Embryo Sac Formation in *Allium thunbergii*, with Transmission Electron Microscope Observation

By

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Abstract. The development of embryo sac in *Allium thumbergii* was investigated using a light and an electron microscope. Two ovules, which are composed of a thin curved nucellus invested with two integuments, are formed in each of three ovarian locules which are formed in a ovary. The ovule seems to be campylotropus. The nucellus has no parietal cells and is tenuinucellate. The embryo sac is formed according to the bisporic eight nucleate *Allium* type of development. Not a few organelles occur in the cells in the meiosis and in the developing embryo sacs. In the cells constructing a 7-celled mature embryo sac, mitochondria become to be distinguished from plastids in their internal structure and in a stain property of their matrix. There are not a few plastids with a starch grain in these cell. Furthermore, lipid grains are viewed in these cells, though they are not viewed in the cells during the meiosis and in the developing embryo sacs. These cells in the 7-celled mature embryo sac are outlined with a thin wall constructed of the substance stained with ruthenium red in the thin sections. In the embryo sac where one of synergids has already degenerated, the thin wall is disappeared and instead of the wall, a cell membrane is formed along the disappeared wall. In the boundary between the cells of egg apparatus and central cell, the formation of cell wall for dividing them is lag behind. Along the boundary, two cell membranes are in close contact with each other, though the interruptions in the contacts occur. Some interruptions contain the substance stained with ruthenium red and others do not. It seems that the formation of cell membrane and cell wall in the unfertilized egg cell or/and the fertilized egg cell starts from the formation of the thin wall constructed of the substance stained with ruthenium red in the 7-celled young mature embryo sac.

Introduction

The genus *Allium* is usually treated as a genus of the family Liliaceae (s. l.). But Hutchinson (1959) assigned *Allium* to the Amarylidaceae and Dahlgren *et al.* (1985) assigned it to the family Alliaceae which is a family established to divide the Liliaceae (s. l.) into many small families. Although in *Nothoscordum*, *Muilla* and *Brodiaea* of the Alliaceae, their embryo sacs are formed according to the monosporic eight nucleate *Polygonum* type of development, in the members of *Allium* hitherto examined, except for *Allium mutabile* (after Davis, 1966), their embryo scacs are formed according to the bisporic eight nucleate *Allium* type of development (Dahlgren & Clifford, 1982; Dahlgren *et al.*,

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1985). Unfortunately, the embryo sac formation of *Allium thunbergii* remains to be investigated. So, we investigated the formation pattern, using the traditional light microscopic technique. Furthermore, we have intended to investigate the pattern using a transmission electron microscope, as such an investigation has become to perform occasionally from 1960's. Although the developing embryo sac of *Allium* has examined using the transmission electron microscope, the structure of the mature embryo sac unfortunately has not examined yet (Willemse & van Went, 1984). This paper reports on the developmental pattern and the cytological structure of the embryo sac in *Allium thunbergii*.

Material and Methods

Many flowers at various stages of their development were collected from many individuals of *Allium thunbergii* growing wild at Kiyosato in Yamanashi Prefecture at appropriate intervals extending from August to September in 1987 and 1988. Furthermore, some individuals collected at Kiyosato and at Hakone-machi in Kanagawa Prefecture were transplanted and grown on the campus of the Yokohama National University. The materials for the electron microscope observation were mostly obtained from them. The materials for the light microscope observation were fixed in FAA (formalin-acetic-alcohol). They were embedded in paraffin (m. p. 57–60 C), after they were dehydrated in ethyl alcohol-*tert*. butyl alcohol series. These embedded materials were sectioned at about 6 μm thick, and these sections were stained with Heidenhain's iron alum hematoxilin and fast green FCF combination. For the electron microscope observation, a few ovules were carefully excised as a cluster from each of the ovaries under a binocular and they were fixed in 2.5% glutaraldehyde buffered with 0.1 M phosphate buffer. Some of them were fixed in 2.5% buffered glutaraldehyde that contained 0.1% ruthenium red for staining pectin of cell wall. Furthermore, they were post-fixed with 1% osmium tetroxide in 0.1 M phosphate buffer. These fixed materials were dehydrated in a graded alcohol series and embedded in low viscosity epoxy resin (Spurr, 1969). Thin sections were stained with 1% uranyl acetate and lead citrate and examined under a JEM-100CX electron microscope.

Observation

(1) Ovule

The ovary is divided into three locules, each with two ovules. The placentation is axial. The ovule is composed of a small nucellus invested with two integuments. The angle between the axis of funicle and the axis of nucellus is evidently more than 90° in the ovule where a megasporocyte has just entered the meiosis (Fig. 1A) and it becomes about 90° in the ovule where the meiosis has been over (Fig. 1B). Thus, the ovule gradually and slowly curves. Ulti-

mately, the micropyle which is composed of the inner integument alone faces down toward the placenta in the ovule with a mature but unfertilized embryo sac (Fig. 1D). During the ovular curvature, the nucellus also curves (Figs. 1C, D) and it terminates in the embryo sac. The ovule of *A. thunbergii* seems to be campylotropous rather than anatropous. When the ovule begins to curve or when the meiosis begins in the megasporocyte, the two integuments attain to the tip of the nucellus (Fig. 1A). In the median longitudinal section of the young ovule (Figs. 1A, B), characteristically, the outer integument formed from the lower region of the funicle is much thicker than that formed from the upper region.
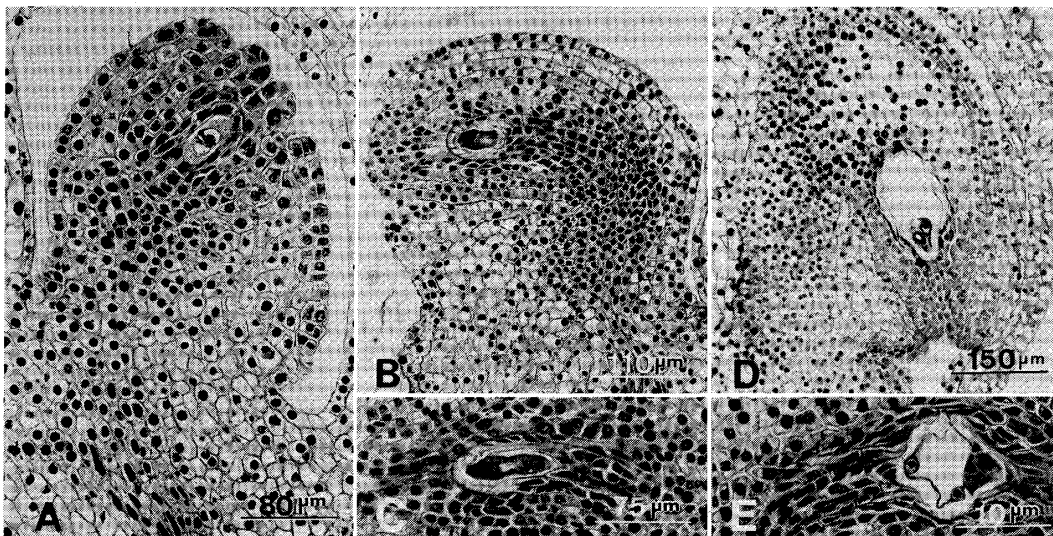

Fig. 1. Development of ovule. A. Young ovule with megasporocyte. B. Ovule with
four nucleate embryo sac. C. Slightly curved nucellus with four nucleate embryo
sac. D. Ovule with mature embryo sac. E. Curved nucellus with mature em-
bryo sac.

(2) Embryo sac formation

An archesporial cell which differentiates directly below the epidermis at the nucellar tip develops into a megasporocyte (Fig. 2A) without any mitotic divisions. It becomes larger and undergoes the first division of meiosis (Fig. 2B) to produce two dyad cells (Fig. 2C), which are separated with a transverse wall. The micropylar cell of the two soon aborts (Fig. 2E) and the second division of meiosis, without the accompanying formation of a wall, produces two free haploid megaspore nuclei in the chalazal dyad cell (Figs. 2D, E). That is, the chalazal dyad cell becomes functional. Two successive mitotic divisions then take place in the functional dyad cell, yielding a mature embryo sac (Fig. 2H). That is, the 7-celled embryo sac matures through the stages of four-nucleate embryo sac (Fig. 2F) and eight-nucleate embryo sac (Fig. 2G) in order. The mature embryo sac is composed of an egg apparatus, three antipodal cells and two polar nuclei. The egg cell is of nearly the same size as the two
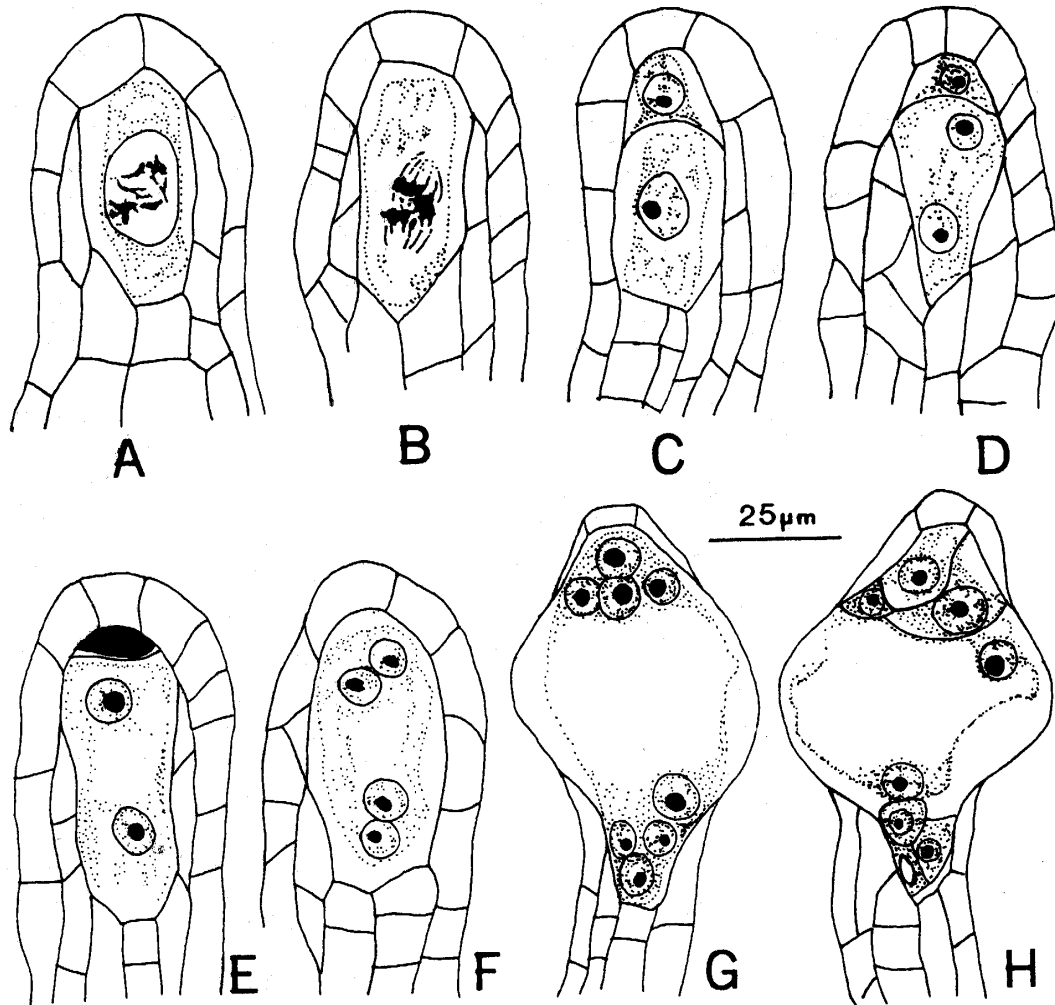
Fig. 2. Development of embryo sac. A. Megasporocyte. B. Megasporocyte at meta-
phase of Meiosis I. C. Two dyad cells, each with one nucleus. D. Functional
chalazal dyad cell with two nuclei and nonfunctional micropylar dyad cell. E.
Two-nucleate embryo sac with degenerated micropylar dyad cell. F. Four-nucleate
embryo sac. G. Eight-nucleate embryo sac. H. 7-celled mature embryo sac.

synergids and it is much larger than each of the antipodal cells. In the egg
cell, its nucleus surrounded by the cytoplasm occupies its chalazal region and a
vacuole does its micropylar region. In both the synergids, conversely, their
nucleus is situated at their micropylar region and a vacuole is situated at their
chalazal region. The synergids have not a filiform apparatus on their surface.
However, the micropylar end of the mature embryo sac is covered with a thick
wall, which probably is produced owing to the degeneration of the epidermal
cells at the nucellar tip during the development of embryo sac. One of the
two polar nuclei is situated near the egg apparatus and the other does near the
antipodal apparatus.

(3) Organelle

There are not a few organelles in the cells in meiotic process (Fig. 3A) and in the developing embryo sacs. In particular, such organelles as mitochondria and plastids which are enclosed with a double membrane are frequent. However, they are very small and their internal structure still develop very poorly. Because of low degree of their differentiation, it is very difficult to distinguish mitochondria from plastids. However, if it is assumed that the slightly oval organelles whose matrix has slightly higher stain property are plastids and the others are mitochondria, the plastids are one forth or one fifth as many as the mitochondria of which more than 150 are counted in a median longitudinal section of the cells in meiotic process and of the poly-nucleate premature embryo sacs. Although mitochondria and plastids are usually scattered all over the cytoplasm of cells in meiotic process and of premature embryo sacs, occasionally they concentrate near the nucleus. The rough endoplasmic reticurum (RER) occurs commonly. There are about ten Golgi apparatus in a median longitudinal section of the cells in meiotic process and of the premature embryo sacs. Each of them is composed of three to five cysternae and a few vesicles near them.

In every cell constructing a 7-celled mature embryo sac, both mitochondrion and plastid become larger than those in the premature embryo sac. Cristae are elaborated in the mitochondrion and thylakoids develop in the plastid, though there is not much difference in size between the mitochondria and the plastid yet. Many of plastids in the egg cell and synergid of the embryo sac in which one synergid has already degenerated have a starch grain or grains in their matrix (Fig. 3D), though such plastids are very meager in the other cells of the embryo sac. Many lipid grains are contained in the cytoplasm of egg cell (Figs. 3B, C) and of the central cell, though they are not viewed in the cytoplasm of the cells in meiotic process and of the premature embryo sac. Cysternae constructing a Golgi stack occasionally become six or more (Fig. 3C) and the diameter of each cysterna becomes larger. The RER occurs commonly all over the cytoplasm of the cells constructing the mature embryo sac.

(4) Cellular envelope
(a) Plasmodesma

Cells constructing a young ovule where the megasporocyte has not entered the meiosis yet are meristematic and their wall is very thin. Plasmodesmata occur in the wall of an archesporial cell which has just differentiated in the nucellus (Fig. 4A). That is, the archesporial cell is kept in contact with its surrounding nucellar cells by the plasmodesmata. In the ovule where the meiosis has just begun in the megasporocyte, the wall thickness of the nucellar cells surrounding the megasporocyte begins to increase in their lateral wall in contact with the megasporocyte (Fig. 4B). In the cell wall of the cells in meiotic process and in the wall wholly enclosing the developing and the mature embryo
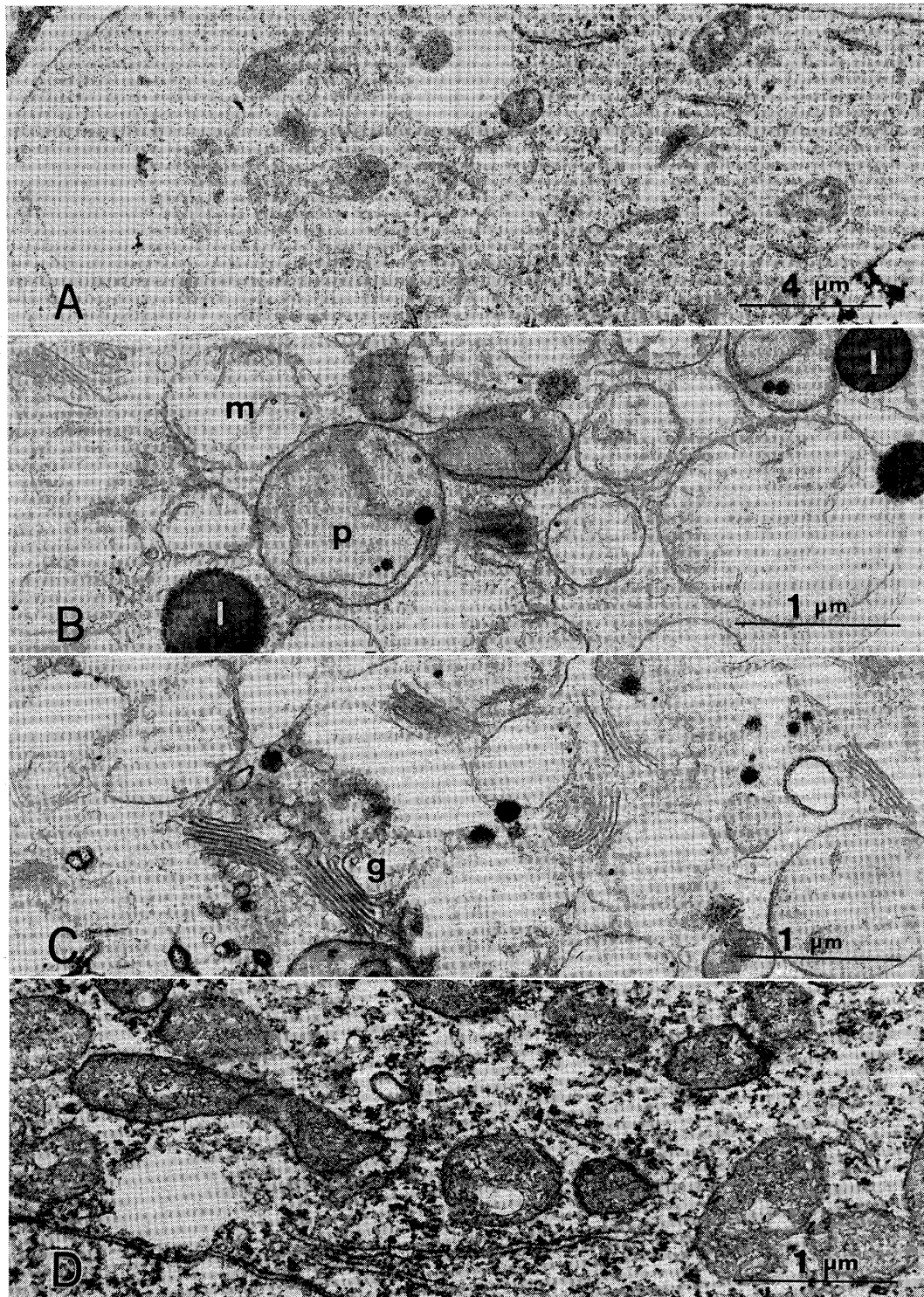
Fig. 3. Organelles. A. Megasporocyte. B. Egg cell. Mitochondria (m), plastids (p) and lipid granules (1) are frequent. C. Egg cell. Golgi apparatus (g) are frequent. D. Synergids. Plastids with a starch grain are frequent.
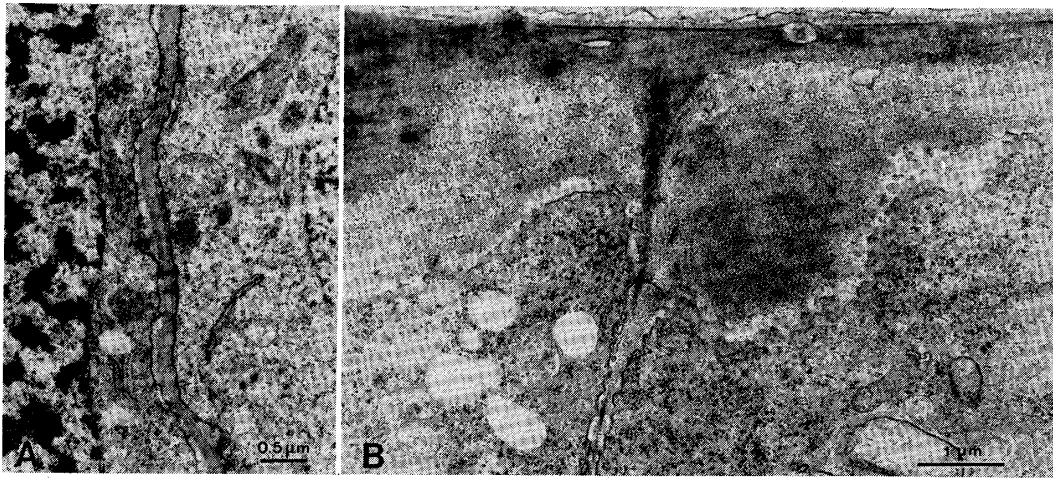
Fig. 4. Plasmodesmata and cell wall of nucellar cell. A. Plasmodesma between archesporial cell and its neighboring cell. B. Wall thickening of nucellar cells adjacent to the megasporocyte.

sacs, plasmodesmata are absent after the onset of this thickening.

(b) Envelope of cells constructing mature embryo sac

The cells constructing a 7-celled mature embryo sac in which two synergids still remain to degenerate are not partitioned by a well-organized cell wall (Figs. 5A, B). The substance strongly stained with ruthenium red accumulates
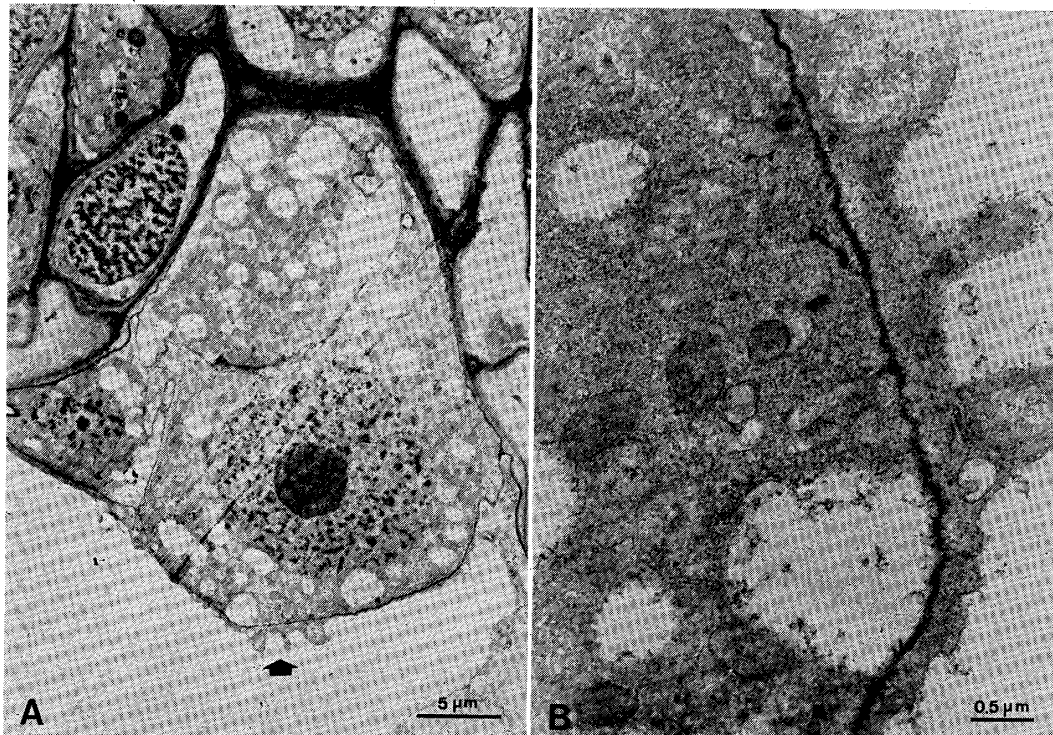

Fig. 5. Antipodal apparatus in 7-celled mature embryo sac. A. Three antipodal cells. B. Thin wall of substance stained with ruthenium red. (magnified figure near arrow in A.)
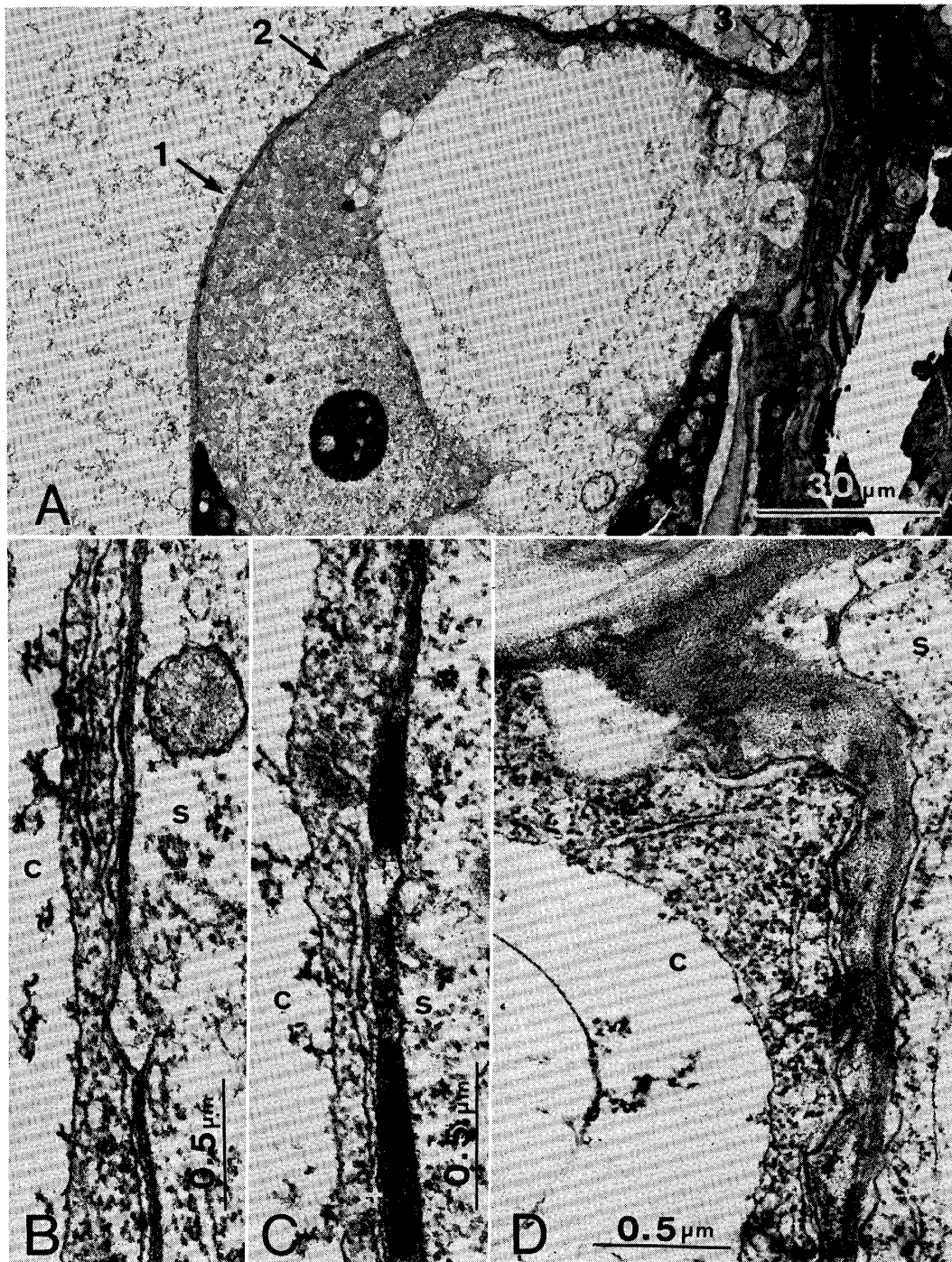
Fig. 6. Synergid in embryo sac where one of synergids has already degenerated. (c:
central cell, s: synergid) A. Persisting synergid. B. Cell membrane of synergid
in close contact with that of central cell. Note the interruption in the contact.
(magnified figure near arrow 1 in A.) C. Interruption in the contacts between
cell membrane of synergid and that of central cell. Note partial occurrence of
the substance stained with ruthenium red in the interruption. (magnified figure
near arrow 2 in A.) D. Cell wall between the membrane of synergid and that
of central cell. (magnified figure near arrow 3 in A.)

along the position where a cell wall is expected to be formed (Fig. 5B). As a result, a thin wall constructed of this substance surrounds these cells. In the sections, each cell of the embryo sac is viewed to be bordered by the thin wall. That is, each of the cells is merely partitioned by the thin wall strongly stained with ruthenium red. Occasionally, the egg cells with a partial lack of the wall at their chalazal end are viewed. Furthermore, a cell membrane also is absent in every cell constructing the 7-celled embryo sac.

However, the cell wholly bordered by the thin wall stained with ruthenium red is absent in the embryo sac in which one of the synergids has already degenerated. In the egg cell, persisting synergid and the central cell of the embryo sac, a cell membrane is formed along the position where the thin wall strongly stained with ruthenium red has been situated. As a result, the membranes wholly enclose these cells (Figs. 6A-D). The cell membrane of the cells constructing the egg apparatus is in close contact with that of the central cell at and near their chalazal end (arrow 1 in Fig. 6A, Fig. 6B). But, at the portion away from their chalazal end, the cell wall intervense between two cell membranes (arrow 3 in Fig. 6A, Fig. 6D). Several interruptions in the contacts between two membranes are present along the cell membranes that link the position where two membranes are in close contact with each other with the position where the wall intervenes between two membranes (arrow 2 in Fig. 6A, Fig. 6C). Some interruptions (Fig. 6C) contain the substance which is strongly stained with ruthenium red, while the others do not (Fig. 6B).

Discussion

The ovule of *Allium thunbergii* is composed of a thin nucellus invested by two integuments. The nucellus has not a parietal cell or cells between the megasporocyte and the epidermis at the nucellar tip. In the section which is cut along axis of the young ovule and which contains the funicle, characteristically, the lower outer integument is much thicker than the upper. Because of the difference in the integument thickness, probably, the ovule gradually and slowly curves to attain to the anatropous condition with a curved nucellus, namely the campylotropous condition. The embryo sac of this species is formed according to the bisporic eight nucleate *Allium* type of development. These embryological features almost coincide with the embryological features of the genus *Allium* summarized by Dahlgren *et al.* (1985) based on the knowledge obtained from the previous investigations.

According to De Boer-De Jeu (1978), in the cells in meiotic process and in the developing embryo sac of the genus *Allium*, there are no plastids with a starch grain or grains, but there are lipid granules, which increase in number after the meiosis has been over. In *Allium thunbergii* examined here, lipid granules occur in the egg cell and synergids, but they do not occur in the cells in meiotic process and in the developing embryo sac. It is well known that there

are plasmodesmata in the wall of cells in meiotic process and in the wall wholly enclosing the developing embryo sac (Willemse & van Went, 1984). But in this species examined here, plasmodesmata occur only in the wall of the archesporical cell and they are not viewed in the wall of cell in meiotic process and of the developing embryo sac. This seems to be related with the wall thickening of the nucellar cell in contact with the cells in meiotic process and with the developing embryo sac. It is known for almost all angiospermous plants hitherto examined that the distribution of organelles changes together with the development of embryo sac (Willemse & van Went, 1984). During the formation of embryo sac of *A. thunbergii*, however, the change of organelle distribution is not viewed conspicuously. In this species the development of the embryo sac seems to be greatly influenced by the changes of nucellar cells. The physiological activity of the egg cell and synergids seems to be high, because the mitochondria, plastids and dictyosomes become larger and become more complicate in structure. It is well known in an unfertilized mature embryo sac and in a sac in which a fertilization has just take place that there is a partial lack of a cell wall along the boundary between cells of an egg apparatus and central cell (Willemse & van Went, 1984). In *A. thunbergii*, also, the same condition is viewed along the boundary between the cells of egg apparatus and the central cell. Furthermore, the interruptions in the contacts between the cell membrane of cells constructing the egg apparatus and that of the central cell are present and the substance stained with ruthenium red is contained in some interruptions. Such interruptions are known for cotton (Jensen, 1965) and *Crepis* (Kuroiwa, 1989). Furthermore, in *A. thunbergii*, each cells of the mature embryo sac is enclosed with the thin wall stained with ruthenium red before the formation of their cell membrane. These substances seem to be related to the formation of the cell wall and the cell membrane in the mature embryo sac or fertilized embryo sac.

摘　　要

　ヤマラッキョウ (*Allium thunbergii*) の胚珠と胚嚢の形成過程を従来の光学顕微鏡的観察に加えて，電子顕微鏡を用いて観察を行った。胚珠は3室に仕切られ各子房室に2個ずつ作られ，湾曲した小形の珠心を2枚の珠皮が被う倒生胚珠 (campylotropous ovule) である。珠心は薄層型 (tenuinucellate) で，パリエタル細胞 (parietal cell) は存在しない。胚嚢は2胞子性8核ネギ型に従って形成される。細胞内小器官は大胞子母細胞の細胞質の中にも多数見られるが，大変未熟なものが多い。そのためにミトコンドリアと色素体の区別をつけがたい小器官が多い。完成した胚嚢の構成細胞では，小器官の区別も可能なものが多くなり，色素体にはデンプン粒を持つものも少なからず見られるようになる。リピッド顆粒は，減数分裂中の細胞や発達中の胚嚢ではほとんど見られないが，卵細胞や中央細胞では普通に見られる。完成した胚嚢を構成する7細胞は，細胞膜や細胞壁で囲まれていることはなく，ルテニウムレッドで濃く染色される薄い層状物質で囲まれている。助細胞の1個が退化した胚嚢の卵装置構成細胞と中央細胞には細胞膜が形成され，それぞれの細胞を囲んでいる。細胞壁も，卵装置構成細胞と中央細胞の境界部分を除いて，形成されている。この

境界部分では隣接する細胞の細胞膜同志が密着しているが，部分的に離生部が見られる。離生部にはルテニウムレッドで染色される物質が含まれているものと含まれていないものがある。7細胞で構成される完成した若い胚嚢で見られるルテニウマレッドで濃く染まる物質の形成が，卵細胞または受精卵における細胞膜と細胞壁の形成の出発点と思われる。

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