REVIEW — THE ORIENTATION OF CELLULOSE MICROFIBRILS IN THE CELL WALLS OF TRACHEIDS IN CONIFERS*
A model based on observations by field emission-scanning electron microscopy

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SUMMARY

We examined the orientation of cellulose microfibrils (Mfs) in the cell walls of tracheids in some conifer species by field emission-scanning electron microscopy (FE-SEM) and developed a model on the basis of our observations. Mfs depositing on the primary walls in differentiating tracheids were not well-ordered. The predominant orientation of the Mfs changed from longitudinal to transverse, as the differentiation of tracheids proceeded. The first Mfs to be deposited in the outer layer of the secondary wall (S₁ layer) were arranged as an S-helix. Then the orientation of Mfs changed gradually, with rotation in the clockwise direction as viewed from the lumen side of tracheids, from the outermost to the innermost S₁ layer. Mfs in the middle layer of the secondary wall (S₂ layer) were oriented in a steep Z-helix with a deviation of less than 15° within the layer. The orientation of Mfs in the inner layer of the secondary wall (S₃ layer) changed, with rotation in a counterclockwise direction as viewed from the lumen side, from the outermost to the innermost S₃ layer. The angle of orientation of Mfs that were deposited on the innermost S₃ layer varied among tracheids from 40° in a Z-helix to 20° in an S-helix.

Key words: Cell wall, cellulose microfibrils, conifers, cortical microtubules, FE-SEM, tracheids.

INTRODUCTION

The physical properties of the plant body are closely related to the structure of cell walls. The cell walls of tracheids are composed of a primary wall and a secondary wall. The primary wall is the cell wall that is formed during the expansion of the surface of the cell and the secondary wall is the cell wall that starts to form just before or immediately

*) Dedicated to the memory of the late Professor Jun Ohtani (1937–2003) who pioneered observations of the three-dimensional anatomy of wood by SEM.

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Associate Editor: Nigel Chaffey
after the cessation of expansion of the cell’s surface. Since the basic study by Kerr and Bailey (1934), who studied transverse sections under polarized light, it has been generally accepted that the secondary wall of tracheids has a three-layered structure. The S₁ layer is the thin outer layer of the secondary wall that exhibits birefringence in transverse sections; the S₂ layer is the thick middle layer that does not exhibit birefringence in transverse sections; and the S₃ layer is the thin birefringent inner layer. The cellulose microfibrils (Mfs) in the S₁ and S₃ layers are generally considered to lie almost perpendicular to the cell axis, while the Mfs in the S₂ layer are oriented at a small angle relative to the cell axis.

The early application of transmission electron microscopy (TEM) to studies of the Mfs in the cell walls of tracheids in the early 1950’s provided some details of the orientation of Mfs (Wardrop & Dadsell 1953; Wardrop 1954, 1957, 1958; Harada et al. 1958). Wardrop (1964) and Wardrop and Harada (1965) proposed a model for the orientation of Mfs of differentiating and differentiated tracheids. This model was modified by Harada and Côté (1985) and became widely accepted. According to this model, the primary wall consists of loosely aggregated Mfs, which are oriented more or less longitudinally in the outer part and transversely in the inner part of the primary wall. By contrast, the Mfs that form the secondary wall are well ordered, and they run parallel to one another and the secondary wall can easily be distinguished from the primary wall by its texture. The S₁ layer, the outer layer of the secondary wall, consists of several lamellae in which the orientation of Mfs corresponds to alternating S- and Z-helices. The helix, when observed from the outer face of the cells, is designated an S-helix when it is a left-handed helix and a Z-helix when it is a right-handed helix relative to the longitudinal axis of the cell. When we observe Mfs from the lumen side of the cell, the helical directions of Mfs are reversed. The Mfs of the S₂ layer are steeply oriented in a Z-helix. By contrast, the Mfs in the S₃ layer form S-helices and are similar to those in the S₁ layer. The presence of two transitional layers (S₁₂ and S₂₃), in which the orientation of Mfs shifts gradually, has been proposed between layers S₁ and S₂ and between layers S₂ and S₃, respectively. These progressive changes are considered to contribute to the semi-helicoidal structure of the cell wall (Neville & Levy 1984; Roland et al. 1987; Vian & Reis 1991).

There have been a number of studies of the orientation of Mfs in the S₁ layer and these studies focused on the observations on mature tracheids that had been macerated (Wardrop 1957; Harada et al. 1958; Dunning 1968, 1969). However, the identification of the outermost surface of the S₁ layer was tentative in these early studies. Dunning (1968, 1969) observed changes in orientation of Mfs in latewood tracheids, which had been split open, of longleaf pine (Pinus palustris). He concluded that transitional layers, namely, S₁₂ and S₂₃ layers, could not be distinguished from the S₁ and S₂ layers, respectively, because of successive changes in the orientation of Mfs. This concept has been supported by some studies, which observed the inner surfaces of differentiating tracheids (Imamura et al. 1972a; Abe et al. 1991, 1992). Dunning also reported that the S₁ layer included more than twelve lamellae, in which the orientation of Mfs shifted by 270° in a clockwise direction and in a counterclockwise direction from the outermost to the innermost surface of the S₃ layer. However, there has been no confirmation of such complicated changes in the orientation of Mfs in the S₃ layer to date.
The orientation of Mfs in the $S_2$ layer has been studied in greater detail than that of Mfs in other layers because the $S_2$ layer is the thickest layer and its properties influence physical characteristics of wood and paper (Heyn 1969; Chafe 1974; Scallan 1974; Meylan & Butterfield 1978; Ruel et al. 1978; Hirakawa & Fujisawa 1995; Hirakawa et al. 1997; Booker & Sell 1998; Butterfield 1998; Donaldson 2001). The existence of a tangentially lamellated structure (Chafe 1974; Scallan 1974; Ruel et al. 1978), or radially agglomerated structures (Sell & Zimmermann 1993; Booker & Sell 1998) has been reported within the $S_2$ layer. Kataoka et al. (1992) reported that Mfs in the $S_2$ layer crossed each other at an angle of less than 15° in developing tracheids of Cryptomeria japonica, Pinus densiflora and Chamaecyparis obtusa.

Although TEM allows an analysis of specimens at high resolution (several Å), the various dimensions of the specimen are restricted to several millimeters. Therefore, it is rather difficult to observe progressive changes in the orientation of Mfs in differentiating tracheids by TEM. By contrast, scanning electron microscopy (SEM) is an excellent tool for observations of the structure of relatively large specimens, with dimensions of several centimeters. However, SEM does not allow resolution of the details of Mfs on a scale of several nanometers (Hirakawa & Ishida 1981a, b). Kataoka et al. (1992) combined SEM and a replica method to overcome the disadvantages of SEM. They measured the angles subtended by individual Mfs that had been deposited on the innermost surface of tracheids during the formation of the secondary wall.

The development of SEM with higher resolution, namely, field emission-scanning electron microscopy (FE-SEM), has allowed us to examine details of the structure and orientation of Mfs in cell walls of tracheids at a resolution approaching that of TEM. Detailed observations of the orientation of Mfs in woody plants have been reported (Hirakawa & Ishida 1984; Prodhan et al. 1995a, b; Booker & Sell 1998; Yoshida et al. 2000; Awano et al. 2000, 2001; Hosoo et al. 2002; Sano 2004). We have reviewed our earlier reports of pattern of newly deposited Mfs in cell walls, as determined by FE-SEM (Abe et al. 1991, 1992, 1994, 1995a, b, 1997), and we now propose a model of the progressive changes in the orientation of Mfs in the primary and secondary walls of the differentiating tracheids of conifers (Fig. 1). In the review of our earlier work, we paid particular attention to the $S_3$ layer, which has not been investigated in sufficient detail. In this report, we describe that the $S_{12}$ and $S_{23}$ layers ontogenetically belong to the $S_1$ and $S_3$ layers, respectively, in view of the successive changes in the orientation of the Mfs.

Fig. 1. A hypothetical model for the orientation of cellulose microfibrils (Mfs) as viewed from the lumen side of tracheids in the cell walls of tracheids of conifers. P = primary wall; $S_1$, $S_2$, $S_3 = S_1$, $S_2$, $S_3$ layers, respectively.
Advantages of FE-SEM

One of the major advantages of FE-SEM is that it allows the examination of relatively large specimens. In our series of consecutive studies, we observed the orientation of Mfs by FE-SEM in radial files of specimens that contained cambial cells and differentiating and mature xylem (Fig. 2). In Figure 2, there are four, thirteen and one cell(s) in the process of forming the $S_1$, $S_2$ and $S_3$ layers, respectively, in the radial file. The ability to examine large specimens allows us to measure the angles subtended by Mfs on the innermost surfaces of the tracheids of several annual rings simultaneously (Abe et al. 1992).

The orientation of Mfs within a tracheid and, in particular, the orientation of Mfs in the $S_2$ layer, is usually influenced by the presence of bordered pits (Okumura et al. 1973). To determine the extent of the influence of bordered pits on the orientation of Mfs, we examined the orientation of Mfs on the inner surface of tracheids during formation of the $S_2$ layer, in which Mfs are oriented longitudinally with respect to the cell axes. The orientation of Mfs tended to become flatter between pit apertures than it was in non-pit regions (Fig. 3a & b). The orientation of Mfs remained under the influence of bordered pit apertures for as much as 70 μm.

To avoid complications due to the orientation of Mfs due to the presence of bordered pits, we must select an appropriate region in the cell wall for observations of the orientation of Mfs, in particular, those in the $S_2$ layer. Insufficient attention has been paid to
Fig. 3. Field emission-scanning electron micrographs showing the orientation of Mfs in a tracheid in which the $S_2$ layer is being formed. – a: Region between two pit apertures that are 20 $\mu$m apart. Scale bar = 5 $\mu$m. – b: Region between two pit apertures that are 60 $\mu$m apart. Scale bar = 5 $\mu$m. – c: Higher magnification view of the middle part of Figure 3b. — Scale bar = 1 $\mu$m. Arrows indicate the orientation of Mfs.
these issues in previous TEM studies. Using FE-SEM, we can observe changes in the orientation of Mfs over a relatively large area because FE-SEM allows observations from the macroscopic level to the nanometer level in a single sample. Thus, FE-SEM allows the accurate analysis of the orientation of Mfs in tracheids because the influence of bordered pits can be excluded.

**The primary wall**

Wardrop (1958) reported that the orientation of Mfs changed from longitudinal to transverse, relative to the cell axis, from the outer to the inner part of the primary wall of macerated tracheids of *Pinus radiata*. Imamura *et al.* (1972b) reported a similar change from longitudinal to transverse in the orientation of Mfs during formation of primary cell walls in *Pinus densiflora*. Wardrop (1964) and Harada and Côté (1985) proposed a model for the organization of the primary wall, in which loose aggregates of Mfs are oriented approximately longitudinally in the outer part and transversely in the inner part of the primary wall. Wardrop (1958) interpreted the differences in the orientation of Mfs between the inner and outer surfaces of the primary wall of the macerated tracheids in terms of the multi-net growth hypothesis that was originally proposed by Roelofsen and Houwink (1953). According to this hypothesis, Mfs are originally deposited in a direction transverse to the cell axis and, during cell enlargement, their orientation shifts passively in the longitudinal direction.

In our studies, we noted that newly deposited Mfs on the innermost surface of the primary walls of tracheids were not well ordered and that their arrangement varied during differentiation in *Abies sachalinensis* (Abe *et al.* 1995b). The predominant orientation of Mfs changed from longitudinal to transverse with respect to the direction of the cell axis (Abe *et al.* 1995b, 1997). If the orientation of deposited Mfs does not change during cell enlargement, the Mfs of the primary wall of mature tracheids should be oriented approximately longitudinally with respect to the cell axis in the outer part of the primary wall and transversely with respect to the cell axis in the inner part (Fig. 1). Our observations suggest that it is not necessary to adopt the multi-net growth hypothesis to explain the difference in orientation of Mfs between the outer and inner parts of the primary wall in tracheids.

Harada and Côté (1985) suggested the existence of a lamellar structure in primary walls in which Mfs are sparsely deposited. Such a structure would require the parallel arrangement of Mfs that are deposited simultaneously. In our studies, we never observed a highly ordered arrangement of Mfs during the formation of primary walls and we were unable to confirm the existence of any lamellar structure.

**The S1 layer**

The deposition of well-ordered Mfs that are considered to form the secondary wall starts just before the cessation of the radial expansion of tracheids (Abe *et al.* 1997). The Mfs that are deposited first in the secondary wall are oriented in an S-helix in the tracheids of *Abies sachalinensis* (Abe *et al.* 1991, 1997). Similarly, as reported by Kataoka *et al.* (1992), the Mfs that are deposited on the inner surface of differentiating tracheids forming the outermost S1 layer have S-helices in *Cryptomeria japonica*, *Pinus*...
densiflora and Chamaecyparis obtusa. The orientation of Mfs shifts gradually from an S-helix to a Z-helix from the outer toward the inner part in the S1 layer, in a clockwise direction as viewed from the lumen side (Abe et al. 1995a, 1997; Fig. 1). Because of the continuity of the changes in the orientation of Mfs, no transitional layer (S12 layer) between the S1 and S2 layers was clearly distinguishable from the S1 layer.

It has been reported that transitional layers cannot be clearly distinguished from the S1 layer and the S3 layer on the basis of the observation of changes in Mf orientation (Dunning 1968, 1969; Imamura et al. 1972a; Abe et al. 1991, 1992; Kataoka et al. 1992). On the other hand, transmission electron micrographs on oblique transverse sections stained with KMnO4 revealed an ‘arced’ pattern in the cell wall of tracheids and wood fibers, indicating the progressive change in the orientation of Mfs (Prodhan et al. 1995a, b; Singh et al. 2002a; Brändström et al. 2003), and these ‘arcs’ were distinguishable from the S1 layer. Such different results between FE-SEM and TEM may be due to the different distribution of lignin contents in the cell wall, because KMnO4 mainly stains lignin (Singh et al. 2002b). To determine whether transitional layers such as S12 and S23 belong to the S1 and the S3 layers or not, further ontogenetic studies are necessary.

Several authors have proposed that the S1 layer has a ‘crossed fibrillar texture’, due to alternation between an S-helix and a Z-helix in the S1 layer (Wardrop 1964; Wardrop & Harada 1965; Harada & Côté 1985). However, no such structure was observed in the tracheids of Abies sachalinensis and the orientation of Mfs changed progressively in one direction only. Brändström et al. (2003) also found clear evidence for the absence of a ‘crossed fibrillar structure texture’ with Mfs in alternating S- and Z-helices in the tracheids of Picea abies.

In specimens collected during a season of active cambial growth, we found that approximately one-fifth of tracheids in the process of the formation of the secondary wall in a radial file were forming the S1 layer (Fig. 2). During their deposition, Mfs do not always form a complete lamellar structure without any gaps between Mfs because the orientation of Mfs shifts progressively as they are deposited.

The S2 layer

The S2 layer consists of Mfs that are organized in a steep Z-helix with a high degree of parallelism (Fig. 1). In the orientation of Mfs in the S2 layer in a steep Z-helix, there is a difference of about 15° among the orientations of individual tracheids. Such angles in differentiating earlywood tracheids differ among species. The angle is 3°–14° relative to the cell axis in Abies sachalinensis (Fig. 4a), 9°–21° relative to the cell axis in Larix kaempferi (Fig. 4b), 17°–32° relative to the cell axis in Picea jezoensis (Fig. 4c). In specimens of Abies sachalinensis collected during active cambial growth, we found that close to three-quarters of tracheids in the process of the formation of the secondary wall in a radial file were forming the S2 (Fig. 2).

The S2 layer is the thickest layer of Mfs that are oriented in a steep Z-helix in the cell walls of tracheids (Wardrop 1964; Harada & Côté 1985). Studies with the light microscope indicated that angles of Mfs in the S2 layer differed among individual tracheids and depended both on the tree and the species of tree (Butterfield 1998). Kataoka
et al. (1992) measured angles between individual Mfs in tracheids in the S2 layer in Cryptomeria japonica, Pinus densiflora and Chamaecyparis obtusa. They reported crossing angles of 10° to 12° in Cryptomeria japonica, 12° to 14° in Pinus densiflora, and 13° to 16° and 11° to 14° in the radial walls and tangential walls, respectively, of
Chamaecyparis obtusa. These values are similar to those that we obtained in Larix kaempferi, Picea jezoensis and Abies sachalinensis but the deviations are smaller.

According to the variation in angles of Mfs in the S\(_2\) layer, there may be narrow spaces continuing in radial direction in the S\(_2\) layer. As lignin is deposited in such spaces, the radially agglomerated structures, which Sell and Zimmermann (1993) and Booker and Sell (1998) reported, may be observed on transverse surfaces, fractured under tensile stress.

The S\(_3\) layer

In the S\(_3\) layer, the orientation of Mfs shifts from a steep Z-helix to an S-helix in a counterclockwise direction, as viewed from the lumen side, from the outer toward the inner part of the layer (Fig. 1). The orientation of Mfs deposited on the innermost S\(_3\) layer varies among tracheids from an angle of 40° to the cell axis in a Z-helix to an angle of 20° in an S-helix. The most frequently observed orientation of Mfs in the innermost surface of tracheids ranged from 70° to 80°, from 60° to 70° and from 40° to 50° in the S-helices of Larix kaempferi, Picea jezoensis and Picea abies, respectively (Abe et al. 1992). However, Mfs in Z-helices were occasionally observed on the innermost surfaces in tracheids in the late part of the annual ring. The Mfs in Z-helices on the innermost surfaces might be the result of the incomplete rotation of Mfs within the S\(_3\) layer.

Liese (1960, 1963) reported that the dominant arrangement of Mfs in the S\(_3\) layer was an S-helix in Picea abies, Larix decidua and Sequoia gigantea. However, S- and Z-helices were observed at equal frequencies in Pinus sylvestris. Our model of the S\(_3\) layer is consistent with the information provided by Liese, except in the case of Pinus sylvestris. By contrast, Kataoka et al. (1992) reported that Mfs were found at equal frequency in Z-helices and S-helices within the S\(_3\) layer in differentiating tracheids of Chamaecyparis obtusa. These observations suggest that the orientation of Mfs in the innermost S\(_3\) layer might differ among tree species.

Wardrop (1964) reported that the thickness of the S\(_3\) layer varied considerably among species. Liese (1963) reported that the thickness of the S\(_3\) layer ranged between 0.07 and 0.08 μm in Pinus sylvestris and Picea abies. Saiki (1970) reported that the thickness of the S\(_3\) layer ranged between 0.04 and 0.22 μm in Pinus densiflora, Cryptomeria japonica, Chamaecyparis obtusa, Larix kaempferi and Thuja orientalis. During active cambial growth in Abies sachalinensis, approximately 6% of tracheids in a radial file that were forming the secondary wall were in the process of forming the S\(_3\) layer (Fig. 2). By contrast, Kataoka et al. (1992) reported that about one-third of the tracheids were forming the S\(_3\) layer in Chamaecyparis obtusa, during active cambial growth. These differences might be related to differences in the thickness of the S\(_3\) layer. The duration of the deposition of the S\(_3\) layer and the rate of change in the orientation of Mfs determine the structure of the S\(_3\) layer.

The tracheids of the compression wood of conifers lack an S\(_3\) layer (Yumoto et al. 1983). Moreover, the absence of the S\(_3\) layer is the first anatomical change that is noticeable during the transition from normal to compression wood (Yumoto et al. 1982; Yoshizawa 1987; Yoshizawa et al. 1992). By contrast, the S\(_3\) layer in the wood
that is formed on the side that is opposite to the compression wood (opposite wood) is thicker than that in the normal wood in conifers (Timell 1973, 1986). We reported that there was no shift in the orientation of Mfs from the $S_2$ to the $S_3$ layer in latewood tracheids that were formed at the end of the growing season in *Larix kaempferi* (Abe *et al.* 1992). These observations suggest that the development of the $S_3$ layer might be influenced by a tree’s environment. It is likely that the development of the $S_3$ layer is affected by factors such as tree species, the season during which the tracheid develops, and the tree’s environment.

In our studies, we observed that the Mfs in the $S_3$ layer formed bundles (Abe *et al.* 1991, 1992, 1994). We observed Mfs being deposited in bundles in the gaps between previously deposited microfibrillar bundles. Our results suggest that the Mfs in the $S_3$ layer aggregate to form bundles and that a complete lamella without any gaps between Mfs is not formed. The $S_3$ layer is the thinnest layer in the secondary wall of tracheids (Liese 1963; Saiki 1970). The rate of rotational change in the orientation of Mfs is probably too rapid to allow Mfs to cover the entire inner surface of a tracheid. Indeed, Liese (1963), Harada and Côté (1985) and Abe *et al.* (1991, 1992, 1994) observed that Mfs were loosely arranged in the $S_3$ layer. Such an arrangement might be caused by the non-rectilinear nature of Mfs in the $S_3$ layer. The Mfs change their orientation rapidly as they are synthesized during formation of the $S_3$ layer and, thus, they tend to be bent in one direction.

### Possibilities for the control of the orientation of microfibrils

Our observations by FE-SEM revealed that the orientation of newly deposited Mfs changes progressively during formation of the primary and secondary walls of tracheids. The orientation of Mfs is one of the most important factors that determine the physical properties of wood. In particular, the angles at which Mfs are oriented in the $S_2$ layer play a significant role in determining the strength of wood (Barnett & Bonham 2004). It has been suggested that it might be possible to control microfibril angles genetically (Donaldson & Burdon 1995; Hirakawa & Fujisawa 1995). Such control cannot be achieved until we understand fully those factors, such as specific genes, that determine the angles of Mfs in the $S_2$ layer.

When we consider the possible biological mechanisms for control of the orientation and deposition of Mfs in higher plants, it is clear that cortical microtubules, which are components of the cytoskeleton, must play an important role (Ledbetter & Porter 1963; Hepler & Palevitz 1974; Gunning & Hardham 1982; Seagull 1991; Shibaoa 1994; Chaffey 2000; Nick 2000; Baskin 2000; Baskin 2000; Chaffey *et al.* 2002), although conflicting evidence has been presented by Emons *et al.* (1992, 2002). In conifers, cortical microtubules are orientated similarly to Mfs during the formation of the cell wall in tracheids of normal wood (see Funada 2000, 2001, 2002; Funada *et al.* 2000, 2001) and compression wood (see Furusawa *et al.* 1998). Cortical microtubules might control the movement of cellulose-synthesizing complexes (terminal complexes) in the plasma membrane. The details of the mechanism that controls the orientation of cortical microtubules are not yet fully understood but recent molecular approaches have yielded valuable information. For example, the change in a single amino acid in tubulin resulted in a
change in the orientation of cortical microtubules in the epidermal cells of *Arabidopsis thaliana* (Thitamadee *et al.* 2002). Other factors that control the orientation of cortical microtubules during formation of the cell wall will probably be characterized in the near future. The identification of such factors might be important in the creation of ‘new’ woods with desirable commercial and industrial properties.

**ACKNOWLEDGEMENTS**

The authors thank Professor K. Fukazawa, Dr. T. Fujii, Dr. Y. Hirakawa and Dr. Y. Kataoka for their valuable comments. This work was supported in part by a Grant-in-Aid for Scientific Research (no. 14560127) from the Ministry of Education, Science and Culture, Japan.

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