CELL WALL DEVELOPMENT OF *MICRASTERIAS AMERICANA*, ESPECIALLY IN ISOTONIC AND HYPERTONIC SOLUTIONS

KATSUMI UEDA AND SACHIYO YOSHIOKA

Biological Laboratory, Women's University, Kitauoya, 630 Nara, Japan

SUMMARY

The cell wall development of *Micrasterias americana* was investigated by light and electron microscopy. From digestion experiments with pectinase and cellulase, and from fluorescence spectra in Calcofluor and Coriphosphin solution, it was concluded that pectic substances were the main component of the young developing cell wall and that cellulose was synthesized after the daughter hemicell was well developed. In 0.16 M mannitol, wall materials accumulated and were incompletely incorporated into the wall at the region where wall growth would be expected. The plasma membrane was in close contact with the cell wall at the sinus, and this contact was assumed to prevent penetration of wall material at this region, resulting in the accumulation of wall material at regions other than the sinus. The cellulosic wall layer was formed after the production of pectic substances in the 0.16 M mannitol. In 0.3 M mannitol neither a definite wall layer of cellulose nor a pectic wall was produced, presumably due to extensive dilution of the wall materials in the plasmolysed space between the cell wall and the plasma membrane. Under normal circumstances, the shape of the daughter cell is assumed to be determined by the shape of the developed primary wall, which is induced by precocious differentiation of the wall at the sinus.

INTRODUCTION

It is well established from light-microscopic observations that the cell wall of growing hemicells of *Micrasterias* is unevenly thickened in isotonic solution (Kiermayer & Jarosch, 1962; Kiermayer, 1964; Kobayashi, 1973). Kiermayer (1964) assumed that the uneven thickening of the wall reflects the non-uniform capacity of the surface of the plasma membrane to secrete wall material, and he tried to interpret the normal process of cell wall formation mainly from the standpoint of the capacity of the plasma membrane to secrete wall material. To interpret this process more precisely, both the chemical nature and the ultrastructure of the cell wall of *Micrasterias americana*, especially in isotonic and hypertonic solutions, were examined in the present investigation.

MATERIALS AND METHODS

*Micrasterias americana* was cultured in Waris solution at 20 °C with 13 h of illumination under fluorescent light (2000 lx) and 11 h of darkness per day. The growing cells were transferred into Waris solution containing 0.16 or 0.3 M mannitol, later simplified to 0.16 or 0.3 M.
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0.3 M mannitol. After growing for 1, 2, 3, 6, 12, or 24 h in the mannitol solutions, the cells were examined by light and electron microscopy.

A fluorescence microspectrophotometer, Nikon SPM-F1, was used to obtain the fluorescence spectrum from the cell wall treated with Coriphosphin or Calcofluor. For experiments involving digestion of the cell wall, 5% pectinase (Sigma) and 5% cellulase (Onozuka R-10 cellulase, Kinki Yakult) solutions were used. The enzymes were dissolved in Waris solution containing 0.16 M mannitol, and the pH was adjusted to 5.5.

For electron microscopy, cells were fixed for 30 min with 3% glutaraldehyde solution (buffered with phosphate to pH 7.3) containing the same concentration of mannitol as culture media, washed for 30 min with the buffer solution, and postfixed with 1% osmium tetroxide solution (pH 7.3). After washing with water, cells were dehydrated through graded acetone solutions, and embedded in Epon. Thin sections were stained with lead citrate and examined with an Hitachi HU-12A electron microscope.

OBSERVATIONS

Light-microscope observations

After nuclear division, a cell is divided by a septum across the isthmus, resulting in the formation of 2 cells. From each of these, a daughter hemicell begins to grow, first as a hemispherical bulge, which develops 3 and then 5 lobes and finally assumes the form of the mother hemicell.

When growing cells at the 3-lobed or early 5-lobed stage were transferred to 0.16 M mannitol and cultured for 1 h, the walls of growing daughter hemicells partially thickened. Waris (1950, 1951) assumed from observations of the staining property with dyes and the behaviour towards acid and alkali that the thickened wall consists of a pectic substance. When cell culture was continued for 4 h in the

Fig. 1. A cell grown for 4 h in 0.16 M mannitol. A new wall layer (arrow) is seen beneath a thickened wall (t). ×300.

Figs. 2–4. Fluorescence of walls of growing cells in 0.005% Coriphosphin solution ×130.

Fig. 2. Early stage of development.

Fig. 3. Middle stage of development. Fluorescence of the cell wall of daughter hemicells is not detectable.

Fig. 4. Late stage of development. The cell wall of daughter hemicells fluoresces weakly.

Figs. 5–7. Fluorescence of walls of growing cells in 0.02% Calcofluor solution ×110.

Fig. 5. Early stage of development.

Fig. 6. Late stage of development. The cell wall of daughter hemicells fluoresces weakly.

Fig. 7. Cells cultured for 5 h after the full growth. The walls of daughter hemicells fluoresce strongly, and the intensity of fluorescence is strongest at the sinus.

Fig. 8. Daughter hemicells fixed just after transfer to 0.16 M mannitol; the daughter cell walls are thin. ×2000.

Fig. 9. Daughter hemicell grown for 1 h in 0.16 M mannitol; the daughter hemicell walls are thickened. ×3000.

Fig. 10. Enlargement of a part of Fig. 9. Fibrils, small vesicles (arrows) and vacuolate vesicles (v) are seen in the thickened wall. ×16000.

Fig. 11. Dictyosome with many large vesicles (Le) at its periphery in a growing cell cultured for 1 h in 0.16 M mannitol. ×40000.
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O-16 M mannitol, a new wall layer appeared between the thickened wall and the protoplast. Such a cell is shown in Fig. 1, in which the arrow indicates the new wall layer and t the thickened wall.

The thickened wall was positively stained by 0.01% neutral red and 0.01% ruthenium red solutions. It fluoresced in 0.03% Coriphosphin solution, the maximum peak of the fluorescence spectrum being 620 nm (Fig. 12, A). A 1% pectin solution with 0.03% Coriphosphin also fluoresced with the maximum at 620 nm (Fig. 12, B).

After treatment with 5% pectinase for 3 h at 35 °C, the cell wall, including the thickened part, of the daughter hemicell was disorganized. Treatment with 5% cellulase did not cause the wall to become disorganized. These facts suggest that the growing cell wall consists mainly of pectic substances. The cell walls of daughter hemicells which had just grown to the size of mother hemicells were not disorganized in 5% pectinase solution, but were disorganized by treatment with 5% pectinase plus 5% cellulase. In a dilute solution of Coriphosphin (0.005%), fluorescence of the cell wall of growing daughter hemicells was hardly visible (Figs. 2, 3). The wall of a daughter hemicell which had grown fully to the size of the mother hemicell fluoresced weakly in the dilute Coriphosphin solution (Fig. 4), but the intensity of the fluorescence increased gradually as the cell matured. In the dilute Coriphosphin solution, the fluorescence intensity of pectin greatly decreased, and the maximum peak of the spectrum shifted to a shorter wavelength of about 530 nm (Fig. 12, D). Cellulose
fluoresced strongly in both dilute (0.005%) and concentrated (0.03%) Coriphosphin solutions with the maximum peak at 525 nm (Fig. 12, C).

The fluorescence spectra of the wall of a full grown daughter hemicell in the dilute Coriphosphin solution are shown in Fig. 13. The spectrum obtained just after ultraviolet irradiation had its maximum peak at 570 nm and a shoulder at about 525 nm (Fig. 13, A). The intensity of fluorescent emission at 570 nm decreased as the cell wall was continuously irradiated with ultraviolet light. The spectra after 3 and 6 min of ultraviolet irradiation are shown by curves B and C, respectively of Fig. 13.
A mother hemicell fluoresced strongly in 0.005% Coriphosphin solution. The position of the fluorescent maximum changed as shown in Fig. 14, that is, the peak found initially at 570 nm shifted to 525 nm, after irradiation for 6 min with ultraviolet light. There were at least 2 substances in the cell wall, one emitting fluorescent light at 570 nm and the other at 525 nm. The latter wavelength corresponds to that of fluorescent cellulose in the same solution. As the cell wall of daughter hemicells consists mainly of pectic substance and the maximum peak of the fluorescent light due to pectin shifts from 620 to 530 nm according to the concentration of the Coriphosphin solution, the emission at 570 nm may be derived from the pectic substance. The shift of the maximum wavelength from 570 to 525 nm with the mother hemicell is interpreted in terms of quenching of the 570-nm light by the ultraviolet light, causing the peak at 525 nm to become visible in the spectrum curve. The newly matured daughter hemicell contained so little cellulose that the peak at 525 nm did not appear after quenching of the 570-nm light.

The detection of cellulose by fluorescence with Calcofluor has been described by Nagata & Takebe (1970). When growing daughter hemicells were treated in Calcofluor solution (0.02%), the walls fluoresced very weakly (Fig. 5). The intensity of the fluorescent light gradually increased after the daughter hemicells had reached full size (Figs. 6, 7), and the intensity was always stronger at the sinus. Fluorescence from the cell wall in Calcofluor solution showed maximum intensity at 520 nm, which was the same wavelength as the fluorescence from cellulose in the same solution. These observations on fluorescence strongly suggest that cellulose synthesis in the walls of daughter hemicells in *Micrasterias americana* begins at a late stage of their development.

**Observations with the electron microscope**

Cells at the early stages of the development were not plasmolysed in 0.16 M mannitol solution. Fig. 8 shows 2 daughter hemicells which were fixed just after

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Fig. 15. Daughter hemicell grown for 2 h in 0.16 M mannitol. Fibrils and vesicles are accumulated between the wall and plasma membrane. Accumulation occurs at the regions from which wall growth would be expected. There is none at the sinus (arrows). × 1500.

Fig. 16. Daughter hemicell at a late developmental stage grown for 2 h in 0.16 M mannitol. Arrow indicates the new wall produced between the original wall and the plasma membrane. × 1000.

Fig. 17. A new wall of uniform thickness formed independently of the original wall. × 11000.

Fig. 18. A new wall cut obliquely. Groups of microfibrils are seen. × 29000.

Fig. 19. Daughter hemicell grown for 3 h in 0.16 M mannitol. A new wall layer (arrow) is seen under the wall which has seemingly thickened. × 2000.

Fig. 20. Dictyosomes with no large vesicle at their peripheries in the same cell shown in Fig. 19. × 45000.

Fig. 21. Part of a daughter hemicell at a late stage of development grown for 6 h in 0.16 M mannitol. A thick new wall layer is seen beneath the original wall which is thin and less electron-dense. × 1500.
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they were transferred to 0.16 M mannitol. The cell wall of the daughter hemicells was thin, and rather uniform in thickness. After growing for 1 h in 0.16 M mannitol, the cell wall of the daughter hemicell seemingly thickened (Fig. 9). It was constructed of fine fibrils, small vesicles and vacuolate vesicles with few fibrous inclusions (Fig. 10). Vesicles were abundant near the plasma membrane, and the fibrils were mostly located near the surface of the wall. These vesicles may come from the protoplast through the plasma membrane, as Tutumi & Ueda (1975) suggested, and they may contribute to the impregnation of pectic substances into the cell wall, because the thickened wall is mainly constructed of pectic substances as described above. In cells at this early developmental stage, dictyosomes with many large vesicles at the margins of several distal cisternae were seen (Fig. 11). Dictyosomes of the same appearance were reported in cells in normal culture medium without mannitol and were interpreted as dictyosomes which were actively producing wall material (Ueda & Noguchi, 1976). The production of wall materials or pectic substances by the dictyosomes in cells cultured for 1 h in 0.16 M mannitol may occur normally as in cells maintained in the normal culture medium, since there was no visible difference between dictyosomes of cells from the 2 different culture media.

When cells with 3- to 5-lobed daughter hemicells were cultured for 2 h in 0.16 M mannitol, the cell wall of the daughter hemicells apparently thickened at the corners of lobes (Fig. 15). The thickening seemed to be due to dense accumulation of fibrils and vesicles between the original cell wall and the plasma membrane. The accumulation of fibrils and vesicles occurred so as to push the plasma membrane inwards. Only at the sinus of the daughter hemicells (Fig. 15, arrows) did the plasma membrane lie close to the cell wall. The thickness of the cell wall could be clearly defined at the sinus owing to its high density and to the absence of accumulation between the wall and the plasma membrane. The boundary between the original cell wall and the accumulation was not always so clear at regions other than the sinus. When a cell at the later stage of development was cultured for 2 h, a new cell wall layer appeared between the original cell wall and the plasmolysed protoplast of the daughter hemicell (Fig. 16, arrow). Fig. 17 shows a dense original wall and a less-dense new wall

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Fig. 22. Part of a cell at a late developmental stage grown for 6 h in 0.16 M mannitol. Pores traverse the new wall produced during growth. ×15000.

Fig. 23. Daughter hemicell cultured for 24 h in 0.16 M mannitol. A small daughter hemicell is fully matured and surrounded with the new thick wall. ×1200.

Fig. 24. Enlargement of enclosed part of Fig. 23. The original wall is almost completely decomposed to very fine fibrils. ×19000.

Fig. 25. Dictyosome in a daughter hemicell grown for 24 h in 0.16 M mannitol. No large vesicles are seen at the periphery of the dictyosome. ×40000.

Fig. 26. Daughter hemicells fixed just after plasmolysis in 0.3 M mannitol. Arrows indicate the regions of negative plasmolysis, where the wall is somewhat thicker. ×2000.

Fig. 27. Plasmolysed space beneath wall of a hemicell grown for 1 h in 0.3 M mannitol. The space is filled with many fibrils, vacuolate vesicles (v), and concentrated small vesicles (sv). ×10000.
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about 0.3 μm thick. The new wall is shown cut obliquely at higher magnification in Fig. 18, in which many microfibrils, about 15 nm in diameter, are observed. Several parallel microfibrils were bundled in a group, and their arrangement and appearance were closely similar to those reported as cellulosic microfibrils in cell walls of many plants (Frey-Wyssling, 1959; Heyn, 1966; Mix, 1966; Kiermayer & Staehelin, 1972; Kiermayer & Dobberstein, 1973).

When a cell at an early developmental stage grew for 3 h in 0.16 M mannitol, a new wall layer of rather uniform thickness was produced outside the plasma membrane (Fig. 19, arrow). Between the new wall layer and the original cell wall, many vesiculate substances were found. There was a space between the plasma membrane and the new cell wall layer, which is assumed to be a product of sample preparation, because the new wall seen with an optical microscope is always in contact with the plasma membrane. Dictyosomes in this cell had ceased to produce large vesicles, and consisted of 8–11 cisternae (Fig. 20). These features of the dictyosomes were the same as those found in full-grown cells which had almost finished wall synthesis in normal culture medium (Ueda & Noguchi, 1976).

During growth for 6 h in 0.16 M mannitol, the electron density of the new wall layer gradually increased, while that of the original cell wall decreased (Fig. 21). Many small pores were produced traversing the new wall as in the wall of cells grown in normal culture medium, but these pores did not traverse the original wall (Fig. 22).

Prolonged culture in 0.16 M mannitol caused no marked alteration in appearance of the wall of daughter hemicells. Fig. 23 shows part of a cell cultured for 24 h. The area enclosed by a rectangle in Fig. 23 is shown enlarged in Fig. 24. The original cell wall was almost completely broken down to very fine fibrils. This may indicate that the wall is always autolysed by its enzymes. In normal growth, this decomposition must be essential for the incorporation and synthesis of new wall material. The dictyosomes in the cell usually consisted of 11 cisternae, and large vesicles were not seen around them (Fig. 25).

Just after plasmolysis with 0.3 M mannitol, a few fine fibrils and small vesicles were observed between the cell wall and the plasma membrane (Fig. 26). The cell wall was thicker at the regions of negative plasmolysis (indicated by arrows). When
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cells at early stages of development were cultured for 1 h in 0·3 M mannitol, vesicles and fibrils accumulated in the whole of the space between the cell wall and the plasma membrane (Fig. 27). This accumulation pattern was different from that of cells grown in 0·16 M mannitol, because in the latter the accumulation was found only at the corners of lobes, showing apparent thickening of the wall. The outline of the original wall was very clear. Vacuolate vesicles (v) appeared to be almost empty and randomly scattered in the space, while small vesicles (sv) were concentrated in several areas. In these cells large vesicles were seen at the periphery of the dictyosomes, but their number was fewer than in cells of similar developmental stages grown in normal culture medium without mannitol. In cells at late stages of development cultured for 1 h in 0·3 M mannitol, fine fibrils were arranged in concentric layers upon the plasma membrane of the daughter hemicell (Fig. 28, arrow). In such cells, few vesicles were observed in the space between the cell wall and the plasma membrane of the daughter hemicell.

When cells at early stages of development were cultured for 3 h in 0·3 M mannitol, the new wall layer was formed on the plasma membrane in some cells (Fig. 29). The wall layer was not of uniform thickness, and many vesicles were seen in it. In other cells, the outline of the new wall layer was obscure owing to loosening and separation of the constituent fibrils (Fig. 30). In cells at a late stage of development cultured for 3 h, concentric layers of fine fibrils and vesicles covered the plasma membrane (Fig. 31). These fibrils were abundant near the plasma membrane, but sparse at regions distant from it. They closely resembled microfibrils of cellulose in dimensions and appearance. The only difference between the 2 kinds of fibrils was that the former were more loosely packed than the latter. In these cells, dictyosomes were composed of 10 or 11 cisternae, at the periphery of which small vesicles were visible (Fig. 32).

Cells cultured for 24 h in 0·3 M mannitol showed a similar accumulation pattern of fibrils and vesicles in the space between the cell wall and the plasma membrane to cells cultured for 3 h.

**DISCUSSION**

In 0·16 M mannitol, pectic substances are incorporated into the cell wall of daughter hemicells at early stages of cell development, resulting in partial thickening of the wall, as Kiernayer & Jarosch (1962), Kiernayer (1964) and Tippit & Pickett-Heaps (1974) have described. After growing for 3 h in the same solution, another new wall of cellulosic nature becomes visible on the surface of the plasma membrane, and the wall continues the thickening for several hours. These observations suggest that the production of wall materials such as pectic substances and cellulose may occur extensively even in the 0·16 M mannitol solution. This is also confirmed by the finding that dictyosomes actively produce vesicles in cells under the same conditions. Schnepf (1961) investigated the gland cells of *Drosophyllum*, from which water had been withheld, and concluded that water shortage does not affect the production of secretion vesicles from dictyosomes but affects the discharge process. In *Micrasterias*, withdrawal of water from cells with solutions of 0·16 and 0·3 M did
not prevent migration of the vesicles towards the cell wall through the plasma membrane.

In 0.16 M mannitol, the vesicles and fibres secreted outside the plasma membrane are not pressed by the plasma membrane to the wall, because the cell is in a state of incipient plasmolysis. It seems probable that pressure, by which the plasma membrane presses the vesicles and fibrils to the wall, is necessary for normal synthesis of the cell wall. Without the pressure which may force the vesicles and fibrils into the cell wall, normal wall is not formed, but a swollen and incomplete wall accompanied by vesicles and fibrils is produced. As the vesicles are continuously supplied from the cytoplasm, the incomplete wall grows large and pushes the plasma membrane inwards, causing the partial thickening of the cell wall observed light microscopically. In 0.3 M mannitol, the plasma membrane is widely separated from the cell wall. In this wide space, vesicles and fibrils which may be wall material and enzymes derived from dictyosomes are dispersed. Because the diluted wall material and enzymes in the wide space may be of almost no use for normal wall synthesis, the wall of daughter hemicells at early stages of cell development would not be thickened in 0.3 M mannitol.

Cellulose is synthesized in the later stages of cell development in normal culture medium. The observation that cells at the late developmental stage formed new cellulosic wall without accompanying wall thickening due to pectic substances in 0.16 M mannitol, may mean that the cell at this developmental stage had almost finished production of pectic substances and started production of cellulose. In the daughter hemicell in which growth had almost stopped at the 3-lobed stage in 0.16 M mannitol, the cellulosic wall layer was formed after pectin production. This indicates that the production of pectin and cellulose is independent of the shape of the daughter hemicell, even in 0.16 M mannitol. In 0.3 M mannitol, microfibrils of cellulosic nature were synthesized at the surface of the plasma membrane of the strongly plasmolysed cells at late stages of development. They did not form a definite wall, but were diffusely spread. The space between the plasma membrane and the cell wall caused by plasmolysis may be too wide for the cellulose-fibrils to form a tight wall layer.

Mix (1966) has proposed that the young and permanent walls of *Micrasterias* correspond to the primary and secondary walls of higher plants respectively. From the microfibrillar construction and the cellulosic nature, the new wall layer produced in 0.16 M mannitol may be regarded as the secondary wall, and the original pectic wall as the primary wall. Mix (1972) classified the cell walls of Conjugatophyceae into 3 types, each type of wall consisting of at least the primary and secondary walls. However, we could not usually see these two kinds of wall in contact with each other in old cells of *Micrasterias americana*. Only the secondary wall was observed, the outer and inner parts of which were frequently electron-dense. As seen in Fig. 24, the primary wall was broken down rather quickly after formation of the secondary wall. Therefore, it is natural that only the secondary wall remains in old cells, even if the primary and secondary walls are clearly detected in a growing daughter hemicell.
How does the bulge-shaped daughter hemicell differentiate the lobes in normal circumstances? First, pectic substance must be produced within the large vesicles which are pinched off from the cisternae of the dictyosomes (Kiermayer, 1970; Tutumi & Ueda, 1975; Ueda & Noguchi, 1976). These vesicles may migrate towards the cell wall and fuse with the plasma membrane, then secrete their contents outside the plasma membrane. In other plants, the origination, migration and secretion processes of the vesicles are similar to those in *Micrasterias* (Northcote & Pickett-Heaps, 1966; Schnepf, 1969). Next, pectic substances and enzymes for wall synthesis must be accumulated at the particular regions. These substances were indeed accumulated in 0.16 M mannitol at the region where wall expansion would be expected. Kiermayer (1964) assumed a 'fixation-zone' where the wall and plasma membrane were so firmly joined with the aid of some plasma element such as papillae that wall material could not be secreted in this zone, resulting in thickening of the wall at regions elsewhere. Although such a connecting element between the wall and the plasma membrane could not be seen electron microscopically in the present investigation, the concept of a fixation-zone would be useful. As shown in Fig. 15, the cell wall and the plasma membrane are in contact with each other at the sinus, and the wall at the sinus is thicker than at the edges of the lobes. That the cell wall fluoresces strongly at the sinus suggests that the wall at the sinus is already considerably differentiated and may contain some special substances even in the growing cells. It is possible, therefore, that the binding substance between the wall and plasma membrane is included within this differentiated wall. The binding substance would prevent vesicles originating from the Golgi apparatus from passing through the plasma membrane at the sinus. The vesicles or their contents may pass through the plasma membrane at regions other than the sinus, from where the lobes could originate using substances included in the vesicles. As shown in Fig. 10, many vesicles are seen just outside the plasma membrane. These vesicles themselves weaken the contact of the wall and the plasma membrane, and would further help the passage through the plasma membrane of vesicles produced later. Once lateral lobes have been formed, the wall begins to differentiate at the centre of each lobe; this would induce the binding substance. In this way, the number of lobes may be multiplied.

The secondary wall becomes detectable at late stages of development when the mature cell shape has almost completely developed. Sometimes, 2 layers of the primary and secondary walls were not recognized in cells at late developmental stages. In these cells, cellulose would be incorporated in the primary wall in such a manner that the boundary between the primary and secondary walls is not sharply delimited.

Thus, it may be said that the shape of the daughter cell is determined by the shape of the developed primary wall, which is induced by precocious differentiation of the wall at the sinus, and that the role of the secondary wall may be to toughen the wall through the presence of cellulotic microfibrils.
REFERENCES


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