Development of Scutellar Epithelial Cells during Rice Embryogenesis, Studied by Chemical Fixation and Freeze-substitution Methods

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Abstract: The whole surface of rice embryo was continuously covered with a cuticular layer until 4 days after anthesis. The cuticular layer became intermittent at only the abaxial side of scutellum at 5 days and disappeared completely at 7 days. By the extinction of this cuticular layer, it was possible to distinguish scutellar epithelial cells from epidermal cells on the other side of the embryo. Therefore, scutellar epithelial cells began to establish a peculiar nature from around 5 days. Epithelial cells, in this time, were still rich in cytoplasm and showed anticlinal divisions. At the end of the anticlinal divisions, epithelial cells gradually altered to an elongated profile in shape. Tiny ingrowths of cell walls were often observed at the surface of cell walls. The walls of epithelial cells were lengthened at a right angle to the outer surface and thickened about half the length inward from the surface, showing unique characteristic of scutellar epithelial cells. Thereafter, the number of lipid bodies and amyloplasts increased in the cells and vacuoles accumulated their contents and transformed to protein bodies. As the embryo developed into the dormant stage, lipid bodies were displaced near the cell walls. By using freeze-substitution method, it was noticed that membrane systems are smooth and the inside of mitochondria or plastids is more electron-dense in comparison with chemical fixation. The presence of thin ingrowths and thickening of cell walls was also observed by freeze-substitution method.

Key words: Cuticular layer, Freeze-substitution, Oryza sativa, Scutellar epithelial cell, Tiny cell wall ingrowth, Ultrastructure.

In general, grass embryos adjoin endosperm in dry seeds. The portion where an embryo attaches to endosperm is a scutellum. Thus, the outer epidermal cells of the scutellum, scutellar epithelial cells, are in close contact with starchy cells of endosperm. During seed germination, scutellar epithelial cells absorb carbohydrates and amino acids released by the dissolution of storage substances such as starch and proteins inside endosperm cells. Scutellar epithelial cells are elongated at right angles to the surface of scutellum and show a very characteristic palisaded shape. The ultrastructure of scutellar epithelial cells in dry seeds and that during germination have been previously reported in rice and barley. On the other hand, it is well known that callus induction occurs from scutellar epithelial cells in rice seeds. Recently it was reported that somatic embryos are induced directly from them. It was also demonstrated by two separate media methods that scutellar epithelial cells are capable of retaining on active...
state for a long time, having abundant cytoplasm without its marked degeneration\(^\text{12}\). Scutellar epithelial cells are thus said to have an extremely dynamic nature, showing their polyfunctionality. When and how may these scutellar epithelial cells be initiated during embryogenesis? To date, no report gives a detailed description of the initiation and development of epithelial cells, although many papers have described the morphological events of rice embryogenesis\(^{4,7,13,16}\). In this report, we have tried to analyze ultrastructural changes in scutellar epithelial cells associated with the developmental process of rice embryos.

We also used a freeze-substitution method in our experiments. By this method cells are frozen rapidly and fixed physically, and the produced image is more near alive conditions than that of chemical fixation. It has been reported that membrane systems of samples fixed by the freeze-substitution method are very clear and the inside of organella is more electron-dense and more homogeneous as compared with that of chemical fixation\(^{6,10,19}\). So we can avoid misunderstanding on artifact when we use this method to study ultrastructure of plant cells. However, this method has limitations as good fixation ranges are only several microns from the surface to touch liquid propane. However, it is possible to take out only a young embryo separating from the endosperm during embryogenesis, because the endosperm is still immature and soft at this developmental stage. Scutellar epithelial cells are located on the surface of embryo. Consequently, it is possible to obtain a comparative- ly good preservation of the epithelium prepared by rapid freezing and the substitution method. Cuticular layer was detected by the I\(_2\) KI-H\(_2\)SO\(_4\)-AgP method\(^{39}\).

In this study, we made obvious ultrastructural changes during the differentiation in epithelial cells of rice scutellum, by the aid of chemical fixation, freeze-substitution method and ultra histochemistry.

**Materials and Methods**

*Oryza sativa* (cv. Nipponbare) plants were cultivated in the field of Aichi-ken Agricultural Research Center and used in the experiments. Samples were collected from superior spikelets of the plants during 4-17 days after anthesis (DAA).

Samples were fixed in a mixture of 3% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2) for 5.5 h at 25°C. After washing in a rinse buffer (0.2 M cacodylate buffer, pH 7.2), they were post-fixed in 2% osmium tetroxide buffered in 0.1 M cacodylate buffer for 3 h at 4°C. Then, they were dehydrated in a graded alcohol series and embedded in Epon 812 resin.

For light microscopy, 1 µm-thick sections were cut with glass knives on Reichert Ultracut N microtome (Reichert-Nissei) and stained with Toluidine Blue.

For electron microscopy, ultrathin sections were cut with diamond knives on the microtome. The sections on grids were stained with 2% aqueous uranyl acetate for 20 min followed by lead citrate for 5 min.

For cuticle detection, sections were mounted on gold grids, stained with 1 : 1 mixture of 0.5% aqueous I\(_2\) + 1.5% KI solution and 60% H\(_2\)SO\(_4\) for 20-30 min and washed in distilled water. They were then treated with 10% aqueous Na\(_2\)S\(_2\)O\(_3\) solution, washed in distilled water again and reacted finally with 1% aqueous silver proteinate solution for 30 min in the dark (I\(_2\)-KI-H\(_2\)SO\(_4\)-AgP)\(^{39}\). They were photographed on H-600 transmission electron microscope at 100 kV.

For freeze-substitution, rice embryos were carefully separated from the surrounding tissues and adhered on small platinum loops using Tissue-Tek (Miles Scientific Inc.). Samples on the loop were rapid-frozen in liquid propane (precooled by liquid nitrogen) using the KF 80 immersion cryofixation system (Reichert-Jung) and transferred into a CS auto-cryosubstitution apparatus (Reichert-Jung). Samples were preserved in anhydrous acetone with 2% osmium tetroxide at -80°C for 48 h and then the temperature of acetone was increased to 0°C by 4°C/h. Samples were removed from the loop in acetone, dehydrated in anhydrous acetone again and embedded in Epon 812 resin.

**Results**

1. **Observation by light microscope**

At the rice embryo 4 DAA, the coleoptile had already begun to differentiate and the shoot apex appeared as a dome which was rather flat (Fig. 1). Epidermal cells and sub-
epidermal cells were readily distinguishable on the upper side of embryos. Modified endosperm cells were seen with degenerated contents between scutellar epithelial cells and endosperm cells that will store starch. At 5 DAA, the embryo was entirely covered with a single layer of epidermal cells (Fig. 2). The cell surface of scutellar epithelium was smooth in appearance.

At 7 DAA, the epithelial surface had a rough outline compared to that at 5 DAA. Modified endosperm cells degenerated completely at this stage and there remained only the cell wall, producing a space of about 50 µm width (Fig. 3). The space was maintained till about 12 DAA, but finally vanished. As a result, epithelial cells closely adhered to endosperm cells (Fig. 6).

During 7 DAA to 10 DAA, the embryo rapidly increased in volume and the surface area of scutellum expanded simultaneously. The length to width ratio of scutellar epithelial cells was about 1.5 : 1 at 5 DAA, but it became about 2 : 1 at 9 DAA (Fig. 4). After that, this ratio further increased more and more, reaching about 2.5 : 1 at 17 DAA (Fig. 6).

2. Observation of chemically fixed materials by electron microscope

a) Cuticular layer

The whole surface of embryo 4 DAA was surrounded with a thin electron-dense layer which was stainable by uranyl acetate and lead citrate (Fig. 7). On the site where this electron-dense layer was supposed to appear, deposits of silver granules were observed by the I$_2$KI-H$_2$SO$_4$-AgF method of Holloway$^9$, detecting the presence of epoxides in the layer (Figs. 17 to 19). Therefore, this result is indicative that this electron-dense layer stainable by the ordinary method is a cuticular layer. At 5 DAA, this cuticular layer became discontinuous at the abaxial side of embryos (Figs. 8 and 20), and almost disappeared at 6 DAA (Fig. 9). Then this abaxial side differentiated to scutellar epithelial cells. On the contrary, at the adaxial side of the embryos 5 DAA, the cuticular layer became thicker (Fig. 18). Hereafter, the cuticular layer at the adaxial side of embryos existed at about the same consistency as that of dry seeds. Consequently, the scutellar epithelial cells were possible to easily distinguish from cells of the other organ surface by the absence of the cuticular layer.

b) Cell wall and cell shape

Cell walls on the outer surface of scutellar epithelium were of a thin layer at 4 DAA and 5 DAA (Figs. 7 and 8), but increased in thickness with time. Microtubules were observed around the cell walls (Fig. 8).

By 8 DAA, anticlinal divisions occurred at the epithelial cells (Fig. 11). After anticlinal divisions had terminated, the cell turned to elongated forms (Fig. 12). The superficial walls were electron dense 8 DAA when compared to those at the early stage (Figs. 11 and 12).

After becoming an elongated shape, the longitudinal cell walls began to thicken gradually from the surface directly contacted with endosperm (Fig. 12). Afterwards the thickening activities of cell walls attained about half the length of epithelial cells (Figs. 14 and 16).

Small curving ingrowths of the cell wall were frequently observed in scutellar epithelial cells, especially during 7 DAA to 10 DAA (Figs. 10 and 13). The plasma membrane along the thickened cell walls was wavy, with a tiny loop of cell wall (Fig. 15).

Plasmodesmata were observed between the adjoining epithelial cells, and between the epithelial cells and the underlying parenchyma cells. However, they were not presented between the epithelial cells and the endosperm cells (Fig. 11).

c) Vacuole, lipid body and plastid

Small vacuoles were scattered within scutellar epithelial cells at 4 DAA (Fig. 7). These vacuoles increased in volume during 5 and 6 DAA (Fig. 9). Electron-dense substances began to accumulate in the vacuoles from about 8 DAA (Fig. 11). Besides, at 17 DAA, most of the vacuoles converted into protein bodies containing heavy electron dense materials (Fig. 16).

Lipid bodies were hardly observed in the epithelial cells 4 DAA (Fig. 7), but the number increased gradually thereafter as embryos enlarged, and they scattered all over the cells (Fig. 12). These lipid bodies were surrounded by a single membrane (Fig. 10). When the longitudinal walls of epitelial cells ceased thickening activity at the distal half, lipid bodies were disposed as they lined up near cell walls (Fig. 14). Although the inside of the lipid bodies was comparatively electron-dense.
Explanation of figures

Figs. 1-6. Light micrographs of rice embryos and the scutellum surface at the abaxial side (Magnification is the same as Fig. 6 where the bar is 100 \( \mu m \)).

Fig. 1. An embryo 4 DAA. Coleoptile (C) arises from the adaxial side of an embryo. Shoot apex (SA) is recognized as a dome. Endosperm cells directly surrounding the embryo (End) begin to degenerate.

Fig. 2. Five DAA. Mono-layered, epidermal cells formed at the abaxial side of an embryo. There is heavy accumulation of starch in endosperm cells (SEnd).

Fig. 3. Seven DAA. Endosperm cells degenerated completely, accompanied by a space (Sp) about 50 \( \mu m \) between scutellum and starchy endosperm.

Fig. 4. Nine DAA. Scutellar epithelial cells (arrow) of scutellum which develop to an elongated form.

Fig. 5. Eleven DAA. More prominent elongation of the epithelial cell.

Fig. 6. About 17 DAA. Absence of the space between the scutellum and the starchy endosperm.

Fig. 7-16. Transmission electron micrographs (TEM) of scutellar epithelial cells of rice embryos prepared by chemical fixation and regular staining.

Fig. 7. Four DAA. Cuticular layer (arrow) seen as an electron-dense continuous layer on the outermost surface of epithelial cells. Epithelial cells are cytoplasmic rich (The bar is 5 \( \mu m \)).

Fig. 8. Five DAA. Discontinuous cuticular layer (arrow) (The bar is 1 \( \mu m \)). Microtubules (arrow head) are present near cell wall.

Fig. 9. Six DAA (The bar is 5 \( \mu m \)). Cuticular layer seriously collapsed.

Fig. 10. Seven DAA. Cuticular layer disappeared completely (The bar is 1 \( \mu m \)). Lipid bodies (L) are surrounded by a single membrane. The tiny ingrowth of cell wall is observed (arrow).

Fig. 11. Eight DAA. Anticlinal division of epithelial cells (The bar is 5 \( \mu m \)). Phragmoplast (arrow) can be seen at the center of cells.

Fig. 12. Nine DAA. Thickening of the vertical walls of epithelial cells which begin from the surface (The bar is 5 \( \mu m \)).

Fig. 13. Nine DAA. Tiny wall ingrowth (arrow) (The bar is 1 \( \mu m \)).

Fig. 14. Twelve DAA. Many lipid bodies near cell walls (arrow) (The bar is 5 \( \mu m \)). Plastids have the compound type of starch grains. Vacuoles have electron dense materials.

Fig. 15. Twelve DAA. Thickening of cell walls (The bar is 1 \( \mu m \)). Partly they formed loops (arrow).

Fig. 16. Seventeen DAA. Amyloplasts (A), protein bodies (Pb) and numerous lipid bodies (L) in the epithelial cells (The bar is 5 \( \mu m \)).

Figs. 17-20. TEM stained by I\(_2\), KI-H\(_2\)SO\(_4\)-AgP schedule (They are same magnification. The bar is 0.5 \( \mu m \)).

Fig. 17. Four DAA. The surface of an embryo at the adaxial side. Opaque silver granules deposit on the cuticular layer.

Fig. 18. Five DAA. The surface at the adaxial side.

Fig. 19. Four DAA. The surface at the adaxial side.

Fig. 20. Five DAA. The scutellum surface at the adaxial side. The cuticular layer is discontinuous.

Figs. 21-32. TEM of scutellar epithelial cells 8 DAA (Figs. 21-31) and 10 DAA (Fig. 32) which were prepared by the freeze-substitution method.

Fig. 21. The plasma membrane and tonoplast of vacuoles (V) which are smooth in appearance (The bar is 2 \( \mu m \)). Lipid bodies (L) are reduced.

Fig. 22. Mitochondria (M), plastids (P) including starch grain and microbody (Mb) (The bar is 0.5 \( \mu m \)). Microtubules (Mt) are presented along the cell wall.

Figs. 23-28. TEM of serial ultra thin sections, showing the tiny cell wall ingrowths (Magnification is the same, as Fig. 30 where the bar is 0.5 \( \mu m \)).

Fig. 29. Tiny cell wall ingrowth on outer surface of scutellar epithelium (Magnification is the same as Fig. 30).

Fig. 30. Tiny ingrowth of cell wall between the abutting epithelial cells (The bar is 0.5 \( \mu m \)).

Fig. 31. Microbody (Mb) and plasma membrane (Pm) (The bar is 1 \( \mu m \)). The leaflet of plasma membrane at the protoplasm side is more electron-dense than that at the cell wall side.

Fig. 32. Ten DAA. Thickening of vertical cell walls of scutellar epithelial cells (The bar is 1 \( \mu m \)).
until about 12 DAA (Fig. 15), the density decreased at about 17 DAA (Fig. 16).

Plastids were generally observable in the epithelial cells. Starch grains appeared inside plastids almost everyday after anthesis, the number increased, and plastids gradually transformed into amyloplasts. Starch grains of the compound type occurred in most of amyloplasts 17 DAA (Fig. 16).

d) Other organelle

There was often a big nucleolus in the nucleus of the epithelial cells, but heterochromatin was almost absent (Fig. 9). Beside this, there was no large variation in the morphology of nucleolus until 17 DAA when our samplings were completed.

A number of mitochondria, rough endoplasmic reticula and microbodies were observed within the epithelial cells. Mitochondria had well-developed cristae inside. These cristae were observed even at 17 DAA (Fig. 16). Rough endoplasmic reticula were arranged parallel to the cell walls. Golgi bodies were numerously observed. There was a dense distribution of ribosomes. Following these changes in epithelial-cell organelles, however, there was a characteristic reduction in the volume of ground cytoplasm as a result of the large proportion inside the cell occupied by lipid bodies and amyloplasts.

3. Freeze-substitution method

In photomicrographs of samples prepared by the freeze-substitution method (Figs. 21 to 32), the plasma membrane and tonoplast were very clear and smooth in the appearance, although they were sinuous in the chemically fixed materials. The plasma membrane was evidently illustrated as continuous bilayers comprising dark-light-dark tram-lines. The layer was more electron dense at the cytoplasm side than that at the inner face of cell wall (Fig. 31). The tonoplast was also double layered and the inside layer was more dense than that at the cytoplasm side.

Vacuoles contained homogeneous substances (Fig. 21). The insides of mitochondria were more electron dense. The contents in plastids were more dense, also. Starch grains in plastids were not stained (Fig. 22). However, the double membrane structure of envelopes of mitochondria and plastids was less clearly presented with the freeze-substitution compared to chemical fixation.

Microbodies were surrounded by a single membrane (Fig. 31). Microtubules disposed around cell walls were clearly observed (Fig. 22).

Lipid bodies were surrounded by a single membrane contracted with prolonged treatment of acetone during freeze-substitution and the contents were unstained and remained as white portions (Fig. 21). In spite of that, they were spherical shape in the chemical fixation.

It was possible to observe the curving small ingrowth of cell walls even through freeze-substitution. The plasma membrane of the ingrowth part was more electron-dense at the cytoplasm side than at the inner side of ingrowth. So it was understood that this ingrowth is as a continuity of cell wall (Fig. 30). The ingrowth of the cell wall was examined in detail. Figs. 23 to 28 are photomicrographs made from a serial section of the small ingrowth. The circle made by small ingrowth was not completely closed (Fig. 26). It was found that the portion of cytoplasm surrounded with the ingrowth continued to the peripheral cytoplasm in the cell where the sections were made. Besides, it was also noted that the inner portion surrounded by the ingrowth has a complicated morphology, showing several branches of ingrowth (Fig. 29). Ingrowth were also present on the cell wall between the abutting cells of epithelium (Fig. 30).

By 10 DAA, thickenings of their boundary cell walls between the abutting epithelial cells were observed with freeze-substitution. The plasma membrane in this portion was smooth again (Fig. 32).

Discussion

1. Initiation of scutellar epithelial cells

Epidermal cells at the abaxial side of rice scutellum take an elongated shape at right angles to the embryo surface from about 9 DAA. The epidermal cells begin to transform into a unique cell layer as scutellar epithelial cells at 9 DAA15). However, we observed at 5 DAA the difference in epidermal cells between the scutellum and the other portions in embryos. One of the clear characteristics is the disappearance of the cuticular layer at the abaxial side of scutellum.

It is well known at this stage during rice
embryogenesis that scutellum, radicle and other principal organs begin to differentiate, accompanying the coleoptile surrounding a shoot apex. After appearance of these organs, the superficial cells of scutellum begin to transform to the characteristic figure as epithelial cells.

2. Transport function of scutellar epithelial cells during embryogenesis

It is reliable that the endosperm cell layer directly surrounding a young wheat embryo becomes a source of nutrients for embryo development through autolysis. Maize endosperm cells near embryos help the transportation of substances into embryos. In rice, as the endosperm cells in contact with the embryo are different from the endosperm cells including amyloplasts, it is considered they have the function of nutrient transportation during early periods of embryogenesis.

Endosperm cells near the embryo begin to degenerate at about 4 DAA. The function of such endosperm cells seems to terminate at the end of the globular embryo atage before 4 DAA. After that, a space of about 50 µm width is found around the embryo. Consequently it is considered that the embryo floats in liquid nourishment. As the whole surface of the embryo is covered with a cuticular layer except at the abaxial side of scutellum, it is rather difficult to transport nutrients from the surrounding tissues besides the route through scutellar epithilium. As the cuticular layer inhibits the transportation of substances, its disappearance may advance the movement of substances. It is probable that, at the later stage of growing embryo, nutrient pathways are mainly via both suspensor and scutellar epithilium.

Scutellar epithelial cells have many mitochondria, Golgi bodies and rough endoplasmic reticula. Epithelial cells are considered to conduct a remarkable physiological activity during embryogenesis.

There are many tiny ingrowths on the cell wall of scutellar epithelial cells. Those structures could be observed even by the freeze-substitution method, so they are not artifact. The tiny ingrowths seem to play a role in absorption similar to that of cell wall ingrowths which were well revealed in the function. The thickening of both the surface wall and longitudinal wall of the scutellar epithelial cells is considered to facilitate transportation of nutrients.

Our results, therefore, support that scutellar epithelial cells have a substantial role in the transportation of nutrient substances into rice embryos during embryogenesis.

In this paper we make clear the developmental process of scutellar epithelial cells of rice embryos with ultrastructure level by both chemical fixation and the freeze-substitution method, and suggest their transport role of nutrients during embryogenesis. It is obvious that epithelial cells have a very unique feature.

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References


