondria is lower in electron density than that of pro-plastids. The organelle which is enclosed with an obscure membrane and of which the matrix is higher in electron density almost contain starch grain. Organelles that it is impossible or difficult to judge whether they are developed into plastids or whether they are developed into mitochondria relatively decrease in a chalazal dyad cell (Fig. 2A). Mitochondria do not change greatly in size, while plastids become larger and almost all of them become to contain starch grain. In a cytoplasm of the functional megaspore, quite a number of organelles enclosed with a double membrane become identified either as plastids or as mitochondria (Fig. 3A). Quite a number of plastids contain some starch grains. Occupation ratio of mitochondria and plastids in the haploid cells increases as the meiosis progresses (Fig. 5); the occupation ratio of plastid is higher than that of mitochondorion at each stage of development, and also

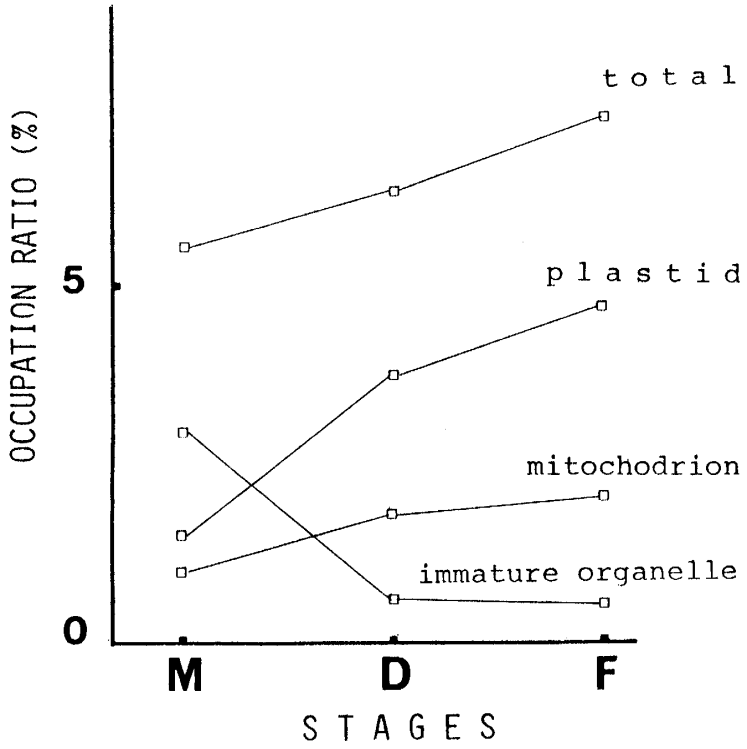

Fig. 5. Occupation ratio (%) of plastid and mitochondrion in cytoplasm of megasporocyte (M), chalazal cell of dyad (D) and functional megaspore (F). The "immature organelle" indicates the occupation ratio of the organelle which is too immature to identify either as plastid or as mitochondrion. The "total" indicates a total of occupation ratio of plastid, mitochondrion and immature organelle.

the ratio increase of plastid together with the progression of meiosis is higher than that of mitochondrion. This seems to show that the metabolic activity is elevated together with the progression of meiosis.

4. Distribution of Organelles within Cell at Meiotic Stage

Although Golgi apparatus and lipid granules are of immature state and they are distributed nearly uniformly in the cells at the meiotic stage, organelles such as plastids, mitochodoria and concentric ERs are not distributed uniformly in the nonvacuolate megasporocyte (Fig. 1A) and the chalazal nonvacuolate cell of the dyad. These cells roughly seem to be constructed of three areas as follows:

Area I: micropylar area containing the nucleus. This area occupies about one-thirds on the micropylar side of the megasporocyte and the chalazal dyad cell.

Area II: chalazal area containing the concentric ER. This area occupies about one-thirds on the chalazal side of the megasporocyte and the chalazal dyad cell.

Area III: intermediate area between Area I and Area II. In this Area, there are many organelles enclosed with a double membrane.

In Area I, organelles are few since the large nucleus occupies nearly all this area. In Area II, vacuoles and organelles enclosed with a double membrane are not many near the concentric ER. Vacuoles are produced in this area, as the concentric ER is gradually replaced with the stratified ER (Fig. 1B). In the vacuolate megaspore and in the chalazal vacuolate dyad cell (Fig. 2A), the nucleus slightly shifts its position to the center of the cell. Together with shifting of the nucleus, many organelles enclosed with the double membrane also shift their position to the micropylar end of the cell. Vacuoles disappear in the cell with a dividing nucleus (Fig. 2B).

In the functional megaspore (Fig. 3A), there is a nucleus at a little micropylar part of its center. Organelles are uniformly distributed throughout the cytoplasm. The concentric ER occurs only at the chalazal end. We have not observed it at the micropylar end. The ER counts from five to seven in the functional megaspore. These numbers are greater than the number (1−2) of the ER counted in the megasporocyte or in the chalazal dyad cell. Vacuoles are produced throughout the cytoplasm of functional megaspore together with maturation of the spore. The vacuolization seems to become more active at the post-meiotic stage than at the meiotic stage. An increase of the concentric ER in number seems to have something to do with the formation or structure of a two-nucleate embryo sac.

5. Non-functional cells such as micropylar dyad cell and three micropylar tetrad cells

The megasporocyte and the chalazal cell of a dyad divide unequally to

become a small cell and a large cell, because the nucleus is located at a little micropylar part of the center of these cells. The micropylar cell of the dyad divides equally to become two micropylar cells of tetrad. These non-functional cells strikingly resemble one another in number, distribution pattern and developmental immaturity of their constituent organelles (Figs. 2A, 3A). The same concentric ER that is found in the chalazal cell of the dyad and in the functional megaspore occurs in these non-functional cells where vacuoles are nearly absent (Fig. 3A), and it disappears together with vacuolization of these cells. The organelles in these cells are much fewer than those in the functional cells such as the chalazal dyad cell and the functional megaspore. The whole cytoplasm of these non-functional cells become higher in electron density than that of the functional cells together with the progression of vacuolization in these non-functional cells.

Discussion

Kapil & Bhatnagar (1981) and Bouman (1984) gave a full account of the information which had been obtained from the electron microscopy of the megaspore ontogeny in Angiosperms and which was available at that time. Judging from their accounts, the following seems to be facts common to every megaspore ontogeny of the angiospermous taxa electron-microscopically examined.

1. A megasporocyte and a functional cell of the dyad and the tetrad found during an embryo-sac development of the monosporic type have some polarity between their micropylar pole and their chalazal pole.
2. A cell wall wholly enclosing a megasporocyte, a dyad or a tetrad has fibrillar material and has high electron density.

We could confirm these facts during the megaspore ontogeny of *T. hirta*. We principally discuss with respect to the polarity and the cell wall in our paper.

1. Polarity

Within the megasporocyte, the chalazal cell of the dyad and the functional megaspore of *T. hirta*, plastids and mitochondria are not distributed uniformly, although some of the plastids are not distinguished without misidentification from the mitochondria because of their immaturity in structure in the megasporocyte. Besides these organelles, Golgi apparatus and lipid granules have attracted much attention in the process of megaspore formation of other species examined (Kapil & Bhatnagar 1981; Bouman 1984). But in *T. hirta*, these are of an immature state and are distributed almost uniformly. The behavior of the concentric ER seems to have something to do with the vacuolation of the functional cells such as the megasporocyte, the chalazal cell of the dyad and the functional megaspore, namely with aging of these functional cells. The occurrence of the concentric ER has been described in the process of the megaspore formation in other species examined as well (Kapil & Bhatnagar 1981). The concentric ER has been considered to be produced as a result of a poor

oxygen supply to the cell at the meiotic stage (Rodkiewicz & Mikulska 1966), and to correlate with the formation of cell plate during meiosis (De Boer-de Jeu 1978) and with the formation of lipid granules (Cresti *et al.* 1985). But in *T. hirta*, there is no evidence that shows the correlation between the presence or absence of the concentric ER and the formation of cell plate, and also that shows intimate relation between the occurrence of the concentric ER and the production of the lipid granules. The concentric ER occurs in the cells which are to form a microspore (Cresti *et al.* 1985), too. This ER seems to be commonly built up in the cell at the meiotic stage, but its function remains to be specified in the future.

2. Cell Wall

The wall wholly enclosing the dyad and the wall enclosing the tetrad directly derives from the wall of megasporocyte. The wall separating two cells of dyad is formed after the heterotypic division, and one of the three walls separating four cells of tetrad are formed after the heterotypic division and the two of them are formed after the homotypic division. The enclosing wall inclusive of the megasporocyte wall has fibrillar material and it is electron-opaque, while the separating wall has no fibrillar material and it is electron-translucent. It is known that callose entirely or partially deposits in these walls of the angiospermous plants (Kapil & Tiwari 1978). The presence or absence of callose may have something to do with the electron-microscopic difference between these cell walls. Unfortunately, however, the detection of callose during the megaspore formation of *T. hirta* has not been performed yet.

The megasporocyte is situated at the top of the nucellar column which is invested with a layer of the nucellar epidermal cells. That is, the megasporocyte, the dyad and the tetrad are respectively contiguous to both the cell of nucellar epidermis and the cell of nucellar column. Plasmodesmata are absent in the boundary between the cell at the meiotic stage and the cell of nucellar epidermis, while they are present in the boundary between the cell at the meiotic stage and the cell of nucellar column. The presence or absence of plasmodesmata and the appearance or disappearance of them have been discussed as to the relation with the developmental type of the megaspore or embryo sac (De Boerde Jeu 1978; Kapil & Bhatnagar 1981). But, it seems to be significant that the megasporocyte is one of the cells of nucellar column. The nucellar epidermal cells are the cells of the dermal tissue-system, while the cells of nucellar column are the cells of the fundamental or ground tissue-system. That is, the unequal distribution of plasmodesmata found in the nucellus of *T. hirta* seems to show that the connection between the cells which belong to the same tissue system is stronger than the connection between the cells, each of which belongs to the different tissue system. But, we could not find the plasmodesmata in wall separating the dyad cells and in any walls separating the tetrad cells.

摘　　要

ユリ科のホトトギス(*Tricyrtis hirta*)の大胞子形成過程を透過型電子顕微鏡を用いて調査した。この種の大胞子母細胞は減数分裂を行い4個の大胞子を作り，その内の合点端の大胞子（機能的大胞子）1個だけが胚嚢の形成を始める。大胞子母細胞と二分子の合点側細胞，機能的大胞子には，細胞内小器官の分布に極性がみられる。特に，液胞化のほとんど進んでいないこれらの細胞には，同心円状の小胞体（concentric ER）が合点極付近に必ず見られる。ホトトギスの珠心は薄層型（tenuinucellate）であるため，大胞子母細胞や二分子，四分子は珠心表皮細胞とこれ以外の珠心細胞に必ず接している。珠心表皮の細胞は表皮系を構成いる細胞であり，表皮以外の珠心細胞は基本組織系の細胞である。つまり，減数分裂期にある細胞は異なる組織系に属する細胞に接している。原形質連絡は，珠心表皮細胞との境界にはみられず，表皮以外の珠心細胞との境界にはかなりよくみられる。原形質連絡の不均一な分布については，大胞子の形成様式（単胞子性，二胞子性，四胞子性）と関係付けて議論されてきた（Kapil & Bhatnagar 1981）。原形質連絡の分布については，そのような議論に加えて，減数分裂期にある細胞が接する珠心細胞がどの組織系に属するかも考慮する必要があると思われる。

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