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Fine Structural Study of Pollen Wall Development at Late Stage of Microsporogenesis in *Panax ginseng*

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인삼의 화분벽 발달에 관한 미세구조적 연구

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ABSTRACT

The ontogeny of pollen wall in *Panax ginseng* was studied with transmission and scanning electron microscopy from early tetrad stage until pollen maturity. Initial indication of exine development is undulation of plasma membrane for the preparation of bacular mound. The first recognizable structure of the pollen wall is the cellulosic primexine which is formed outside of the plasma membrane while microspore tetrads are still surrounded by callose wall. As development proceeds, foot layer and baculum differentiation, callose dissolution and exine formation were progressed. During this process, sporopollenin is deposited into the exine, and then endexine development was followed. The intine, innermost pollen wall layer, is developing form hypertrophic Golgi vesicles. The thickness of exine is very even on all along the pollen wall, but intine thickness of apertural region is thicker than that of nonapertural region. Mature pollen of ginseng is 20 μm in size, tricolpate and shows fine reticulate sculpturing.

Key words : Development, Fine structure, Ginseng, Pollen wall

INTRODUCTION

Two principal layers of pollen wall are intine and exine. The inner, pecto-cellulosic in nature and more or less uniform configuration is intine. The exine is the outer, acetolysis-resistant layer, and also resistant to physical and biological degradation (Bhojwani & Bhatnagar, 1999). Because of this property of the exine, pollen grains are well preserved in fossil deposits for a long periods of time.

Fine structures of pollen wall have been described from many species since last thirty years, but the organizations of macromolecules to produce exine morphology are still unknown. The sculpturing of gymnosperm pollen was known as less regular and complex than that of angiosperms. The exine generally consist of two layers, an outer sculpturing layer called sexine and inner

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non-sculpturing lamellated layer called nexine (Southworth, 1985).

Sexine further comprises roof-like outer layer called tectum, and internal layer of upright rod-like elements, called bacula. Nexine is also comprised as two layers, foot-layer and endexine. The exine is typically four layers consisting of tectum, bacula, foot-layer and endexine (Bhojwani & Bhatnagar, 1999; El-Ghazaly et al., 2001). However, the exine of *Geranium* is characterized by a perforated foot-layer and missing endexine (Stafford & Blackmore, 1991), therefore pollen wall is consisted with three layers-tectum, unbranched bacula and perforated foot-layer (Weber, 1996).

Although the ultrastructure of mature pollen exine and the pattern of developmental changes leading to pollen wall formation have been studied in numerous genera such as *Cosmos* (Dickinson & Potter, 1976), *Helianthus* (Horner & Pearson, 1978), *Artemisia* (Rowley et al., 1981), *Geranium* (Weber, 1996), *Arabidopsis* (Owen & Makaroff, 1995), *Apium* (Weber, 1989), *Ledebouria* (Hess, 1993), *Hibiscus* (Kim & Kim, 1995), *Nelumbo* (Kreunen & Osborn, 1999), Apium (Weber, 1992), no satisfactory explanation for the precise developmental pattern of pollen wall has not yet investigated. And the process of sporopollenin deposition into the exine has not been understood in ultrastructural level during micorsporogenesis.

Therefore, the purpose of this study is to describe fine structural changes of pollen wall development. This research addresses two specific questions. First, what are the ultrastructural similities and/or differences in the origin and development of exine in ginseng comparing other species, second, what cellular or extracellular structures are engaged in the development of exine sculpturing pattern.

MATERIALS AND METHODS

For transmission electron microscopy, anthers of 4-years-old ginseng (*Panax ginseng* C.A. Meyer) were primary fixed in 1% paraformaldehyde-2% glutaraldehyde in 100 mM cacodylate buffer (pH 7.0) for 2 hrs at 4°C. After rinsing in buffer for three times in 20 minutes each, secondary fixation was carried out in several changes (5, 10, 20 min) of fixative containing 2% (w/v) osmium tetroxide, 0.8% (w/v) potassium hexacyanoferrate ($K_4Fe(CN)_6$) in 100 mM cacodylate buffer at pH 7.0 before exposing to final fixative for 1 hr at room temperature. After rinsing in buffer, washing in distilled water was carried out for 10 minutes of three times before dehydration. After dehydration in ethanol series at room temperature, embeddment in Spurr's low viscosity resin (Polyscience) and polymerization were carried out at 60°C for 24 hrs. Ultrathin sections were made using Leica ultramicrotome (Ultracut E) and collected on formvar coated nickel grids (100 mesh). Sections were conventionally double stained with uranyl acetate and lead citrate before observation with JEOL 1200EX-II electron microscope operating at 80 kV.

For scanning electron microscopy, mature pollen grains were collected from dehiscent anther, fixed and washed same as transmission electron microscopy. After complete dehydration in absolute ethanol, isoamyl-acetate substitution was carried out for critical point drying. Hitachi E-1030 ion sputter was used for platinum coating with 50 nm thickness. Platinum coated pollen grains were observed with Hitachi S-4200 scanning electron microscope at 15 kV.

RESULTS

Development of exine, outer layer of pollen wall, starts at the time when the tetrad is still enclosed in the callose wall at tetrad stage. In early tetrads, the microspore plasma membrane is initially straight and in direct contact with callose wall (Fig. 1). The earliest indication of pollen wall development is the thickening of plasma membrane and formation of lamellar structure at the

outside of plasma membrane. At this stage of development, undulation of the plasma membrane in each microspore occur beneath the thickenings of membranaceous structure (Fig. 2).

Thickenings of membranaceous structure and undulation of plasma membrane were widen, and more and more clearly visible at primexine stage (Fig. 3). Membranaceous structures began to change into fibrous structure. Dissolution of callose wall is initiated at this stage (Fig. 4). Young primexine has a lamellar appearance especially on the convex surface of plasma membrane, and undulation of plasma membrane is more progressed, and therefore clearly visible. In late tetrad stage, the formation of primexine, represented the first indication of the pollen wall formation. Primexine shows fibrous appearance (Fig. 5).

During the baculum formation stage (Figs. 6–8), undulated convex surfaces of plasma membrane are changed into bacular mound. Primexine with microfibrilar cellulosic layer beneath the callose wall, has uniformly 50 nm in thickness at early stage of development. Fibro–granular structure of probaculum is began to be developed at the bacular mound for connection with primexine (Fig. 6).

The probaculum gradually developed into the baculum, which is the vertical element in pollen wall. The fibrous primexine still remains, and is easily distinguished from more or less granular callose wall which still envelops the developing microspore (Fig. 7). As development proceeds, the baculum shows lateral extention at bacular mound to form foot–layer. At this stage, the tectum formation was initiated at the outside of the primexine in nonsculpturing region (Fig. 8).

At early stage of exine maturation, the baculum as well as foot–layer is well developed and therefore, exine cavities are formed in–between tectum and ektexine. Remnants of callose dissolutions are observed in the exine cavities (Fig. 9. arrowheads). Tectum is partially spaced at late exine maturation stage (Fig. 9), and therefore the tectum of mature pollen appears as a fine reticulum (Fig. 14).

The tectum and foot–layer show equivalent thickness at the time when pollen grains are mature. Two outstanding features at pollen maturity are the appearance of white lines in the foot–layer, and parallel development of rER with pollen wall at the periphery of cytoplasm (Fig. 10). Tapetal cells are still intact at late exine maturation stage, even tangential cell walls are already degenerated. Pollenkitt droplets (Figs. 11–12. arrows) are characteristically located in exine cavities (Fig. 11).

After development of ektexine–composed of foot–layer, baculum and tectum–endexine follows its development. Increased electron density can be differentiated between ektexine and endexine in electron micrograph. Before beginning of the development of intine which is pecto–cellulosic in nature, exine development was completed. Intine formation was occurred by the active development of hypertrophic Golgi vesicles at the periphery of pollen cytoplasm. Fibrous materials (arrowheads) are begin to deposited at the endexine by fusion of Golgi vesicles with plasma membrane (Fig. 12).

Mature pollen grain shows uniformly thick exine at both apertural and non–apertural region. It shows relatively thin tectum (0.5 μm), well defined bacula, and thick homogenous nexine (1.0 μm). On the other hand, intine has 50 nm thickness in non–apertural region, and 1.7 μm thickness in apertural region. Fibrilar wall structures of endexine are observed in apertural region (Fig. 13). The mature pollen of ginseng, about 20 μm in size, shows tricolpate, semi–tectate and fine reticulate in its external tectum sculpturings (Fig. 14).

DISCUSSION

The primexine is the first–formed component of the pollen wall, and there have been some discussions concerning its chemical nature. From a comparative study of the primexine in *Helleborus* species, electron microscopic configuration is considered as cellulosic (Echlin & Godwin, 1969). And initial indication of exine devel-

opment is the formation of primexine on the plasma membrane (Takahashi & Skvarla, 1990).

More definite information on the chemical nature of primexine has been obtained by cellulase digestion of tetrad during exine development stage in *Lilium* (Heslop-Harrison, 1968). The primexine has been known to be essential for complete and perfect development of exine. Furthermore, the primexine matrix shows a loose and irregular fibrilar texture at early tetrad stage of microspore development in *Rondeletia odorata* (El-Ghazaly et al., 2001). It was also considered that the primexine is the primary location of sporopollenin deposition (Kreunen & Osborn, 1999).

The undulation of plasma membrane, at early tetrad stage for preparing of primexine development, is the genaral feature at early stage of pollen wall biogenesis. In this paper, developmental processes of primexine in ginseng has no significant differences comparing *Helleborus* and *Rondeletia*. The fibrous configurations of early primexine (Figs. 3-5) are considered that its nature is cellulosic. Regular undulation of plasma membrane is also thought to be the site of bacular mound in this study.

Developmental processeses of baculum and foot-layer of *Oenothera biennis* shows columella-like structures with high electron density at late tetrad stage (Takahashi & Skvarla, 1990). The baculum in *Rondeletia* pollen is the first formed wall component synthesized in the fibrilar plasmamembrane coat at early tetrad stage. Later in development, the center of baculum was been obscured by material of the same electron density as sporopollenin.

The foot-layer was scarcely visible at early stage, but it became more pronounced and similar stainability as the tectum. And the contact region of endexine and foot-layer shows white lines in *Rondeletia odorata* (El-Ghazaly et al., 2001), and similar structures were observed in other species (Xi & Wang, 1989; Rowley, 1996). These white lines were considered as the core center of wall components (El-Ghazaly et al., 2001).

At exine maturation stage in ginseng, the white line (Figs. 10, 11) was clearly visible only at the stage of active sporopollenin deposition stage, and therefore it was considered as core site of sporopollenin deposition. This discussion could be supported by the appearance of the white lines in the orbicule which is located in the same thecal fluid at late exine formation stage (unpublished data).

The intine is characterized by a fibrailar appearance which is quite distinct from the homogenous appearance of the exine. It has not been well understood whether first appearance of intine occur before completion of exine formation or any other stage of development (Kreunen & Osborn, 1999). Intine development was known to be occurred after mitosis of microspore (Roland, 1971), however, its formation was initiated before mitosis in *Rondeletia* (Heslop-Harrison, 1968). Although the exact timing of intine initiation is different from species to species of plants, Golgi vesicles are involved its development by exocytosis of the intine precursors into the periplasmic space (Suarez-Cervera & Seoane-Camba, 1986; El-Ghazaly et al., 2001).

Exact stage of intine formation is not clear in ginseng, but hypertrophic Golgi complexes developed in all periphery of pollen cytoplasm after exine formation (Fig, 12). Chemical nature of intine was known to be cellulosic, the inclusions in the Golgi vesicles show fine fibrilar textures (Fig. 12. arrowheads). Electron densities of intine in mature pollen grain are very low comparing high densities of exine (Fig. 13).

The complex exine structures are the storage sites for carbohydrates, proteins, lipids, terpenoids, and phenolics, all compounds of different chemical structure (Wiermann & Gubatz, 1992). The pollen wall between non-apertural and apertural region showed non-uniform thickness in *Brasenia schreberi* (Osborn et al., 1991). This irregular thickness was also observed in *Rondeletia odorata* such as bacula widen, foot-layer becomes thicker, and endexine lamellae develop in non-apertural region. At the apertural region, on the other hand, ba-

cula widen, additional foot-layer is deposited, and endexine lamellae thicken and anastomose (Kreunen & Osborn, 1999). Exine development in *Arabidopsis* under space flight Endeavour STS-54 was investigated and showed no differences comparing ground cultivated species. Otherwise, the microspore cytoplasm became disorganized, no viable pollen was developed in space flight cultivated anther (Kuang et al., 1995).

Pollen wall development begins at early tetrad stage and finished at free spore stage. And ektexine layers mature more or less simultaneously; tectal elements thicken, the baculum widens, and foot-layer thicken. On the other hand, even though structural development showed simultaneity, sporopollenin deposition into the exine showed unsimultaneity (Kreunen & Osborn, 1999). Endexine development occurs after ektexine for-mation (Blackmore & Barnes, 1990), but it is difficult to distinguish between endexine to ektexine. Furthermore, the endexine and foot-layer commonly have similar electron densities (Weber, 1998).

At late of exine formation stage, electron densities of ektexine and endexine show no differences (Figs. 10, 11), but at pollen maturity, endexine shows higher electron density than ektexine at intine formation stage (Fig. 12) and in mature pollen (Fig. 13). The thickness of exine is very even in both apertural and non-apertural region in ginseng , and tectum is relatively thin (0.5 μm) comparing nexine (1.0 μm). On the other hand, the thickness of intine is not even, such as 50 nm in non-apertural region and 1.7 μm in apertural region.

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<국문초록>

인삼의 소포자 발달에 따라 화분벽의 형성과정을 밝히고자 소포자 4분자 시기부터 화분이 성숙되기까지의 전 과정을 투과 및 주사 전자현미경으로 관찰하였다. 화분벽의 발달은 감수분열이 끝나고 소포자 4분자가 callose에 둘러싸여 있을 때 시작된다. 화분벽 발달 초기에는 원형질막이 두터워지고 구불구불해지며 원형질막 바깥쪽에 섬유성 구조물이 나타나기 시작하고 이 섬유성 구조물은 점점 뚜렷하게 나타나고 premexine으로 발달한다. 원형질막의 함입으로 형성된 돌출부와 premexine이 연결되어 단간이 발달하고 성숙화분에서는 endexine에 일시적으로 흰색의 선이 관찰되었다. 표벽발달이 완료되면 hypertrophic Golgi에서 형성되는골지소낭에 의하여 내벽이 발달하고 발아구 부위에서는 내벽이 비후되어 나타났다. 성숙한 인삼화분은 3구형 화분으로서 약 20μm 크기이며 표벽무늬는 세망상형을 나타내었다.

FIGURE LEGENDS

Figs. 1–5. primexine formation stage.

Fig. 1. Undifferentiated microspore, still surrounded with the callose wall (C), shows even plasma membrane (PM) at late tetrad stage. bar = 0.4 μm

Fig. 2. Thickening of plasma membrane and myeline structure were thought to be preparations of exine formation. bar = 0.2 μm

Fig. 3. Undulation of plasma membrane (PM), at early stage, is the initial structural changes of exine development. bar = 0.2 μm

Fig. 4. Fibrous components are appeared in the callose wall. bar = 0.2 μm

Fig. 5. Partial undulation of plasma membrane and primexine (Pr) were clearly distinguishable. bar = 0.2 μm

Figs. 6–8. Baculum formation stage.

Fig. 6. Convex surfaces of plasma membrane undulation are changed into bacular mound (BM) to form probaculum (Pb). Pr: primexine. bar = 0.3 μm

Fig. 7. Baculum is clearly visible. bar = 0.4 μm

Fig. 8. Degradation of callose after formation of baculum at late baculum formation stage. bar = 0.2 μm

Figs. 9–11. Exine maturation stage

Fig. 9. After development of baculum, exine formation was progressed. Note the fibrouse materials (arrowheads) in between exine cavities. bar = 0.3 μm

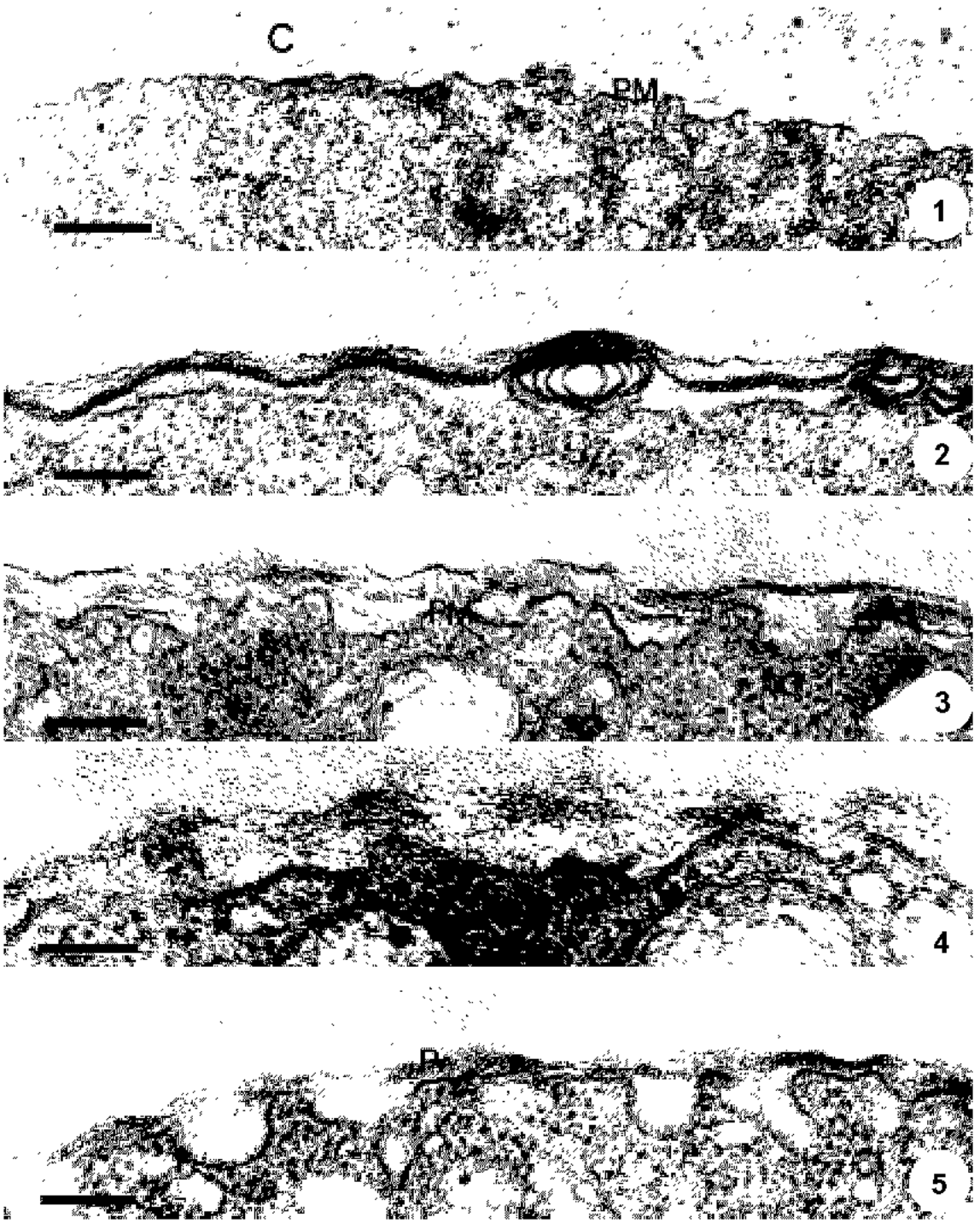
Fig. 10. Maturing pollen shows well developed exine (Es), white lines in between foot-layer and scare remnants of callose. Rough endoplasmic reticulum (rER) are developed parallel with pollen wall. bar = 0.4 μm

Fig. 11. Pollenkitt droplets (arrows) are begin to developed after exine formation. Tapetum (T) is still intact at this stage. bar = 0.5 μm

Fig. 12. Intine formation stage. Hypertrophic Golgi complex (G) are well differentiated for deposition of fibrous materials (arrowheads) for intine formation. Arrows indicate pollenkitt dropletss. bar = 0.3 μm

Fig. 13. Mature pollen. Well developed pollen wall, thick intine in apertural region, prominent nucleus (N) and plastid (P) are typical configuration of mature pollen grain. Note the electron dense inclusion (asterisk) in the cytoplasm of pollen. bar = 1.5 μm

Fig. 14. SEM image of mature pollen grain shows 20 μm in size and tricolpate aperture. Sculpturing pattern of exine is fine reticulate with 0.2~0.6 μm in width of cavities. bar = 3.5 μm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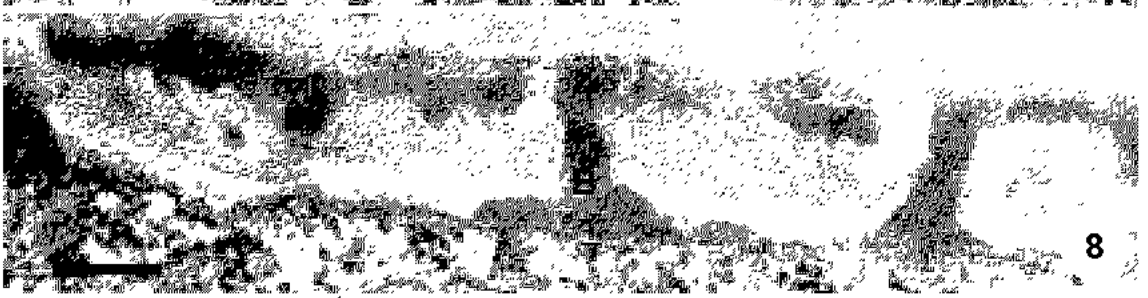

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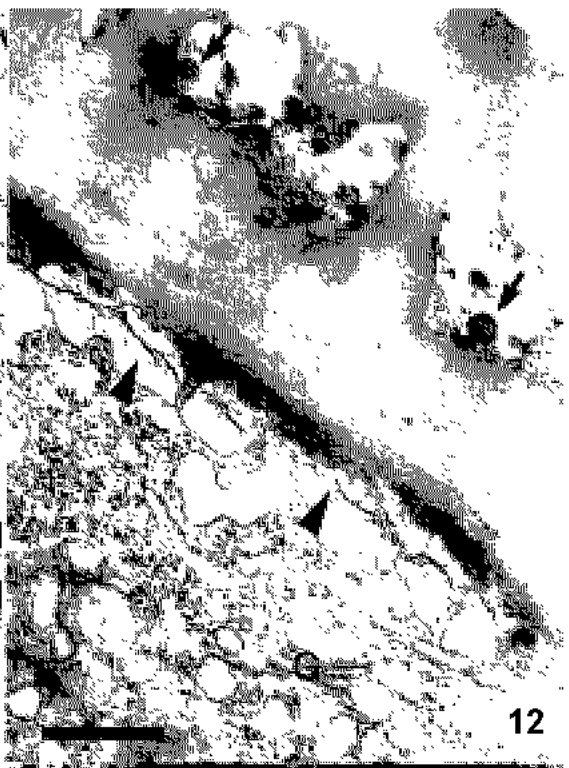
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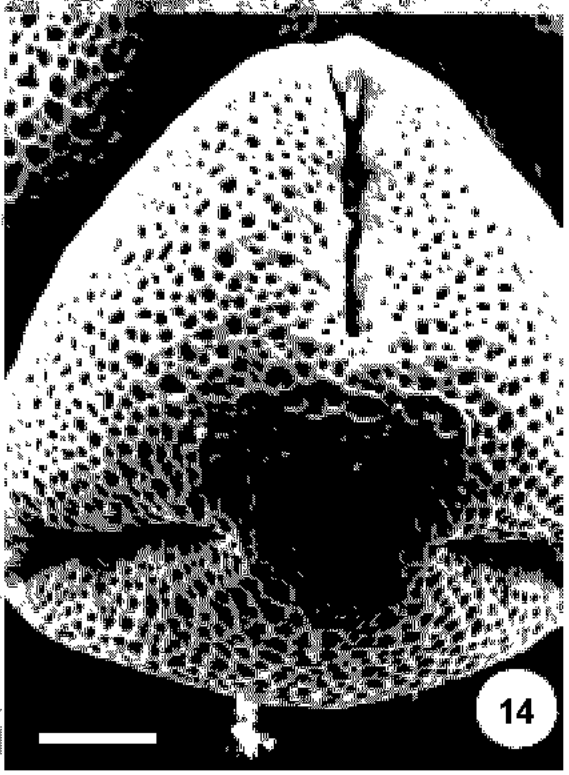
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