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Developmental process of the gametangium in *Pseudobryopsis hainanensis* TSENG (Codiales, Chlorophyceae)

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The developmental process of the gametangium in *Pseudobryopsis hainanensis* was investigated by synchronized culture using light- and electron-microscope. Cytoplasm in ramelli periodically migrates upward and downward in the vegetative stage, accumulating at ramellus bases during dark periods. In the reproductive stage, the cytoplasm aggregates by upward streaming at the site where gametangium formation is expected. Gametangium formation starts by protrusion of the ramellus wall near the aggregated cytoplasm. The gametangium continues to expand for 12 hrs after protrusion. During this period many interphase nuclei migrate from a ramellus into the developing gametangium. A papilla is formed at the distal end of the gametangium. The wall around the papilla is stained by dyes identifying proteins. A plug which isolates the cytoplasm of the gametangium from that of the ramellus is formed in the orifice present in the basal constriction of the gametangium. When a large vacuole occupies the basal portion of the gametangium, nuclei begin to divide for gamete formation.

Key Index Words: *coenocytic green alga*; *gametangium development*; Pseudobryopsis hainanensis; *synchronized culture.*

Ultrastructural investigations have contributed to our understanding of reproductive differentiation in coenocytic green algae (BURR and WEST 1970, MARCHANT and PICKETT-HEAPS 1970, 1971, WHEELER and PAGE 1974, HORI and ENOMOTO 1978). These investigations deal only with those species in which the vegetative cells directly differentiate into the reproductive organ. For instance, when the thallus of *Bryopsis hypnoides* attains reproductive maturity, the side branch is converted into a gametangium by itself (BURR and WEST 1970). The ramellus in *Pseudobryopsis* is morphologically similar to the side branch in *Bryopsis* but the former produces a gametangium on its outer surface. However the ultrastructure in developmental stages of the aforementioned gametangium remains obscure.

In *Pseudobryopsis hainanensis* the main axis is clavate-cylindrical in shape and is surrounded by dense radially arranged ramelli. It originates from rhizome-like creeping filaments at the basal portion. The gametangium born near the proximal portion of the ramellus is provided with a papilla at the distal end and a plug at the base (OKUDA *et al.* 1979). Therefore it is assumed that several morphogenetic regulatories such as positional control of gametangium, local differentiation for papilla and plug formation proceed. The present study clarifies the developmental

process of the gametangium in *P. hainanensis* through light and electron microscopic observations.

Materials and Methods

Experimental organism

Gametophytic plants of *Pseudobryopsis* (mainly clone MK-065*) were used in the present study. This strain, clone MK-065, was obtained at Ayamaru Point, Amami Oshima, Japan on June 2, 1977 and then maintained as unialgal stock culture grown in artificial medium ASP_{12} (PROVASOLI, 1963) at 22°C and 14:10 hr L:D cycle at the Institute of Algological Research, Hokkaido University at Muroran. The gametophytic plants in some experiments were newly collected at the same habitat on May 22–23, 1985 (Fig. 1a). The thalli from these cultured plants were morphologically quite similar to those of the clone MK-065 grown in culture (Fig. 1b).

Culture

Methods of culture and induction of gametangium formation were essentially the same as those described by OKUDA and TATEWAKI (1982). Briefly, plants, precultured at 22°C and 14:10 hr L:D cycle for 3–4 weeks, were re-cultured in a fresh modified medium at 24°C and continuous light. Groups of plants under the same preculture condition synchronously formed gametangia (OKUDA and TATEWAKI 1982). Developmental stages of the gametangia were defined by hours after the beginning of light regime independent of the time when the plants were induced. In the present study, plants were fixed and observed at 3 hr intervals, commencing at the beginning of continuous light regime.

Specimen preparation

Plants were fixed in 0.5% glutaraldehyde

* Although this strain has been reported as *Pseudobryopsis* sp. in our previous papers (OKUDA *et al.* 1979, OKUDA and TATEWAKI 1982), we describe here our strain as a synonym of *Pseudobryopsis hainanensis* TSENG (1936) in accordance with the opinion of KOBARA and CHIHARA (1978), working on the specimen from the same habitat where we collected the material.

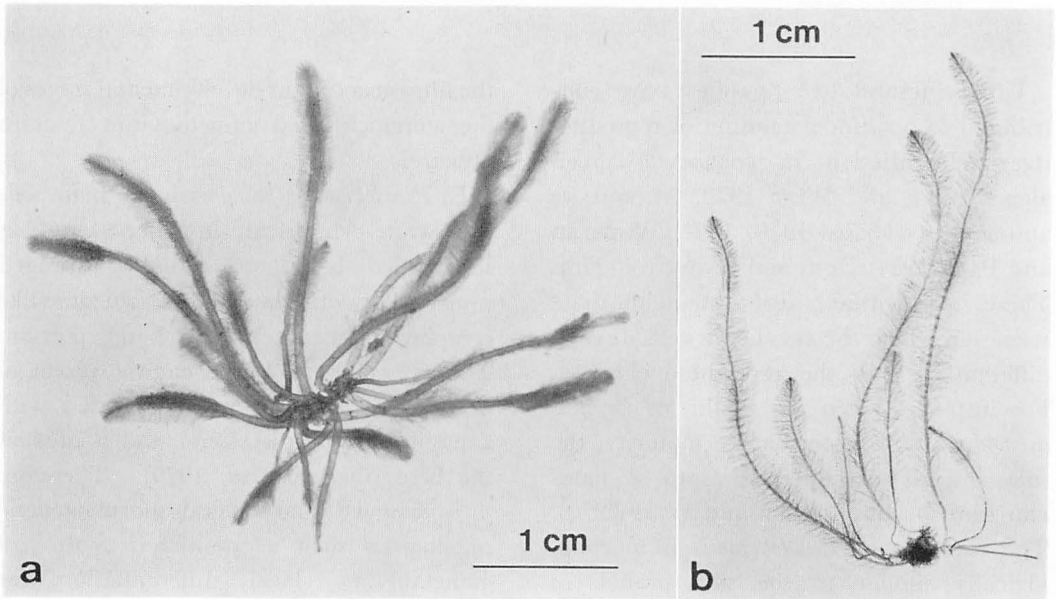


Fig. 1. *Pseudobryopsis hainanensis* TSENG. collected at Amami Oshima Island in 1985 (**a**) and some of them cultured for a month (**b**).

in 10 m*l* culture medium for 5 min. This was followed by 5% glutaraldehyde treatment in 0.1 M sodium cacodylate at pH 7.2 with 50% major salt solution (0.451 M NaCl, 0.052 M $MgSO_4$, 0.018 M $MgCl_2$, 0.008 M KCl and 0.009 M $CaCl_2$) for 25 min at room temperature, and subsequently for 1.5 hr at 5°C. After rinsing briefly in 50% major salt solution, the specimen was washed in a series of 50, 40, 30, 20, 10% major salt concentrations. Then the plants were postfixed with 2% OsO_4 in 0.05 M sodium cacodylate buffer at pH 7.2, for 2 hr. The lateral ramelli were cut off at the base with dissection scissors in cold pure water. These ramelli were dehydrated slowly with an acetone series by 10% increments until 80% at 5°C, then placed at room temperature which followed complete dehydration with absolute acetone. The material dehydrated in fresh absolute acetone was first added dropwise to 1/10 resin diluted with acetone for 2 hr to prevent collapse of cells. Then it was put in a small vial (15 mm × 40 mm) containing 3 m*l* of 1/5 concentration of resin and placed in a sealed box containing 3–4 g well dried Silica gel on a rotator (Penetron Mark IV, Sunkay Laboratories Inc.). After complete evaporation of acetone from this resin mixture, several changes of 100% resin were followed to obtain proper penetration. Finally the resin was polymerized at 70°C for 1 day.

Thin sections were cut with a diamond knife on a Solvall Porter-Blum MT-1, mounted on formvar-coated slot mesh grids, stained with saturated uranyl acetate solution in 50% ethanol for 5 min, and then with lead citrate (Reynolds 1963) for 7 min, and examined in a Hitachi H-300 electron microscope.

Some materials were preserved in 50% ethanol solution at 5°C for several days after fixation with glutaraldehyde and subsequent brief rinsing. These were then stained by either 1% acid fuchsin, 1% amidoblack or 0.5% fast green FCF on a glass slide. Some were treated with 1% pepsin or 1% protease for 30 min before staining.

Result

Gametangium formation

The first visual indication of gametangial differentiation in *Pseudobryopsis hainanensis* is a local accumulation of the cytoplasm around the central vacuole below the apex of a ramellus at 0–9 hr (Figs. 2c, 4a and 4b). Many chloroplasts are seen in this area,

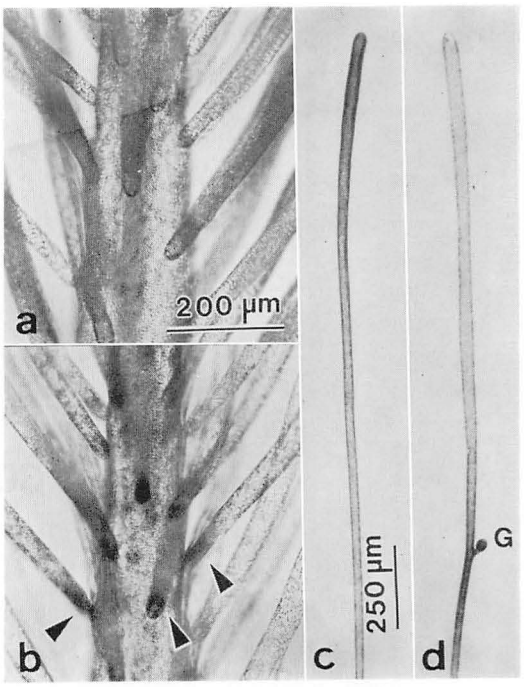


Fig. 2. Changes of cytoplasmic distribution in ramellus. Ramellus bases during light (**a**) and dark period (**b**) in vegetative stage. Arrowheads indicate accumulation of cytoplasm during dark period. Ramelli at 9 hr (**c**) and 18 hr (**d**) of reproductive stage. G, gametangium protruding at the lower part of ramellus. Scale bars in **a** and **c** apply to **b** and **d** respectively.

but the number decreases toward the base. They contain no starch or small grains, if present, until 3 hr (Fig. 4a), but large ones at about 6 hr (Fig. 4b). Then, at 12–15 hr the aggregated cytoplasm gradually migrates downward and accumulates at ramellus bases as that during the dark period in the vegetative stage (Fig. 2b). At 18–21 hr, the cytoplasm (Fig. 3a) migrates a little above the ramellus bases where gametangium formation initiates (Fig. 2d).

The surface view of the initiation of gametangium looks like a hyaloid circle (Fig. 3b), and its lateral view like a convex lense (Fig. 3c). The cytoplasm of this portion is fenestrated due to ER (endoplasmic reticulum) and vacuolar evagination (Fig. 4d) and contains many nuclei (Fig. 4c). Most of the chloroplasts are limited to the regions adjacent to a central vacuole. At about 21 hr the gametangium initial begins to protrude upward (Figs. 3d and 5a), many small vesicles are produced in the cytoplasm just below the wall of the gametangium (Fig. 5d). At 24 hr, vacuoles in the cytoplasm gradually enlarge (Fig. 5d), concomitant with the expansion of a gametangium initial (Fig. 3e) which develops a stalk (Figs. 3f and 3g). At 27 hr, a large vacuole occupies the center of the gametangium and its ramifications intrudes the peripheral cytoplasm (Fig. 5b). At 30 hr, the gametangium completely expands (Fig. 3g) and a gametangial central vacuole shifts towards the basal part (Fig. 5c). The cytoplasm is divided

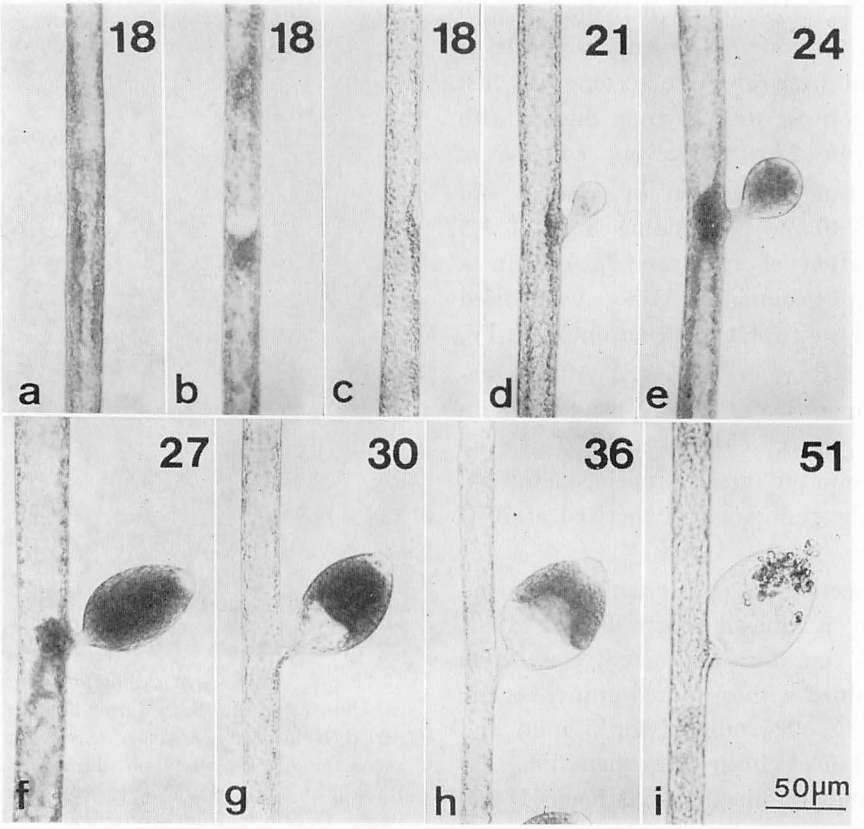


Fig. 3. Light micrographs showing developmental stages of a gametangium. Numerals at the upper right side show the time of the development in hr. Scale bar in **i** applies to all figures.

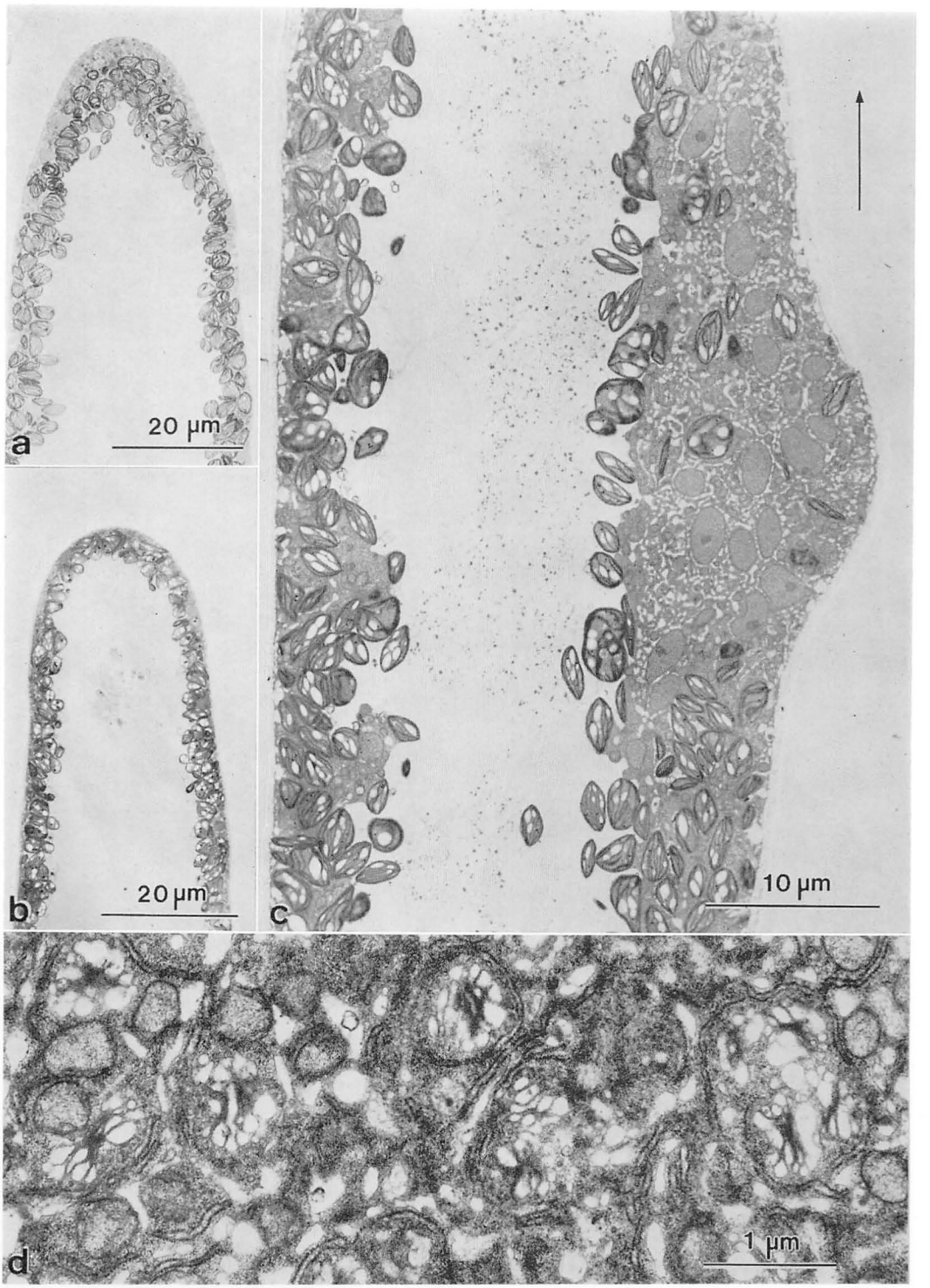


Fig. 4. Electron micrographs of longitudinal sections of ramellus. Apical portion at 03 hr (**a**) and 06 hr (**b**), cytoplasmic aggregation (**c**) and reticulated cytoplasm (**d**) at the site where gametangium formation is expected at 18 hr. Arrow indicates direction of ramellus apex.

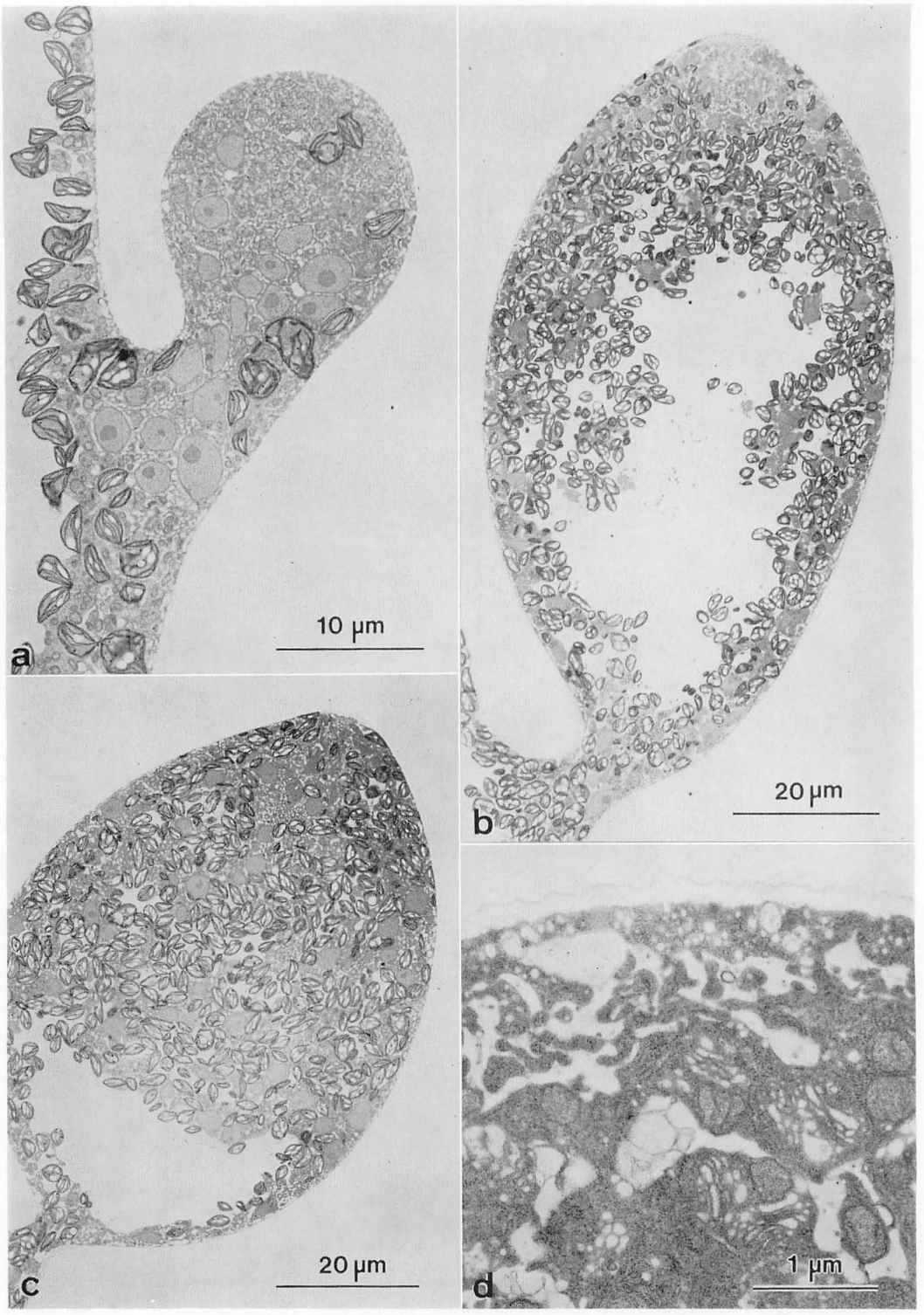


Fig. 5. Electron micrographs showing the developmental stages of a gametangium. **a**, early stage of gametangium formation at 21 hr, showing reticulated cytoplasm containing many nuclei. **b**, gametangium at 27 hr. Note the vesiculate cytoplasm located at the distal portion, from where chloroplasts are excluded. **c**, gametangium at 30 hr. **d**, many small vesicles and dictyosomes in peripheral cytoplasm of gametangium at 24 hr.

into two parts by the gametangial vacuole; a large portion which is later differentiated into gametes and a small one which is later involved in plug formation.

Plug formation

By 30 hr the cytoplasm migrates into the gametangium through the orifice which is plugged at 33–36 hr (Fig. 6e). At 30 hr, many electron dense, fine granules appear in vacuoles present in the stalk (Fig. 6b). At 33 hr, the plug material is deposited centripetally on the inner side of the stalk (Fig. 6a). It is successively deposited on both gametangium and ramellus side of a plug (Figs. 6c and 6d) and eventually results in separating the gametangial cytoplasm from that of the ramellus at 36–39 hr (Fig. 6e).

The forming plug contains electron dense granules and bubble-like globules (Figs. 6c and 6d) which overlie successively and form some ordered arrays (Fig. 6a). Dense vesicles are present in the cytoplasm near the plug (Figs. 6c and 6d), but their role in plug formation is unknown.

Papilla formation

At 27–30 hr, a papilla differentiates at the distal end of a mature gametangium (Figs. 3f and 3g). Sooner or later it becomes more translucent, because the inner side of the cell wall of the papilla is dissolved into a loosely disorganized layer of granules and the ramifying cytoplasm containing many vesicles and multivesicular bodies intrude it (Figs. 7a and 7b). At 33–36 hr, the cytoplasm retracts from the wall of the papilla (Fig. 7c) and the apex becomes somewhat flat (Fig. 3h). Finally the papilla changes to a pore for gamete liberation (Fig. 3i).

The papilla wall is stained by either acid fuchsin, fast green or amido black (Fig. 7d), but not when treated with pepsin or protease before staining (Fig. 7e).

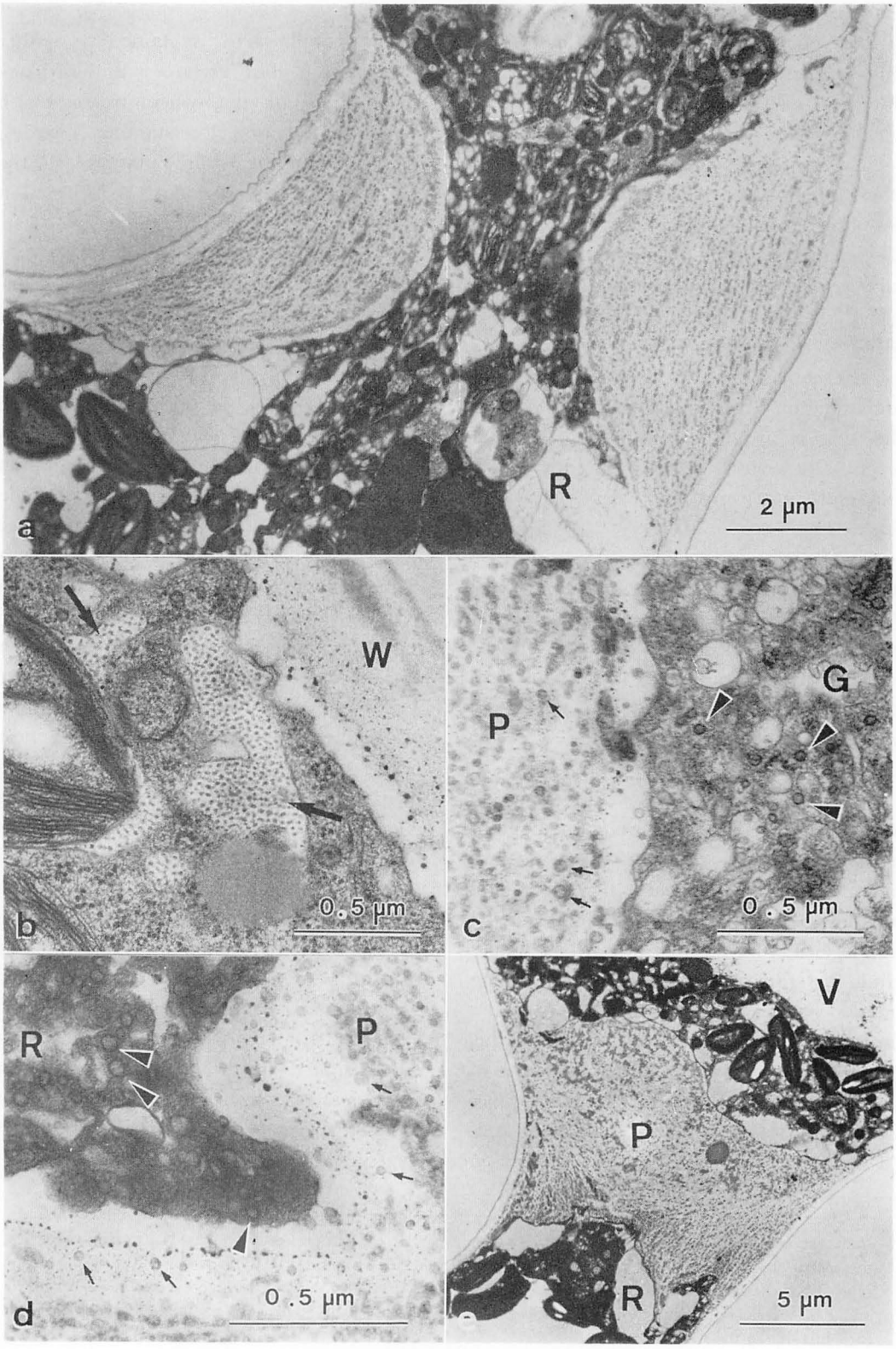
A diagramatic representation of the gametangial development is given in Fig. 8.

Discussion

It has been suggested that the photosynthesis-dependent accumulation of a large amount of starch is prerequisite for the induction of sexual cell division in *Closterium* (ICHIMURA 1971). The accumulation of starch grains in chloroplasts of the ramellus seems to be essential for the cytoplasmic aggregation and subsequent induction of gametangium formation in *Pseudobryopsis hainanensis*.

In the vegetative stage of *P. hainanensis* and *Bryopsis hypnoides* (BURR and WEST 1970) the following periodical changes occur due to cytoplasmic streaming: most of the cytoplasm accumulates at ramellus bases during the dark period, while it is widely distributed in ramelli during the light period, though being more aggregated at ramellus apices. In the reproductive stage of *P. hainanensis* the cytoplasm at the ramellus bases gradually migrates towards the site where the gametangium is to be formed, possibly by upward cytoplasmic streaming. After that, the change in cytoplasmic distribution differs from that in the vegetative stage, because the cytoplasm seems to be trapped at the site where the gametangium is formed so that this cyto-

Fig. 6. Formation of a plug. **a**–**e** show electron micrographs of longitudinal sections of gametangial stalk. **a**, centripetal growth of plug resulting in constriction of cytoplasm at 33 hr. R, ramellus side of the stalk. **b**, dense granules (arrows) in cytoplasmic vacuole at 30 hr. W, stalk wall. **c** and **d**, growing plug (P) at the side of gametangium (G) and ramellus (R) at 36 hr, showing bubble-like globules (arrows) and dense vesicles (arrowheads). **e**, a plug formed completely at 36 hr. V, gametangial vacuole; R, ramellus side of the stalk.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plasm may be subsequently supplied to the developing gametangium. In *Acetabularia* the cytoplasm in the stalk migrates into cap rays when the cap attains a maximum diameter (SCHULZE 1939). This is similar to the migration of the cytoplasm into gametangia in *Pseudobryopsis*, but the gametangial development in *Pseudobryopsis* is accompanied by the cytoplasmic migration, whereas the cap is already formed prior to the migration.

The role assigned to the cytoplasm varies depending on the developmental stage of gametangium and the position of cytoplasm. Thus for non-septate cell differentiation, like gametangium formation in *Pseudobryopsis*, time- and position-dependent functional change of the cytoplasm seems to be important.

In *P. hainanensis* the gametangium can be produced only on the ramellus but not on the main axis. The ramellus hardly produces branches, so that it is functionally different from the main axis. Further, both the main axis and the ramellus never transform into the gametangium. Two steps of differentiation, ramellus formation and gametangium formation, are necessary in reproduction stage of *P. hainanensis*.

Caulerpa okamurae is an example of zero step differentiation. In this alga the entire protoplasmic content of the highly differentiated vegetative thallus is converted into gametes (ISHIWARA *et al.* 1981).

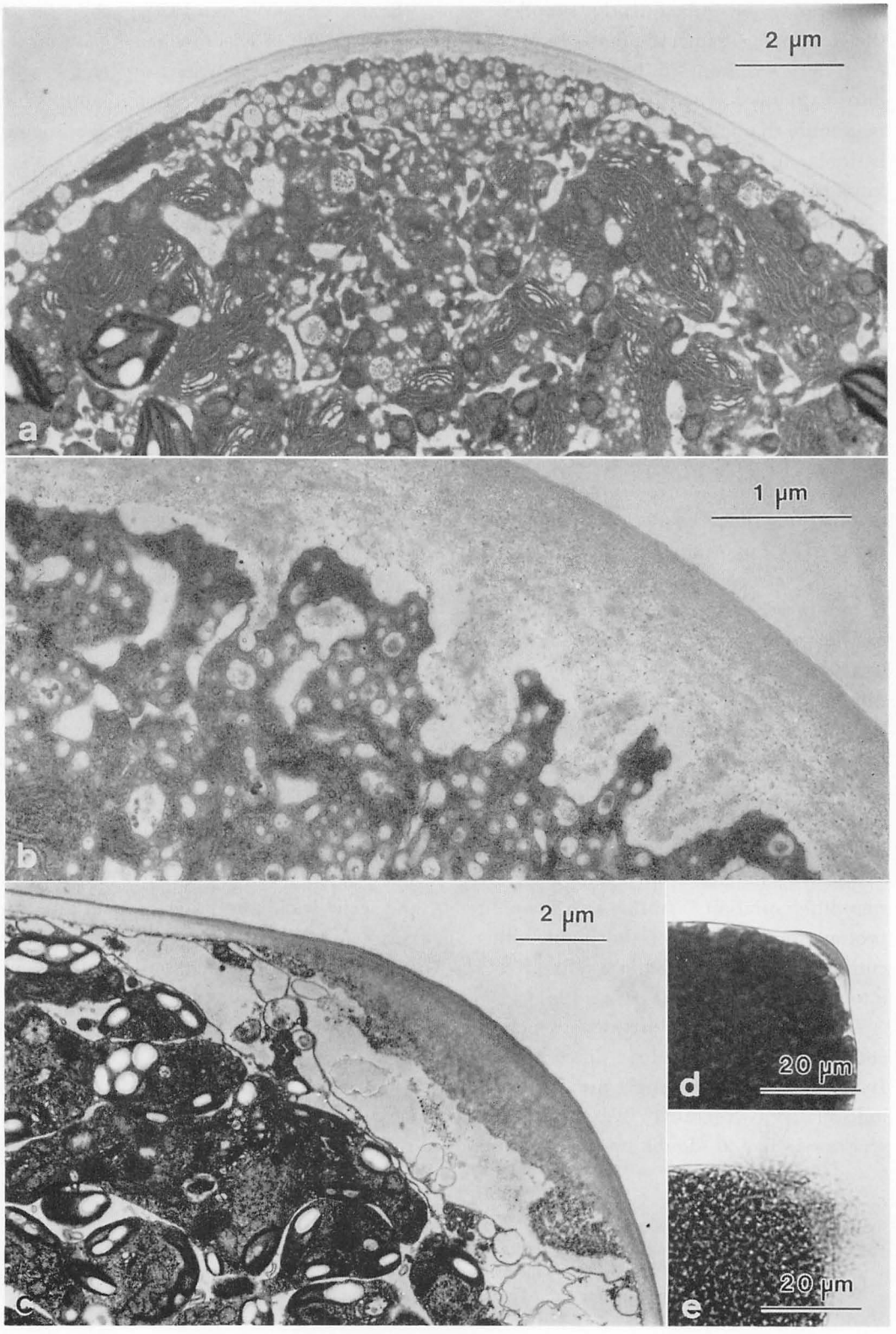
Derbesia and *Bryopsis* are algae which are endowed with one step differentiation. In *D. marina* the sporangia are formed on indistinctive, vegetative filaments, being separated by a double walled septum (SEARS and WILCE 1970); while in *B. hypnoides* the side branches separated by a plug from a main axis are transformed into the gametangia (BURR and WEST 1970). In these species the sporangium formation or the side branch formation is a differentiation step. However such sporangia and side branches often revert to a vegetative stage (SEARS and WILCE 1970, BURR and WEST 1970). Therefore, the differentiation of the reproductive organs of these two algae does not seem to be an elaborate differentiation. In *Codium* the gametangia are formed on an utricle which is produced on the tip of medullary interwoven siphons through two-step differentiation, though the gametangia often revert to vegetative daughter utricles in *C. giraffa* (SILVA 1979).

WERZ (1970) noticed the appearance of ER and small vesicles in the early stage of the development of whorls or cap rays in *Acetabularia*. According to him, ER is specifically associated with the region of the cell wall where hydrolysis takes place. In *P. hainanensis* ER is well developed in the aggregated cytoplasm when the gametangium formation starts. Subsequently, the gametangium protrudes and many small vesicles which seem to contribute to the cell wall formation appear in the peripheral cytoplasm. This suggests that expansion of the gametangial wall follows hydrolysis of the ramellus wall.

When expansion of the gametangium is almost complete, two kinds of local differentiation occur in the gametangium: papilla and plug formations.

WHEELER and PAGE (1974) reported

Fig. 7. Formation of papilla. **a–c** show electron micrographs of papilla at the distal portion of gametangium. **a**, many vesicles and dictyosomes in cytoplasm at 27 hr. **b**, dissolved papilla wall at 30 hr. **c**, swelled papilla wall at 39 hr, showing change in wall structure from homogenous transparent material to granular nature. Gametangium stained with acid fuchsin solution after treatment with protease (**e**) and without protease (**d**).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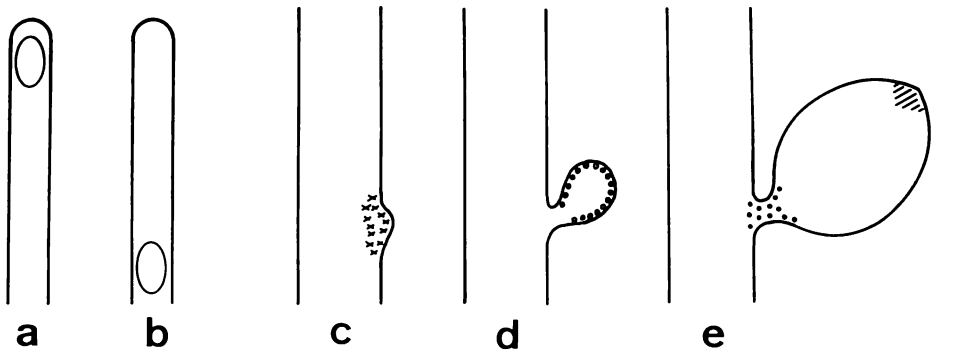

Fig. 8. Diagram showing changes in the distribution and function of cytoplasm during gametangium formation. Cytoplasm absorbing light energy for gametangium formation at upper part of ramellus (**a**) migrates downward and accumulates in ramellus base (**b**). Then the cytoplasm migrates upward to the site expected for gametangium formation. **c–e**. Distribution and function of local cytoplasm. **c**, cytoplasm softening ramellus wall at definite point (xed area). **d**, peripheral cytoplasm (small circles) synthesizing wall of developing gametangium. **e**, apical cytoplasm (oblique lines) degenerating papilla wall and basal cytoplasm (dotted area) secreting plug material.

that in *Derbesia* gametophytes, the degradation of the papilla wall takes place prior to gamete discharge and that many clustered small mitochondria and ER appear in the cytoplasm adjacent to this portion. They have considered that some hydrolytic enzyme is produced in the region and contributes to the wall degradation. In *P. hainanensis* the papilla wall is disorganized and can easily break down at gamete discharge. The papilla wall stained with dyes identifying protein, suggests that the wall contains such protein enzymes.

The development and fine structure of the plug have been described in detail in *Bryopsis hypnoides* (Burr and West 1971) and *Acetabularia acetabulum* (Menzel 1981). The developmental pattern of plug formation and the ultimate fine structure of plugs differ from species to species, although the plug of siphonous green algae may be homologous in structure on the basis of the occurrence of peroxidase (Menzel 1980, 1981). The branch plug in *Bryopsis* is usually composed of three layers: a middle proteinous layer derived from vacuolar protein bodies and two polysaccharide layers, which are structurally similar to the thallus wall (Burr and West 1971). The cap ray plug in *Acetabularia* consists of a voluminous, sponge-like structure, gaps between which are filled with condensed material (Menzel 1981). Unlike *Bryopsis* and *Acetabularia* the gametangial plug in *Pseudobryopsis* consists of electron transparent material with many dense, bubble-like globules. The cytoplasm involved in the plug formation remains near the plug and is never converted into gametes.

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奥田一雄*・榎本幸人・舘脇正和***：多核緑藻ハネモモドキの配偶子嚢の発達過程**

ハネモモドキの配偶子嚢の発達過程を同調培養と光顕及び電顕観察によって解析した。

栄養期の側枝の細胞質の大部分は，明期に頂端部，暗期に基部に集積する。配偶子嚢形成が誘起されると，側枝基部に集積していた細胞質が基部より少し上方の位置に移動する。集合した細胞質は，多数の核と大量の ER を含んでいる。細胞質が集合してくる位置で側枝の細胞壁が突出し，配偶子嚢に発達する。配偶子嚢の成長拡大に伴って側枝にある細胞質が配偶子嚢へ流入する。配偶子嚢の細胞壁に接する細胞質に多数の小胞が出現する。配偶子嚢の頂端部の細胞壁は電子密度が増加し，小突起となる。隔壁（plug）は，配偶子嚢と側枝の間の細胞壁に壁物質が蓄積することによって形成され，配偶子嚢の細胞質を側枝から分離する。配偶子嚢の細胞質の大部分が上方に集合し，下方部に大きな液胞が占めるようになった時，配偶子形成のための核分裂が始まる。

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