Cell Wall Architecture Prerequisite for the Cell Division in the Protoplasts of White Poplar, *Populus alba* L.

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White poplar (*Populus alba* L.) protoplasts were investigated at 0, 3, 10, 20 and 30 d after regeneration to visualize the cell wall architecture prerequisite for cell division. The 10 day-old cells just before cell division developed a thin wall layer with uneven deposition of cell wall materials and were saved from bursting by suspension in low osmotic medium. The three dimensional architecture of the cell wall, as revealed by rapid-freezing and deep-etching electron microscopy, in 10 day-old cells, constituted thin innermost lamellae on the plasma membrane along with highly extended microfibrillar networks. These results suggest that the deposition of thin lamellae is important not only for cells to withstand bursting but also to induce cell division. The present investigations give the first account of the visualization of the three-dimensional architecture of regenerated cell wall right before cell division.

Key words: Cell division — Cell wall — Deep-etching — Innermost lamella — *Populus alba* L. — Protoplasts.

Generally, it is difficult to culture plant-regenerable protoplasts of woody plants because they grow slowly (Ochatt and Power 1992). It is known that it takes longer in woody plants than herbaceous plants for the cell wall to form and the cell division to initiate (ex. tomato, tobacco; Ochatt and Power 1992, Morgan and Cocking 1985, Niedz et al. 1985, Shahin 1985, Tan et al. 1987, Zapta et al. 1981). The time taken for cell division after regeneration could be dependent on the nature of the regenerated cell wall, which might be different between woody and herbaceous plants. However, there has been little information on structural analysis of cell wall regeneration in woody plants.

Galun (1981) and Ochatt and Power (1992) described that cell wall formation and cell division often correlate. However, such an assertion is still open to debate. Meyer and Abel (1975) proposed that a rigid wall is not required for cell division by showing the presence of a non-rigid pseudo-wall during cell division. However, no ultrastructural details of non-rigid wall were given. Schilde-Rentschler (1977) found that the addition of cellulase to tobacco

protoplast cultures inhibited wall formation as well as cell division, and inhibiting added cellulase with cellobiose resulted in initiation of cell division. Zelcer and Galun (1980) showed that the inhibitory effect of coumarin on microfibril formation in tobacco protoplasts arrested cell division, but cell division occurred soon after coumarin removal. These reports suggested that cell wall formation was correlated to cell division. However, we do not yet know the nature of the cell wall right before cell division. Further, it is difficult to understand the importance of cell wall for initiating cell division without ultrastructural information on cell wall regenerated from protoplasts.

We investigated the cell wall architecture during the course of cell wall regeneration from protoplasts to define the nature of the cell walls in *Populus alba* L. before and after cell division. For this purpose, we applied rapid-freezing and deep-etching electron microscopy and visualized the three dimensional correlation between plasma membrane and cell wall. Furthermore, we have tested the osmotic stability of the regenerated wall formed during the course of protoplast regeneration by suspending the cells in low osmotic medium. Finally, we elucidated whether or not cell wall formation is a prerequisite for cell division in cultured protoplasts of white poplar.

Materials and Methods

Plant materials and protoplasts—Mesophyll tissues in poplar (*Populus alba* L.) were used for donor tissues of protoplasts (Sasamoto and Hosoi 1990). The leaves were cut from shoot cultures, which were grown and maintained in a growth chamber (25°C, 16 : 8 light/dark) for approximately one month. Protoplasts were isolated from the cuttings of leaves using 1% Cellulase Onozuka RS (Yakult Co., Ltd., Tokyo, Japan) and 0.25% Pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd., Tokyo, Japan) in 0.6 M mannitol solution. The protoplasts were cultured in modified liquid MS medium (ammonium nitrate free) containing 0.6 M mannitol, 1 μM of 2,4-D and 0.1 μM of BAP. Protoplasts were enumerated with a hematocytometer, and cell densities were adjusted to about 5×10^4 protoplasts ml^{-1}. They were cultured in 24 well plates (Falcon, Nippon Becton Dickinson) at 28°C in the dark.

Ultra-thin sectioning—Specimens were fixed and dehydrated according to Fowke (1982). The specimens were embedded in Spurr's resin, and sectioned with an ultramicrotome (Ultracut E; Reichert, Austria). Sections were stained with 2% uranyl acetate and Reynolds' lead citrate before observation under a transmission electron microscope (model 2000 EX-II; JEOL, Japan).

Rapid-freezing and deep-etching—Specimens after a fixation

Abbreviations: BAP, 6-benzylaminopurine; RFDE, rapid-freezing and deep-etching.

in 3% glutaraldehyde for 2 h, were slowly transferred into distilled water. They were rapid-frozen by contact with a copper block that had been precooled with liquid helium (QF-5000; Meiwa Co., Ltd.). The specimens were fractured at $-150°C$ and etched for 15 min at $-95°C$ using a freeze-fracture unit (BAF 400D; Balzers, Liechtenstein), then rotary-shadowed at 25° with platinum/carbon and coated at 85° with carbon. Subsequently, replicas were cleaned on 50% sulfuric acid containing 5% potassium dichromate, washed with distilled water and picked up on copper grids coated with Formvar.

Determination of osmotic stability of the protoplasts and regenerated cells—Determination of osmotic stability of the cells was performed by modified methods of Meyer and Abel (1975). Protoplasts (2×10^6 protoplasts ml^{-1} in 0.6 M mannitol) and the 3, 10, 20 and 30 day-old cells were transferred into culture medium (1.5 ml) containing 0, 0.3, 0.4, 0.5 M mannitol. They were left for 1 h, at 28°C and then the number of burst or non-burst cells was counted. More than 200 cells in a single well were counted for each experiment, and experiments were repeated at least 3 times for each stage of cell development.

Measurement of cell-expansion—The cells typical to each stage (0, 3, 10, 20 and 30 d after protoplast regeneration) were picked up with a micro manipulator (Shimadzu Co., Ltd.) and transferred into 1.5 ml of distilled water. They were left for 1 h, at 28°C, and then both their length and width were measured. These experiments were repeated at least ten times for each stage of cell development.

Results

Development of poplar protoplasts—The growth and development of the mesophyll-derived protoplasts of white poplar (*Populus alba* L.) were observed. The first cell division occurred in about 10 d, but the number of divided cells was only 4–5 cells/a well. Thus, a large number of cells were at the stage just prior to cell division. Cell wall deposition was further confirmed by polarizing microscopy (ORTHOPLAN-POL, Leitz) in 10 day-old cells, but not in 3 day-old cells. The number of divided cells increased abruptly in 20 d from the initiation of protoplast culture (400 cells well^{-1}) and was a hundred times that at 10 d. At 30 d, individual cells showed more than two divisions, resulting in colony formation. It is difficult and meaningless to estimate the number of cell divisions at this stage.

Osmotic stability of the protoplasts and regenerated cells—We investigated the osmotic stability of the regenerated cells to clarify how strong the cell wall is at each developmental stage of cell wall regeneration. Primarily, the osmotic stability of the cells showed different responses to individual mannitol concentrations at each stages (Fig. 1). The 10, 20 and 30 day-old cells did not burst, as they were suspended in protoplast-culture media without mannitol. Thus, their wall structures were strong enough to withstand bursting. On the other hand, almost all of the protoplasts and 3 day-old cells showed bursting when suspended in media without mannitol. However, the 3 day-old cells were slightly more tolerable than the protoplasts when suspended in 0.4 and 0.5 M mannitol.
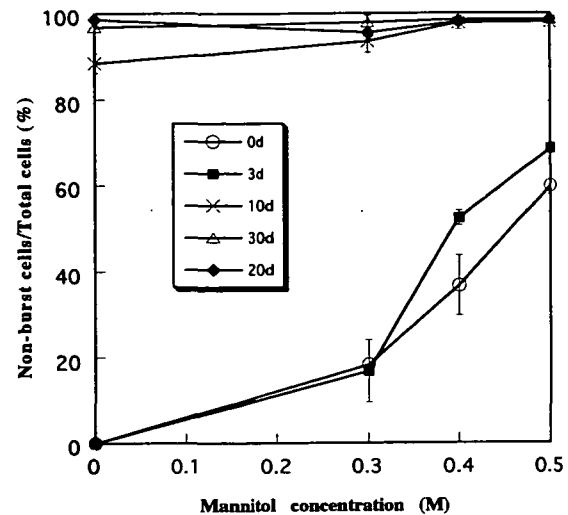

Fig. 1 Osmotic stability of the protoplasts and cells. Cells were transferred into culture medium with 0, 0.3, 0.4 and 0.5 M mannitol and left for 1 h at 28°C, and the percent non-burst to total cells (more than 200 measurements) calculated. These experiments were repeated at least 3 times for each cell stages. Error bars represent the standard deviation. O: freshly isolated protoplasts. ■: 3 day-old cells. ×: 10 day-old cells. △: 20 day-old cells. ◆: 30 day-old cells.

Ten day-old cells are of two types, divided and non-divided cells. We collected both types separately using a micro manipulator and then tested the osmotic stability of the cells. It was shown that both types of cells were capable of withstanding bursting. Furthermore, no substantial change in cell diameter dependent on the swelling was recorded.

The architecture of cell wall regenerated from protoplasts—The structure of cell wall regenerated from the mesophyll-derived protoplasts of white poplar (*Populus alba* L.) was observed by thin-sectioning electron microscopy. The freshly isolated protoplasts as well as 3 day-old cells were spherical in shape and no deposition of cell wall materials was observed (Fig. 2a, b). At 10 d of protoplast culture, it was found that the wall materials were deposited scatteredly along and outside the plasma membrane (Fig. 3a, b). The deposition of cell wall materials at this stage was not even along the surface of protoplast. At 20 d of protoplast culture, some cells had an enlarged nucleus (data not shown), and wall thickness had increased relative to 10 day-old cells. The cells at this stage showed a loosened wall with many spaces upon higher magnification possibly because of low density of cell wall materials (Fig. 4). At 30 and 45 d of protoplast culture, the cells were characterized to have formed large colonies with asymmetric division (data not shown). The cell wall materials were densely deposited compared to that of 20 day-old cells (Fig. 5). However, the thickness of the cell walls varied little between 20 and 30 or 45 day-old cells.
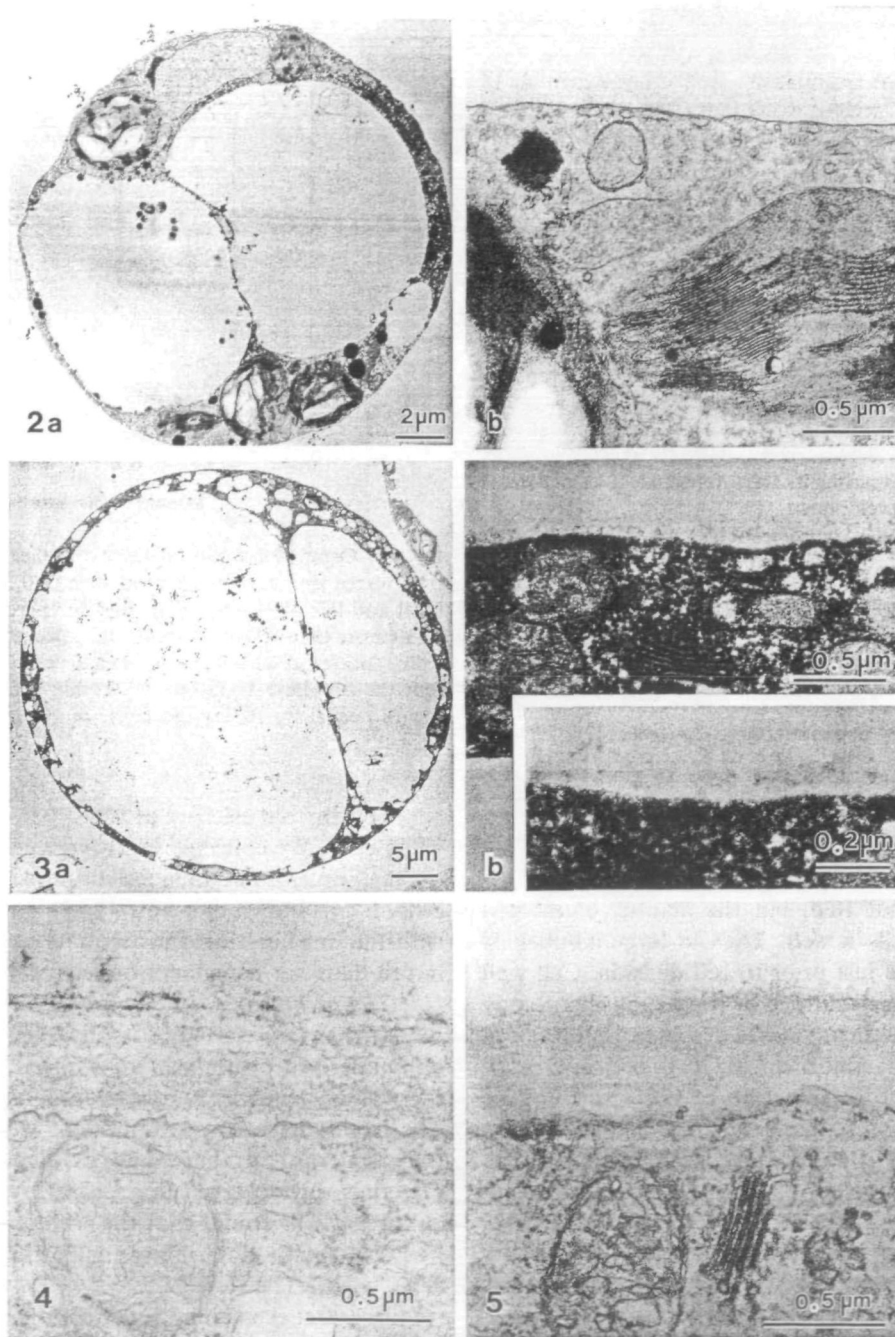
Fig. 2 Three day-old cell. (a) The plastids have changed from an oval to round shape. (b) Higher magnification of part of Fig. 2a. Deposition of cell wall is not observed.

Fig. 3 Ten day-old cell. (a) A highly vacuolated and spherical protoplast. (b) Sporadic deposition of cell wall materials can be seen along the plasma membrane. Inset shows the deposition of cell wall materials at higher magnification.
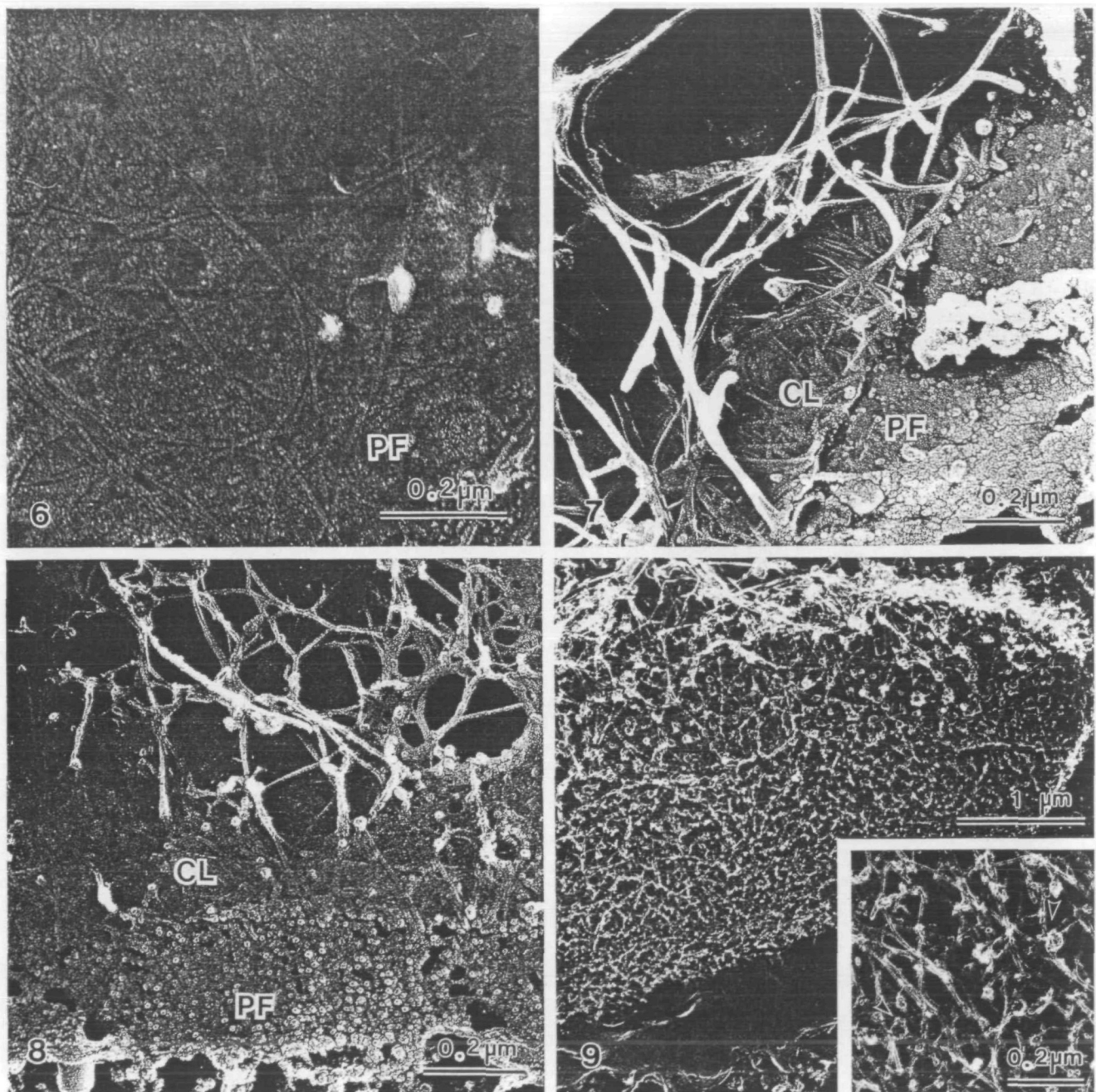
Fig. 4 Twenty day-old cell. Porous and loosened wall can be seen.

Fig. 5 Thirty day-old cell. The cell wall is much denser than at 20 d.

The rapid-freezing and deep-etching electron microscopy disclosed the deposition of cellulose microfibrils in 3 day-old cells (Fig. 6), contrary to the result obtained by sectioning. The microfibrils were oriented randomly in close

Fig. 6   RFDE image of 3 day-old cell. Some microfibrils are deposited sporadically on the surface of the plasma membrane (compare with Fig. 2 that shows no deposition of microfibrils). PF: protoplasmic face of the plasma membrane.

Fig. 7   RFDE image of 10 day-old cell. The plasma membrane is completely covered with thin microfibrillar lamella. All microfibrils outside the lamellae are extended and loosened, resulting in the formation of a network architecture with large meshes. PF: protoplasmic face of the plasma membrane. CL: cellulosic lamellae.

Fig. 8   RFDE image of 20 day-old cell. The diameter of the meshes in the network structure decreased because of the dense and increased deposition of microfibrils. All microfibrils outside the lamellae are extended and loosened, resulting in the formation of a network architecture with small meshes. PF: protoplasmic face of the plasma membrane. CL: cellulosic lamellae.

Fig. 9   RFDE image of 30 day-old cell. The fine network architecture is seen throughout the cell wall. Some granular substances are also observed in the cell wall (arrowhead in the inset). Thin lamellae can not be observed because the cell is covered with a thick cell wall layer.

contact with the plasma membrane and the surface of the plasma membrane was not covered completely with microfibrils. This demonstrated that the plasma membrane is supported partially by microfibril deposition. No microfibrils were extended outside the cell. Figure 7 shows the wall architecture of 10 day-old cells. The right side of the figure shows the PF-face of the plasma membrane. Dense deposition of microfibrillar lamellae can be seen in the middle of the figure. The plasma membrane was completely covered with lamellae at least two microfibrils thick, each lamella with a different orientation. The left side of the figure shows the microfibrils deposited previously as lamellae. These microfibrils seemed to be loosened and extended markedly during cell swelling, thus making large spaces often more than 300 nm in size in the cell wall. The mean diameter of the spaces at this stage was 101 nm. The plasma membrane was always covered with new lamellae. The new cell wall lamellae which deposited tightly on the surface of the plasma membrane were extremely difficult to observe by ultra-thin sectioning, because they were too thin to be visualized. After 20 d of protoplast culture, the deposition of cell wall materials increased, with microfibrillar networks of mean diameter 33 nm seen in the entire wall, and the size of

the mesh decreased gradually because of the dense and increased deposition of microfibrils (Fig. 8). The plasma membrane at this stage also was covered with thin wall lamellae. The microfibrils protruding from the thin wall lamellae increased in number as compared with the protoplast culture for 10 d. After 30 d of protoplast culture, the deposition of the cell wall increased more than that of 20 day-old cells, leaving the network architecture of the cell wall with a mean diameter of 25 nm which is smaller than that of 20 day-old cells (Fig. 9). The fine networks were constructed by the deposition of microfibrils approximately 6–10 nm in width at this stage. Some granular substances were also observed in the cell wall (arrowheads in the inset of Fig. 9).

Discussion

During the course of cell wall regeneration from mesophyll-derived protoplasts in white poplar, the first cell division occurred in 10 d. However, very few cells showed cell division at this point. The 20 day-old cells showed cell division a hundred times more extensive than 10 day-old cells. Thus, 10 day-old cells were regarded as being at the non-
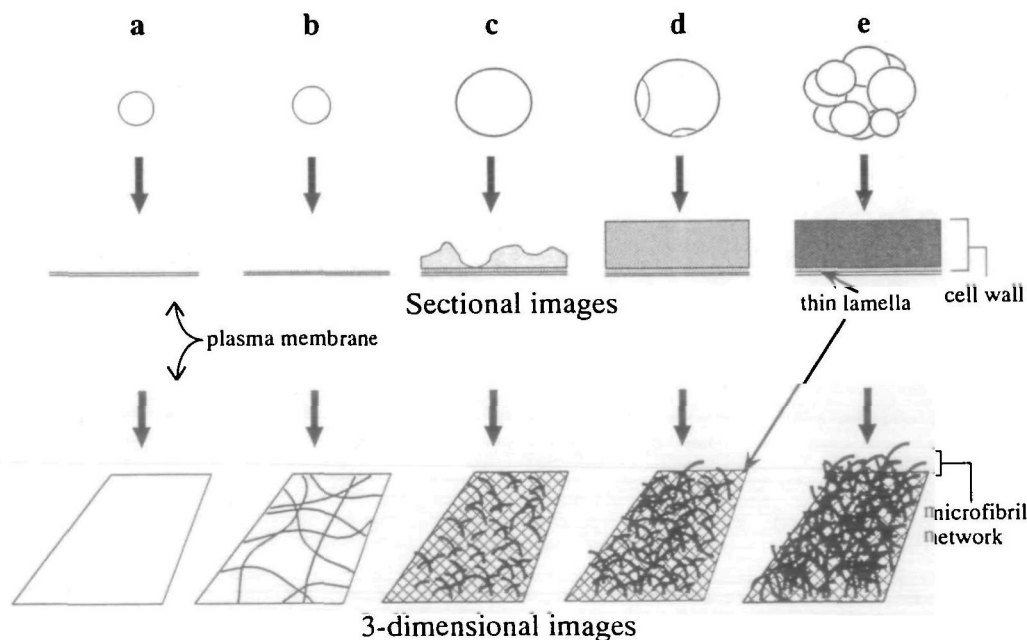
Fig. 10 Schematic illustrations of cell wall regeneration from protoplasts. The developmental stage of regenerated protoplasts is shown in the upper level of the figure. The images taken by ultra-thin sectioning technique are shown in the middle level. The images taken by RFDE technique are shown in the lower level. (a) Freshly isolated protoplasts have no cell wall. (b) Random and sporadic deposition of microfibrils is observed on the plasma membrane in 3 day-old cell using RFDE techniques (lower level). However, they cannot be observed in thin sections (middle level). (c) The scattered deposition of the cell wall materials in 10 day-old cell is shown in the sectional image (upper level). When viewing by RFDE techniques, the plasma membrane is completely covered with thin lamellae, leaving extended microfibrils outside the lamellae (lower level). (d) Thin lamellae closely associated to the plasma membrane can be seen in 20 day-old cell. A number of microfibrils form small networks leaving many spaces (lower level). (e) A large number of microfibrils are deposited densely to form fine networks and cover the thin lamella close to the plasma membrane in 30 day-old cell.

dividing stage.

The test for osmotic stability indicated that both proto-plasts and 3 day-old cells showed a high rate of bursting when suspended in medium containing 0.4 or 0.5 M mannitol. By contrast, 10, 20 and 30 day-old cells did not show bursting when suspended in the same medium. The number of non-burst cells slightly increased at 10, 20 and 30 d upon suspension in medium containing 0.3 M or no mannitol. We separated divided from non-divided cells using a micro-manipulation 10 d after the regeneration of protoplasts and then suspended the cells in different mannitol solutions. Neither cell type showed bursting at this stage. These findings suggest that a substantial change in cell wall nature occurs at between 3 and 10 d. It should be noted that 10 day-old cells right before cell division have already develop-ed a cell wall that can withstand the osmotic pressure gener-ated by 0.4 or 0.5 M mannitol.

Based on the results obtained from the regeneration of cell wall in poplar mesophyll-derived protoplasts, a schematic diagram was produced (see Fig. 10). The upper level of the figure shows cell development. The middle level shows the ultrastructural change of the cell wall based on observation by ultra-thin sectioning. The lower level shows three dimensional change of cell wall architecture based on the visualization of RFDE techniques. The cell walls are not deposited on the surface of the plasma membrane in freshly isolated protoplasts (Fig. 10a). At the very begin-ning of cell wall regeneration in 3 day-old cells, it appeared that no cell wall materials had been deposited based on the observation of ultra-thin sectioning. However, cellulose mi-crofibrils were found to have been deposited randomly on and closely attached to the plasma membrane by RFDE (Fig. 10b). The protoplasts at this stage are not completely covered with microfibrils on the plasma membrane. The sporadic deposition of cellulose microfibrils may explain the difference in osmotic stability between freshly isolated protoplasts and 3 day-old cells after suspension in 0.4 or 0.5 M mannitol solution; that is, 3 day-old cells are osmoti-cally more stable than protoplasts due to the mechanical support of microfibrils (Fig. 1).

The regeneration of random microfibrils in 3 day-old cells is followed by the formation of two different types of cell wall as is found in 10 day-old cells; that is, an inner-most lamellae at least two microfibrils thick is deposited close to the plasma membrane and a network structure of microfibrils extends outside the lamellae (Fig. 10c). The net-work structure does not always cover the plasma mem-brane as is shown in Fig. 2b. The surface of the plasma membrane often lacks the deposition of such structure, which means the structure is not functioning in the mecha-nical support of the cells. The innermost lamellae provide the major structural support to the cells. It is suggested that the lamellae with random microfibrils are too thin to be ob-served in cross sections of the cell wall. The microfibrils

will be passively extended and shifted toward the outside of the lamellae. In short, the microfibrils can be seen as a pro-trusion from the innermost lamellae due to their extension during swelling of regenerated cells.

Most previous attempts to visualize the deposition of microfibrils have utilized the simple freeze fracture tech-nique (Grout 1975, Willison and Cocking 1975, Willison and Grout 1978). Most of the figures in these reports shows microfibrils in close contact with plasma membrane, and the technique is not appropriate for the three dimensional visualization of the cell wall. The first deep-etching study of regenerated cell wall was done by Cooper et al. (1994). However, they showed networks of microfibrils lifted up from the plasma membrane at the very early stage of cell wall regeneration of tobacco protoplasts. No microfibril-lar lamellae were shown to be closely associated with the plasma membrane. In the present investigations, we have found two phases to the regeneration of wall throughout the regeneration of white poplar protoplasts; that is, cellulosic lamellae closely attached to the plasma mem-brane and network structures consisting of microfibrils ex-tended outside the lamellae.

Even at more advanced stages of cell wall regenera-tion, cellulosic lamellae surrounding the protoplasts form-ed with a number of protrusions of microfibrils radiating from the surface of the lamellae, thus the microfibrils make a loose network structure outside the lamellae (Fig. 10d). The wall architecture, which is observed as a loose network of microfibrils by RFDE techniques, is consistent with the loosened wall structure observed by ultra-thin section-ing. The mesh size of the network structures gradually decreases during the regeneration of the cell wall. The mesh size of the regenerated cell wall at more advanced stages of cell development should come close to that of tissue cells in poplar. We do not have such data for poplar tissue cells; however, according to previous reports, the mesh size of onion cell wall is less than 10 nm in diameter, with a rare maximum of 20 nm (McCann et al. 1990) and that of the cell wall of poplar suspension cultures is 18 nm (Itoh and Ogawa 1993). In the present study, 30 day-old cells showed a mesh size of ca. 30 nm mean diameter. This suggests that the mesh size of these cells is not characteristic of the mature cell wall. Furthermore, the network structure of the microfibrils with mesh size of ca. 300 nm diameter in 10 day-old cells could not be involved in the mechanical sup-port of the protoplasts, instead the innermost lamellae is critical for the support. It has been suggested by Richmond (1983) that cell expansion is governed by the innermost lamellae in Nitella. The present results are the first to dem-onstrate that the innermost lamellae play a critical role not only in supporting protoplasts but also inducing cell divi-sion in higher plants.

The relationship between cell wall formation and cell division has been much debated. Galun (1981) stressed the

importance of wall formation as a prerequisite for cell division. Ochatt and Power (1992) pointed out that wall regeneration in protoplasts is a prerequisite for cell division. In fact, structural observations indicated clearly that cytokinesis, but not nuclear division, was inhibited by 2,6-dichlorobenzonitrile which prevented wall formation (Meyer and Herth 1978). The evidence strongly supports a correlation between cytokinesis and wall formation. Then, how do the cells perceive wall formation to initiate cytokinesis? Is a rigid wall necessary for inducing cell division? Meyer and Abel (1975) indicated that non-rigid cell wall can induce cell division. What is the nature of the non-rigid wall? We clarified that non-rigid wall is made of two different structures; that is, the innermost lamellae and a network structure of microfibrils extending from the lamellae. The innermost lamellae are at least two microfibrils thick and critical for inducing cell division. It is hypothesized that the innermost lamellae tightly attached to the plasma membrane can withstand enough turgor pressure that cell division must be triggered indirectly by a physical force via stimulus to the plasma membrane or some unknown mechanism in the cell. Physiological and biochemical analysis of cell wall is needed to elucidate how the innermost lamellae function in cell division.

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