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A SEM Study on the Layer Structure of Secondary Wall of Differentiating Tracheids in Conifer*

By

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走査電子顕微鏡による針葉樹の分化中仮道管の
2次壁層構造に関する研究*

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CONTENTS

Introduction

It is well known that the secondary wall of the normal coniferous tracheid consists of three layers with different nature, i. e., the S 1, the S 2 and the S 3 layer. KERR and BAILEY first gave this idea by the optical microscopy[20]. Afterwards, especially by the transmission electron microscopy (TEM) with the replica technique, the organization of the secondary wall of coniferous tracheid has been studied in detail[5,6,7,8,11,16,18,31,33,35,36,37]. However, hardly all descriptions of the formation and structure of the secondary wall of coniferous tracheids are completely identical.

WARDROP and HARADA suggested that the S 3 layer had zero to six lamellae

which were crisscrossed in a structure and had alternating S and Z helical orientation microfibrils[36]. DUNNING exemplified that the S 3 included at least 12 lamellae in the long leaf pine latewood[5,6]. According to his observation, the S 3 lamellae demonstrate clockwise and counterclockwise rotations. It has been reported that the structure of the S 1 is similar to that of the S 3 in its crisscrossed structure[18,36].

HARADA concluded that intermediate or transition lamellae lay between the S 1 and S 2, and between the S 2 and S 3[11,12]. WARDROP and HARADA stated that transition lamellae between the S 1 and S 2 were observed in mature fibers of hardwood, but these appeared to be somewhat variable in its detailed organization[36]. IMAMURA et al. showed that transitional microlamellae lay between the S 1 and S 2 and between the S 2 and S 3[18]. According to their observation, the S_{12} layer changed gradually in angle, on the other hand, the S_{23} layer changed abruptly. On the contrary, DUNNING stated that transition layers were not ontogenecally separate structures[5,6].

WARDROP and HARADA suggested that all layers of the secondary wall were lamellated and the secondary thickening began near the center of the cell and progressed towards the cell tips, by the polarized light microscopy and the autoradiography[36]. IMAMURA et al. also reported that the progressive development of successive microlamellae was observed in the inner surface of a tracheid forming the S 1 layer[18]. On the other hand, BAIRD et al. stated that the microfibrillar orientation of last deposited wall lamellae was relatively constant over the entire visible length in all developing tracheids in balsam fir[2].

The lamellation of the S 2 layer is a generally accepted feature of the secondary wall by many workers[1,5,6,18,19,33,34,36,37], but CHAFE stated that cellulose microfibrils of the S 2 appeared to be distributed in non-lamellate fashion[4].

In recent years, the scanning electron microscope (SEM) has been used for studying the ultrastructure of the cell wall, especially, the cell wall sculpturing[27,28,29]. These investigations making use of characteristics of SEM appear to be highly evaluated. Advantages of SEM over TEM are firstly that relatively large areas ($\sim$ 1 cm²) of the specimen can be examined[19]. For instance in relation to the present study, in one specimen of the wood sectioned radially, all the differentiating xylem cells can be examined accurately and also all the lumen surface in a differentiating tracheid. In addition, the specimen preparation for SEM is generally simpler and more rapid than that for TEM[19]. However, SEM possesses a lower resolution compared with that obtained with TEM and the specimen is often damaged by the electron beam irradiation[19]. In the point of view of the resolution, TEM is very useful for observing such fine features as orientation of microfibrils of the cell wall in the case of the replica techniques which determined in total actual resolution. On the other hand, the observation of the microfibrillar orientation in detail is also basically possible within the resolving power of SEM. In addition, when vessels in hardwood which has more complicated organization of wall are examined, very effective use of SEM is expected because of many advantages mentioned above although acutually the microfibrillar orientation of the cell wall of hardwood has been examined by SEM[23,26] but not in detail as yet. When

the ultrastructure of specimens is observed by SEM, the specimen coating appears to be most important among many factors concerned with specimen preparations. If it is solved, in future, SEM will be used more frequently for the study of the cell wall ultrastructure conjointly with the high resolving power of this instrument.

The purpose of this work is to obtain more suitable method for observing the orientation of depositing microfibrils in differentiating wood cells by SEM, and then to examine the cell wall formation and structure of woods using it. Further observations of the fine filamentous substances mentioned as supposingly micro-tubules in the previous paper[15] were also made.

Materials and Methods

Wood materials for this study were obtained from a thirty year old Todomatsu tree (*Abies sachalinensis* MAST) grown in a plantation in Tomakomai Experiment Forest, Hokkaido University. They were collected at two weeks intervals during the active period of the cambium in 1978.

Small cubes of fresh materials (about $1 \times 1 \times 1$ cm) containing xylem, cambial zone and phloem were subjected to two different kinds of immersion : 1) the speci-men was plasmolyzed in sacchalose of 0.8 M for a day and 2) it was plasmolyzed and then fixed in glutaraldehyde and osmium tetroxide with the high osmotic buffer for five hours. Two treatments were made in order to detach the cytoplasm from the newly-formed cell wall using plasmolysis[17]. The specimen was fixed in order to prevent from disturbing the newly-deposited microfibrils and contracting by the drying.

Materials plasmolyzed and/or fixed were washed, dehydrated in graded series of ethanol or acetone, dried by the crytical point drying method and then sectioned radially by the razor blade in order to disclose the lumen surface of the cell. After the drying, six different kinds of specimen preparations for making it have the electron conductivity and so on needed, were attempted to employ for SEM observation for reducing the loss of the fine detail of the specimen surface. We will describe later the reasons, for convenience sake in the description.

The method for observing the microtubules was as same as that mentioned in the previous paper[15]. SEM observations were made with JSM-2 at 25 kV.

Results

Specimen preparations for SEM

The method for this study was nearly same as the replica technique[17] till the drying of the successive preparations of specimens. After the drying except for the method of 6), six different kinds of preparations for the conductivity were attempted in order to observe the microfibrillar orientation in detail using SEM: 1) the material was coated with carbon and gold by the vacuum evaporation, 2) it was coated with gold by the ion sputter coating, 3) it was ion-ethched with glow discharge and then coated with gold with it. Ion accelerating voltages of D. C. 500 V with ion currents of 2 mA respectively, were utilized for etching, 4)

it was shadowed with Pt at an angle of approximately 45° to the holizontal in much the same manner as the replica technique and then coated slightly with gold by the ion sputter, 5) in the same manner mentioned above as 4), it was shadowed and then ion-ethched with glow discharge for ranging from 5 to 30 minutes. It was coated with gold by the ion sputter, 6) the conductive stain method with tannic acid and osmium tetroxide[25] was attempted in stead of metal coating before the drying.

1) The vacuum evaporated coating and the ion sputter coating are generally used for SEM. The striation of microfibrils were only revealed roughly but not fine detail. (Photo 1)

2) The ion sputter coating was employed in order to gain the very uniform and more thin layers of the metal, but results were similar to that of the evaporated coating. In addition, there were small pores on the inner surface of differentiating tracheids (Photo 2) and fibrils of the margo structure of bordered pits in conifers were also destructed by the ion sputter coating, while there were no damage in the case of the evaporated coating.

3) Ion etching techniques have been utilized in attempt to reveal the sub-surface structure of the cell in SEM and many researchers have studied the effect of it[10,13,32]. When specimens were etched with D. C. 500 V and 2 mA for 0.5 to 20 minutes, striation of cell wall was revealed at lower magnification of SEM (Photo 3) showing ridge-like features at a higher magnification. (Photo 4)

4) The method that materials were shadowed with Pt in much the same ways the replica technique and then coated slightly by the ion sputter was adequate to observe the microfibrillar orientation of differentiating tracheids in detail by SEM. The microfibrillar orientation of depositing secondary wall was clearly shown (Photos 5, 6 and 7) and the crisscrossed structure which was shown only by the replica technique could be exemplified by SEM. (Photo 8)

5) The cell wall of woods are more easily ion-etched in comparison with the metal (Pt). The specimen surface which was shadowed with Pt was ion etched in order to dig the region which had no Pt coated. In this method, the topography of the cell surface could be outstanding (Photo 9). If the ion etching is employed excessively, the region coated with Pt is also etched, resulting in destruction of the cell surface.

6) The conductive stain method which is made before the drying appears to be not adequate to this study because of the damage of the specimen by the chemical reaction and the electron beam irradiation.

On the basis of the results mentioned above, after the drying, the method of 4) that the material was shadowed with Pt and then coated slightly by the ion sputter coating was highly evaluated for observing the fine detail of secondary walls in this study.

Cell wall formation and structure

This investigation was restricted to the radial wall of non pitted area of differentiating tracheids in earlywoods.

When microfibrils of the inner surface of differentiating tracheids were examined by SEM with a high magnification, they were mostly deposited in parallel to each other, and they were found had a relatively constant distance of about $1000 \sim 2000$ Å or closely arranged. (Photos 7 and 10) In the case of the former, the underlying lamella or layer can be visible easily, but not in the latter. The diameter of microfibrils was about $200 \sim 300$ Å, but it increase by the metal of shadowing and coating, the thickness of which appears to be some n. m., and the length of them was $10 \mu m$ and above.

The structure as if newly-deposited microfibrils were held to be laid down between existing microfibrils, as a result of intussusception growth, was rarely visible. (Photo 11)

In the plasmolyzed sample, microfibrils prior to the lignification were occasionally detached from the wall already formed and attached to the outer surface of the plasmamembrane. (Photo 12 and 13). Such appearances were easily detected by SEM observation on the large area of specimen surface. When these appearances can not be detected, there is a great possibility that the underlying layer or lamella may be recognized to be the last-deposited lamella.

The structure which consisted of lamellae having alternating S and Z helical orientation of microfibrils was termed "crisscrossed structure"[36], but in this study we will term the structure, in which microfibrillar orientation is different in angle between two lamellae, "crisscrossed structure". The crisscrossed structure in such meaning was often present in the S 1, the S 3 and the transition layer, but not found in the S 2 layer except in the region of bordered pits. The microfibrillar angle between two lamellae was changed largely in the transition layer of S_{23} as compared with the other layers. (Photos 8, 14, 15 and 16)

The lamella which appeared to have the thickness of one or two microfibril lay on the underlying layer already packed with substances which might be the hemicellulose in all cells undergoing the secondary wall formation. (Photo 17). The presence of the lamella, as a matter of course, was evident in the S 1, the S 3 and the transition layer because of the presence of the crisscrossed structure. On the other hand, in the S 2 layer the microfibrillar angle of the newly-deposited lamella was nearly same as that of underlying layer or lamella, therefore, there could be found no crisscrossed structure. As shown in photo 18, however, the lamellae were found evidently in the S 2 layer by SEM.

The microfibrillar angle of the inner surface of the cell forming the secondary wall was measured, in turn, in a radial file from the cell forming the S 1 layer to that forming the S 3 layer. Cells undergoing the secondary wall formation observed were about sixteen in number in a radial file. Microfibrils of the S 1 (cells were $3 \sim 4$ in number), the S 2 ($8 \sim 10$) and the S 3 ($2 \sim 3$) lay about at an angle of $90°$, $5° \sim 10°$ and $90°$, respectively, with respect to the cell axis. Cells, microfibrils of which lay at an angle of about $45°$ or ranging from $30°$ to $60°$, were situated between the cell forming the S 1 and that forming the S 2 layer, and between the S 2 and S 3. The change of the spiral inclination was, in turn, shown successively from the S 1 layer to the S 3 layer in photos 19, 20, 21, 22 and 23. It was ascer-

tained by this observation on the inner surface in a radial file that the lamella which appeared to be transition layer was present between the S 1 and the S 2 and between the S 2 and S 3 layer. Furthermore, the microfibrillar orientation of the cell which appeared to be forming the transition layer was examined in detail. Photos 24 and 25 show representative micrographs of transition lamellae between the S 2 and S 3 layer. Photo 24 reveals that the underlying layer is the S 2 layer with its microfibrils at an angle of about 10° with respect to the cell axis and the lamella of transition layer of the S_{23} lies on it. In photo 25, the lamella which has the appearance of flat-S helix with its microfibrils at an angle of about 45°. This micrograph indicates that the lamella to be the S 3 layer is depositing on the transition lamella and two lamellae are crisscrossed each other. The lamella which is newly-deposited and present on the inner surface, consists of microfibrils arranged at a distance of about $1000 \sim 2000$ Å and different in angle from the underlying layer, thus the underlying layer can be visible through the last formed layer. The lamella of the transition layer has the appearance of Z-helix with its microfibrils not S-helix. As cells forming the transition layer, i. e., cells with its microfibrils at an angle of about 45°, are only a few found in a radial file particularly between the S 2 and S 3, the transition layer appears to be very thin.

The inner surface of a differentiating tracheid was examined over a large area in order to ascertain the progressive development of successive lamellae. The reason for that the cell which had the crisscrossed structure was selected for this observation is that the last deposited lamella can be easily visible in it by the authors. The microfibrillar orientation of a great number of differentiating tracheids was examined at regular intervals along the cell axis. The orientation of microfibrils of the newly-deposited lamella was relatively constant over visible short length of the cell. Photo 26 reveales the inner surface of a differentiating tracheid forming the S 3 layer at 20 μm in length along the cell axis. The underlying layer appears to be a transition layer of the S_{23} and there is the crisscrossed structure between the last-deposited lamella and S_{23} layer. Photos 27 and 28 are also showing the inner surface of a cell forming the S 3 layer. The portion in photo 27 is 1.4 mm. far from it in photo 28. There is a difference in appearance between the two at a long distance. Microfibrils are deposited at intervals of about 2000 Å in photo 27, while in photo 28 they are arranged so closely that the underlying layer can not be visible. It may be said that depositing phase of microfibrils in photo 28 is ahead of photo 27.

Orientation of microtubules

It has been reported by SEM that a number of fine fibrillar structures which may be microtubules are present on the inner surface of the plasmamembrane arranging in Z-helix[15]. The orientation of these organelles was examined in detail using SEM, and also studied by TEM using ultra-thin cross sections for making evident whether they were microtubules or not. These structures were not detected by SEM when the cytoplasmic ground substances were packed densely, but they could be visible in the region where the tonoplast was destructed and torn back,

or the cytoplasmic ground substances were not dense. When the inner surface of the plasmamembrane of differentiating tracheids was successively examined in a radial file, they were oriented at an angle of about 90°, 45°, 5°~10°, 45° and 90° with respect to the cell axis. (Photos 29, 30, 31, 32 and 33). They were arranged relatively in parallel to each other or rarely crisscrossed, and might be about 100~200 Å in diameter, 10 μm or above in length. They had various distance ranging 200 Å to 1 μm. They appeared only in the specimen double-fixed with glutaraldehyde and osmium tetroxide. They were detected by TEM with the cross sections in the same specimen as shown in photo 34, in which they are closely attached with the inner surface of the plasmamembrane.

Discussion and Conclusion

Specimen preparations for SEM

Both the vacuum evaporated coating and the ion sputter coating are not adequate for this study. It is needed for the study by SEM that the topography of the cell surface is not lost and the build-up of electric charge on the specimen surface is reduced. In the former, the fine detail of cell surface was lost by the exess of the metal. The ion sputter coating yielded most thin and uniform layer of metal, and thus the observation of microfibrillar orientation in detail is basically possible within the resolving power of SEM. The reason why the fine detail could not be detected appears to be that the ion sputter coating rise artefacts, especially, in soft tissues as biological specimens, due to the thermal damage or the damage of etching to the cell surface by the colliding of atoms. In particular, no lignified cell wall were more damaged in comparison with the lignified cell wall and the membrane of cell organelles by the ion sputter coating. Therefore, even if higher resolving power is able to use, the fine detail of the real cell can not be detected because of the expense of intact topography of the specimen surface.

In the case of the ion etched specimen as 3), it has been known that artefacts as features of cones or bridges were produced on the cell subsurface of soft tissues as red blood cells[10,13,32]. In the case of wood cells, similar artefacts as red blood cells were observed. As the striation of cell wall as the S 1, the S 2 and the S 3 can be revealed though rough and the warty layer which consists largely of a lignin-like material[3] is less etched. There appears to be no marked selection for the chemical composition in the specimen etched by the ion coater.

The method 4) is most sufficient to this study, the reason why the fine detail of the microfibrillar orientation can be detected appears to be that the thin layer of Pt protects the cell surface from the damage by following ion sputter coating without the loss of topography of the cell surface and of the building-up of electric charge.

Methods of 5) and 6) are not adequate to this study because of the damage of the cell surface by the artefact.

Each of method tested except for 4) is not adequate to study the microfibrillar orientation even if using the other SEM instrument having more higher resolution

in comparison with that of the JSM-2, because the fine detail of the specimen surface is lost by the preparation for having the electron conductivity.

It may be probable that the method of 4) can be applied to study the fine structure of some other biological tissues.

Cell wall formation and structure

The reason why the radial wall was investigated is mainly that the micro-fibrillar orientation of the newly-deposited lamella can be examined successively in a radial file and, after this observation we intend to examine the formation and structure of the pit border using the same specimen, comparing with results observed from this study.   In addition, when the cell corner is observed, the lamella having the one or two microfibril thickness is formed continuously from the radial wall to the tangential one, therefore, the radial wall is equal to the tangential each other in its wall organization.   In the latewood, on the other hand, as the width of the radial wall is more reduced in comparison with that of earlywood, observa-tion was not carried out in this study because it is hard to distingush the radial wall from the tangential.   Since the cell forming the primary wall is more dam-aged as compared with that of the secondary wall by the electron beam irradiation, cells forming the secondary wall, especially forming the S 2 to S 3 layer, were mainly examined.   The cell forming the S 1 layer is distinguished easily from that forming the inner primary layer based upon the pit border development about which observation was conducted simultaneously mentioned above,.

It is considered to be important that results gained by SEM in this study are compared and contrasted with that of the TEM study.

The diameter and the length of microfibrils was measured roughly.   Accord-ingly these can not be discussed minutely in this study because the JSM-2 SEM offers resolutions of only about $100 \sim 200$ Å and the metal thickness of the shad-owing and coating can not be measured precisely.

When the way of the deposition of microfibrils was observed, they were ar-ranged closely or at constant distance. (Photos 7 and 8).   This result may support the conclusion of the way of the microlamella in microfibrillar deposition reported by IMAMURA et al[18].   The lamella, microfibrils of which were arranged at constant distance, was gradually changed to that having microfibrils closely arranged. (Photos 27 and 28).   In this point of view, it is concluded that microfibrils are firstly de-posited at a certain constant distance on the existing lamella in turn deposit be-tween them, and then as do in a space resulting in the lamella having the thickness of one or two microfibril.   The lamella, microfibrils of which were closely arranged, were more frequently found in differentiating tracheids in comparison with that at a constant distance.

The interwoven structure was rarely found in the secondary wall (Photo 11), but it can not be distinguished whether this is an artefact or a true structure.   If immediately after depositing a lamella, the hemicellulose packed it rapidly, it is not possible that the intussusception *dóes* occur.   Microfibrils appears to deposit by the way of the lamella in most cases, however, if such interwoven structure

can exist truely, this may be more frequently appeared in the complicated structure as the region of the pit border.

A lamella sheet of newly-deposited microfibrils are sometime observed to detach from the wall on which they are depositing. (Photos 12 and 13). When all last-deposited microfibrils are detached from the wall existed, the underlying lamella or layer will be regarded as the one. When detached lamella is attached again to the wall of the opposite site during the specimen drying, there might be seen a crisscrossed structure. But, these artefacts can be detected by the SEM observation on the large area and pit apartures. When the progress development of successive lamellae is discussed, it must be careful not to misinterpret these artefacts.

The crisscrossed structure was often visible in cells forming the S 1, the S 3 and the transition layer. The difference in angle between neighbouring two lamellae was more evident in the S_{23} layer. WARDROP and HARADA stated that the S 3 layer consisted of lamellae having alternating S or Z helical orientation of microfibrils[36], on the other hand, IMAMURA et al. reported that this layer consisted of lamellae being different in angle[18]. DUNNING indicated that the S 3 layer included at least 12 lamellae with varying angles of orientation[5,6]. In this study, the S 3 layer was found to have lamellae having microfibrils varied in angle not in a particular helical orientation. (Photo 15). This is rather consistent with that of IMAMURA et al.,[18] but it can not be wholly denied that at least 2~3 lamellae each having same orientation are deposited successively in the S 3 layer. The number of lamellae in the S 3 layer can not be found exactly in this study, because this is variable according to the measurement of the diameter of microfibrils. If the lamella is about 200~300 Å in thickness, there may be 5~6 in number in the S 3 layer. By DUNNING's work[5,6], the S 3 layer includes the transition layer of S_{23} which is divided from the S 3 by the authors, therefore the number of lamellae of S 3 appears to be fewer in this study.

The lamella structure is generally considered to consist of microfibrils arranged concentrically and separated by thin layers of lignin and hemicellulose[1,33]. The lamellation of S 2 layer, at both the light microscopic level and the EM level, has been studied by many workers[1,5,6,34,35,36]. It has been reported that the lamella is 600 Å in thickness by the light microscopic study[1], 100~200 Å by the EM[35], and about 35 Å by the study by nitrogen adsorption and EM study[34]. On the other hand, CHAFE stated that microfibrils appeared to be distributed in non-lamellate fashion in the S 2 layer[4]. According to this study, it is evident that lamellae having one or two microfibril thickness are present in the S 1, the S 3 and the transition layer because of the recognition of the crisscrossed structure. Although no crisscrossed structure was recognizable in the S 2 layer except the pitted region, a sheet of microfibrils was occasionally in the S 2 as shown in photo 18. Therefore, a lamella structure must be considered to be existed in the S 2 layer. This means that concentric lamellae are present but not the radial lamellation. This lamellation of S 2 can not be detectable by the cross section using TEM and the LM. The S 2 layer consists of lamellae each of which has probably one or two microfibril with about 100~300 Å in thickness. This observation is consistent with

earlier works of WARDROP[35], and PARHAM and CÔTÉ who reported that the distribution of the lignin is relatively uniform in the S 2[30]. OHTANI and ISHIDA reported that in some hardwoods, all or the part of S 3 layer was absent in vessels[29]. It is of interest that the conception of the lamella is probably variable in wood species.

Lamellae having the microfibrillar orientation at an angle of 30° to 60° are almost always present between the S 1 and S 2 and between the S 2 and S 3 showing in photos 20 and 22. HARADA concluded the existence of transition or intermediate lamellae[11], on the other hand, DUNNING stated that the terms of S 1, S 2 and S 3 were useful ones but the transition or intermediate layer not[5,6]. WARDROP and HARADA stated that the presence of transition lamellae were uncretain in hardwood[36]. In this study, the transition between the S 2 and S 3 occured abruptly, and gradually between the S 1 and S 2. This result is coincident with earlier work of IMAMURA et al. in *Pinus densiflora*[18].

Each layers of secondary wall, i. e., the S 1, the S 2 and the S 3, is termed on the basis of the difference in orientation of microfibrils. In the point of view of the microfibrillar orientation, the secondary wall appears to be grouped into four classes on the assumption that the microfibrillar orientation is the same in one lamella : 1) the microfibrillar orientation is the same among lamellae in Z-helix, S 2, 2) that is a little different in angle among lamellae in S, Z or flat helix, S 1 and S 3, 3) that is a little different in angle among lamellae in Z-helix, S_{12}, and 4) that is much different in angle among lamellae in Z-helix, S_{23}. The opinion of the authors is that lamellae between the S 1 and S 2 and between the S 2 and S 3, each of which is changed in angle keeping Z-helix, should be termed the transition layer or lamella. It should be considered that the transition layer or lamella is easily detected in the case of the cell which has the S 2 layer having more steep-helix as nearly 0° at an angle, for instance, microfibrils of the S 2 layer in *Abies sachalinensis* early wood are oriented at an angle of about 5°~10°, on the contrary, in the pitted (R–T) region of *Larix leptolepis* early wood at an angle of about 40°~ 45°[26], and microfibrils of the S 3 layer are oriented at an angle of about 90° in both species. This result indicates that the transition layer is variable in its organization within a species and between early- and latewoods. However, the presence of the very thin transition layer appears to be detected easily by SEM, because a number of differentiating tracheids can be observed simply, although the uncertainty of presence of transition layer was reported by WARDROP and HARADA[36].

WARDROP[37], and WARDROP and HARADA[36] suggested that the secondary thickening began near the center of the cell and progressed towards the cell tips. More recently, IMAMURA et al. showed the similar result in the cell forming the S 1 layer[18]. On the contrary, BAIRD et al. reported that the microfibrillar orientation of the last deposited lamellae was relatively constant over their entire visible length[2]. As showing in photos 26, 27 and 28 in this study, the appearance of newly-deposited lamellae was relatively constant between two regions at a short distance, but changed at a long distance. The former is consistent with the result of BAIRD et al.,[2] on the other hand, the latter is similar to that of WARDROP and HARADA[36].

IMAMURA et al. showed that three microlamellae which were different in orientation respectively lay at a short distance in the cell forming the S 1 layer[18]. In this study, however, it was not found an example that both lamellae of the S 2 and the S 3 were depositing simultaneously in a differentiating tracheid, in other words, lamellae which were much different in angle respectively were not present. The assumption that microfibrils appear to be deposited firstly at some distance, secondly closely and lastly to be the lamella which has the thickness of one or two micro-fibrils, as IMAMURA et al. reported[18], may coincide with the result of progressive development of lamellae in this study. The direction of the progressive development and the demarcation of new lamellae can not be detected exactly. As it is no doubtful that there is not much difference in microfibrillar angle in a differentiating tracheid, microfibrils may be deposited rapidly till the formation of the lamella.

Orientation of microtubules

Fine fibrillar structures were present on the plasmamembrane, and the orientation of them was coincident with that of microfibrils of secondary walls of which the angle was successively changed from the S 1 to the S 3. The specimen for this observation was coated only ion sputter, but they were not destructed by the treatment. That cellulose microfibrils were considerably damaged by ion sputter coating, on the other hand, cytoplasmic membranes of cell organelles were less destructed, was mentioned above, therefore these fibrillar structures are not cellulose-like materials but cell organelles. In addition, they were observed only in the specimen double-fixed. They were also detected by TEM with the cross section taken from the corresponding materials for the SEM, and the site and dimension of which was consistent with the result by SEM. It is concluded from these observations that these structures are microtubules. FUJITA et al. reported that microtubules in the cell forming the S 1, the S 2 and G layer were about 200 Å in diameter, 5~8 μm in length and well alighned[9]. This is coincident with that of the present study. The role of microtubules has been investigated by many workers[14,21,22,24,38] and some of them concluded as to be an instrumental for localized thickening of cell wall[9,21,22,38]. In this study, also, the orientation of them agreed with that of microfibrils at each stage of cells forming from the S 1 to the S 3. It may be reasonable to assume that they are instrumental for control the microfibrillar orientation. Apart from the mechanism in detail of the control by them, the orientation can be detected easily and surely by SEM.

Therefore, when the orientation of them is examined, that of microfibrils in the secondary wall can be easily detected. It appears that the SEM observation for this subject must be made in future.

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要　　約

分化中の細胞の壁層構成を SEM (JSM-2) 観察するための手法に改良を加え, これを用いてミクロフィブリルの堆積経過を, これに関連する細胞小器官 (ミクロチュービュール) を含めて観察した. 得られた結果は次のとおりである.

I.　SEM 試料の処理方法

　　1.　試料の乾燥までは, TEM のレプリカ法の場合と同様の方法を用いることができる.

　　2.　SEM に常用されている真空蒸着法 (炭素–金), スパッタコーティング法では, このような軟弱試料表面の微小な凹凸の損傷は避けがたい. 乾燥を完了した試料表面に白金による

shadowing を施した後に，軽くイオンスパッタコーティングを行なうことにより，ミクロフィブリル配向の観察を容易にすることができた。

II.　分化中の針葉樹仮道管の壁層構成観察

　　1.　トドマツ早材仮道管の半径壁の壁孔のない部分の壁層形成経過を追跡観察した。

　　2.　S_{23} 又は S 3 においては，ミクロフィブリルは，まず 1000〜2000 Å の間隔で堆積し，さらにその後，逐次この間を埋めるように堆積してゆき，やがてこれらが密に並び，ある一定の方向をもった 1〜2 ミクロフィブリル厚さの薄層―ラメラ―を形成する。その上に同様の経過で次の段階のラメラが形成される。この形成経過と完成した壁の構造については今村らの報告にほぼ一致した。

　　3.　時折，網目構造的 (interwoven) なものが観察されたが，これは隣接する壁孔などによる構造変異の影響なのかもしれない

　　4.　隣接ラメラ間のミクロフィブリル配向角の相異，すなわち交差構造の存在は典型的 S 3 部分以外の部分 (S 1, S 3, S_{12}, S_{23}) で確認された。ラメラ間のミクロフィブリル傾角変化は S_{23} で最大であった。

　　5.　典型的 S 2 にも，ラメラ間の傾角相異がないにしても，1〜2 ミクロフィブリル厚さの薄層―ラメラ―が事実上存在するものと考えられる。

　　6.　1 つの半径面試料上で，S 1 から S 3 までの壁形成段階にある仮道管群を連続的に観察することができた。分化の若い段階のものから順に，傾斜方向，ミクロフィブリル傾角，細胞数，壁層記号を示すと S 又は Z ・90°・2〜3・ S 1 (交差構造)，Z ・60°〜30° ・3〜4・ S_{12} (交差構造, 移行層)，Z ・5°〜10° ・8〜10・ S 2 (交差構造はない)，Z ・30°〜60° ・1〜2・ S_{23} (交差構造著しい, 移行層)，S 又は Z ・90° ・2〜3・ S 3 (交差構造)

　　7.　1 本の仮道管の軸方向約 2 mm の範囲内ではミクロフィブリルに堆積量の著しい相異は観察されず，とくに前項で述べた 2 stage (S 2 と S 3) が同時に観察されることはなかった。

　　8.　前報でその可能性を指摘しておいたミクロチュービュールの存在が，2 次壁形成中の仮道管の原形質膜内表面上に SEM で確認された。壁形成の各 stage におけるミクロフィブリル配向はミクロチュービュール配向に一致した。

Explanation of Photographs

Note: Longitudinal direction of wood is vertical in all photographs. Photos 30, 31, 32, 33, 34 and 35 were taken from the specimens fixed by glutaraldehyde and osmium tetroxide.

Photo 1. The inner surface of the tracheid forming the S 3 layer. This specimen is coated with C and Au by the vacuum evaporation. The striation of the S 3 is showing roughly. (cf. photos 2, 3 and 7)

Photo 2. The inner surface of the cell forming the S 3 layer. This specimen is coated by the ion sputter coating. Small pores are present on the cell surface by artefacts. (arrow) (cf. photos 1, 3 and 7)

Photo 3. The inner surface of the cell forming the S 2 layer. This specimen is ion-ethced and then coated by the ion sputter coating. The striation is showing markedly.

Photo 4. A higher magnification view of an area in photo 3. Ridge-like features are present on the cell surface by the artefact.

Photo 5. The inner surface of the cell forming the S 1 layer. This specimen is shadowed with Pt and then coated by the ion sputter coating. Microfibrils are oriented at an angle of about 90°.

Photo 6. The inner surface of the cell forming the S 2 layer. The specimen preparation is same as that of photo 5. Microfibrils are oriented at an angle about 5°. (cf. photos 1, 2 and 4)

Photo 7. The inner surface of the cell forming the S 3 layer. Microfibrils are oriented closely at an angle of about 90°. The crisscrossed structure is not detected in this cell. (cf. photos 8, 10 and 14)

Photo 8. The inner surface of the cell forming the S 3 layer. Microfibrils which are oriented at an angle of about 100° lie on the S_{23} layer. The crisscrossed structure is clearly shown.

Photo 9. The inner surface of the cell forming the S_{23} layer. This specimen is shadowed with Pt, ion ethced for 15 minutes and then coated by the ion sputter coating. The topography of the specimen surface is showing markedly. The portion of the shadow is dug. (arrows)

Photo 10. The inner surface of the cell forming the S 3 layer. Microfibrils are depositing at intervals of 1000~2000 Å. The underlying layer of the S_{23} is visible.

Photo 11. The inner surface of the cell forming the S 3 layer. Microfibrils are held down between existing microfibrils. (arrow) In this micrograph, microfibrils which are oriented at an angle of about 100° are depositing on the S 3 layer, microfibrils of which are oriented in flat-helix.

Photo 12. The plasmamembrane outer surface and the detached microfibrils in the cell forming the S 2 layer. Microfibrils are attached to the outer surface of the plasmamembrane. They are sheet-like features.

Photo 13.   A higher magnification view of the area in photo 12. Protrusions are present on the outer surface of the plasmamembrane. (arrows)

Photo 14.   The inner surface of the cell forming the S3 layer. Newly-deposited microfibrils are oriented at an angle of 45° with to underlying microfibrils. (cf. photo 15)

Photo 15.   The inner surface of the cell forming the S3 layer. The microfibrillar angle of newly-deposited microfibrils is a little changed as compared with that of the underlying microfibrils. (cf. photo 14)

Photo 16.   The inner surface of the cell forming the S_{12} layer. The crisscrossed structure is clearly shown. (cf. photos 8, 14, 15, 24 and 25)

Photo 17.   The inner surface of the cell forming the S3 layer. Depositing microfibrils are torn back. The lamella as sheet-like features has one or two microfibril thickness.

Photo 18.   Detached microfibrils as sheet-like features in the cell forming the S2 layer. Microfibrils are considerably disturbed.

Photos 19~23 ;   The change of the spiral inclination of microfibrils in a radial file.

Photo 19.   The inner surface of the cell forming the S1 layer.

Photo 20.   The inner surface of the cell forming the S_{12} layer.

Photo 21.   The inner surface of the cell forming the S2 layer.

Photo 22.   The inner surface of the cell forming the S_{23} layer.

Photo 23.   The inner surface of the cell forming the S3 layer.

Photo 24.   The inner surface of the cell forming the S_{23} layer. Newly-deposited microfibribls which are oriented at an angle of about 45°, lie on the S2 layer, microfibrillar angle of which is about 15°. (cf. photo 25)

Photo 25.   The inner surface of the cell forming the S3 layer. Microfibrils in flat-helix are depositing on the S_{23} layer, microfibrillar angle which is about 45°. (cf. photo 24)

Photo 26.   The inner surface of the cell forming the S3 layer. Micofibrils are arranged uniformly in the cell. (cf. photos 27 and 28)

Photos 27 and 28.   Two portions of a differentiating cell forming the S3 layer. The portion in photo 27 is 1.4 mm from it in photo 28. Microfibrils in flat-helix are depositing at regular intervals in photo 27, on the other hand, they are not present in photo 28. The underlying layer as showing in photo 27 is same as the inner layer in photo 28. (cf. photo 26)

Photos 29~34 ;   Microtubules on the inner surface of the plasmamenbrane.

Photo 29.   Microtubules on the plasmamenbrane in the cell forming the S1 layer. They are oriented in flat-helix. (cf. photos 5 and 19)

Photo 30.   Microtubules in the cell forming the S_{12} layer. They are oriented at an angle of about 45°. (cf. photos 16 and 20) A mitochondrion is present. (arrow)

Photo 31. Microtubules in the cell forming the S 2 layer. They are oriented at an angle of about 5°. (cf. photos 6 and 21) The vacuolar membrane is present (arrow), in there microtubules can not be visible.

Photo 32. Microtubules in the cell forming the S_{23} layer. (cf. photos 22, 24 and 25)

Photo 33. Microtubules in the cell forming the S 3 layer. They are few in number as compared with in photos 29~32 and they are rarely crisscrossed. (arrow)

Photo 34. A TEM micrograph showing microtubules. (arrows)

Plate II   Y. Hirakawa and S. Ishida

7    $1\,\mu$

8    $1\,\mu$

9    $1\,\mu$

10    $1\,\mu$

11    $1\,\mu$

12    $5\,\mu$

Plate IV

26

27

1μ

28

1μ

1μ

Plate VI

Y. Hirakawa and S. Ishida

29
1 μ

30
1 μ

31
1 μ

32
1 μ

33
1 μ

34
S2
S1
0.5 μ