Ultrastructural Investigations of Diurnal Periodicity in Cell Wall Formation of Conifer Tracheids

Yoshihiro HOSOO[1]*, Takanori IMAI[2] and Masato YOSHIDA[2]

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Summary

Differences in the innermost surface of developing secondary walls of conifer tracheids are seen from day to night using field emission scanning electron microscopy (FE-SEM). Cellulose microfibrils are clearly evident during the day, while amorphous material is prevalent at night. Immunogold-labeling experiments revealed that the amorphous material contains abundant levels of glucomannans, indicating that the material is matrix containing hemicellulose. Diurnal fluctuation of the tangential strain on the inner bark is also observed, suggesting that the diurnal differences in the innermost surface of developing secondary walls occur, corresponding to the diurnal changes in the volume of differentiating cells. Our further investigation showed that the diurnal differences were affected by light. When day and night in the photoperiodic cycle were reversed, the volumetric changes in differentiating cells and the diurnal differences in the innermost surface of developing secondary walls were also reversed. These findings suggest that there is a diurnal periodicity in the supply of cell wall components to the innermost surface of developing secondary walls, corresponding to the 24-h light-dark cycle.

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Key words: Cell wall, Diurnal periodicity, FE-SEM, Immunogold labeling, Tracheid

Xylem cells in the stem of a tree are essential for supporting the tree body, transporting water, and storing and supplying nourishment. In order to maintain these functions, xylem cells form highly developed cell walls. Xylem Cell walls are classified as primary or secondary walls during the course of their development. Primary walls are the thin walls that form when differentiating cells elongate or enlarge. Secondary walls are the thick walls formed inside the primary wall after elongation and enlargement are complete. Since the stems of trees consist primarily of xylem cells, tree growth is controlled by xylem cell wall formation.

The main components of the wood cell wall are cellulose, hemicellulose, and lignin; some wood species also contain many extractives. Cellulose is a linear homopolymer of β-1, 4-glucans, which associate to form the crystalline entity known as a microfibril[1]. Hemicelluloses are branched polymers composed of various polysaccharides. Lignin is an aromatic amorphous molecule composed of three different monolignols: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. Secondary walls consist of cellulose microfibrils embedded in a lignin-hemicellulose matrix. Cellulose deposition increases cell wall thickness. By contrast, the deposition of hemicellulose and lignin increases cell wall density. Hemicelluloses have many variations and form physical and chemical bonds with cellulose and lignin. They have an important role in building the three-dimensional structures of cell walls. In secondary walls, three layers can be differentiated based on the microfibril angles: the outer, middle, and inner layers (S_1, S_2, and S_3, respectively).

Cell wall formation requires the expression of a number of genes. Characteristic of plants, gene expression is affected by external factors, such as water, temperature, light, and nutrients. Natural environmental factors change diurnally. It is probable that there is also diurnal periodicity in cell wall formation. In order to elucidate the mechanism of cell wall formation, we focused on diurnal periodicity. We have examined the changes from day to night in the cell walls in differentiating tracheids of *Cryptomeria japonica* D. Don. In this paper, our recent studies regarding the diurnal periodicity in cell wall formation are summarized.

SYNTHESIS AND DEPOSITION OF CELL WALL COMPONENTS

The Golgi apparatus plays a central role in hemicellulose biosynthesis[2, 3]. Hemicelluloses synthesized in the Golgi apparatus are transported to the plasma membrane by the Golgi vesicles, and then secreted to the innermost surfaces of cell walls by exocytosis of these vesicles[2-4]. Secreted hemicelluloses absorb water to form a hydrated gel that covers the innermost surfaces of cell walls[5]. The deposition of hemicelluloses starts before the beginning of lignification, and previously deposited hemicelluloses guide lignin polymerization[6]. Cellulose is synthesized by cellulose-synthesizing enzyme complexes called rosette terminal complexes in the plasma membrane and newly synthesized cellulose microfibrils deposited on exposure to the hemicellulose gel. Lignin is deposited in the presence of

[1] Graduate School of Science and Technology, Niigata University

[2] Graduate School of Bioagricultural Sciences, Nagoya University

* Corresponding author: hosoo@gs.niigata-u.ac.jp

hemicelluloses, regardless of the plant species. Monolignols provided to the cell walls diffuse into the hemicellulose gel, which fills the spaces between cellulose microfibrils. Afterward, the hydrophobic cell walls are formed by water removal via the dehydrogenative polymerization of lignin.

DIFFERENCES FROM DAY TO NIGHT IN THE INNERMOST SURFACE OF DEVELOPING SECONDARY WALLS

The recent development of field emission scanning electron microscopy (FE-SEM) allows high-resolution images of cell walls. FE-SEM has been used to observe the orientation of cellulose microfibrils in woody plants[7-10]. Yoshida et al.[11] and Hosoo et al.[12] collected samples during the day and at night from the trunk of *Cryptomeria japonica* D. Don, and observed the innermost surface of developing secondary walls in differentiating tracheids using FE-SEM. The S_2 layer in conifer tracheids occupies a large part of the cell wall, and most of the differentiating xylem cells were S_2-forming tracheids. Therefore, these studies were made on the innermost surface of the developing secondary walls in S_2-forming tracheids. Cellulose microfibrils were clearly observed during the day, while an amorphous material was observed and cellulose microfibrils were not evident at night (Fig. 1). The observed differences from day to night indicate that a diurnal periodicity in the supply of cell wall components to the innermost surface of developing secondary walls exist in conifer tracheids.

DIURNAL DIFFERENCES IN THE AMOUNT OF IMMUNOGOLD LABELED GLUCOMANNANS

What is the amorphous material on the innermost surface of developing secondary walls that is present, specifically, at night? The amorphous material clearly differs from the cellulose microfibrils. Lignin is not produced until the differentiating cells reach a specific developmental stage because it is a product of secondary metabolism. First, the lignification process begins at the cell corners and in the middle lamella after the S_1 layer starts to form. Second, lignin is slowly deposited in primary walls and in the S_1 layer during S_2 layer formation. Finally, it is most actively deposited throughout the secondary walls after the S_3 layer has formed[13-16]. Therefore, the amorphous material is not likely to be lignin. Instead, it is likely that the amorphous material is a matrix containing hemicellulose and lignin precursors.

Immunogold-labeling methods are frequently used to study plants and microorganisms. The localization of xylans in plant cell walls has been studied using immunogold labeling[4, 17]. Recently, the localization of xylans in differentiating xylem in Japanese beech was investigated by transmission electron microscopy (TEM) and FE-SEM[18, 19]. Maeda et al.[20] studied the localization of glucomannans in the cell walls of differentiating tracheids in *Chamaecyparis obtusa*

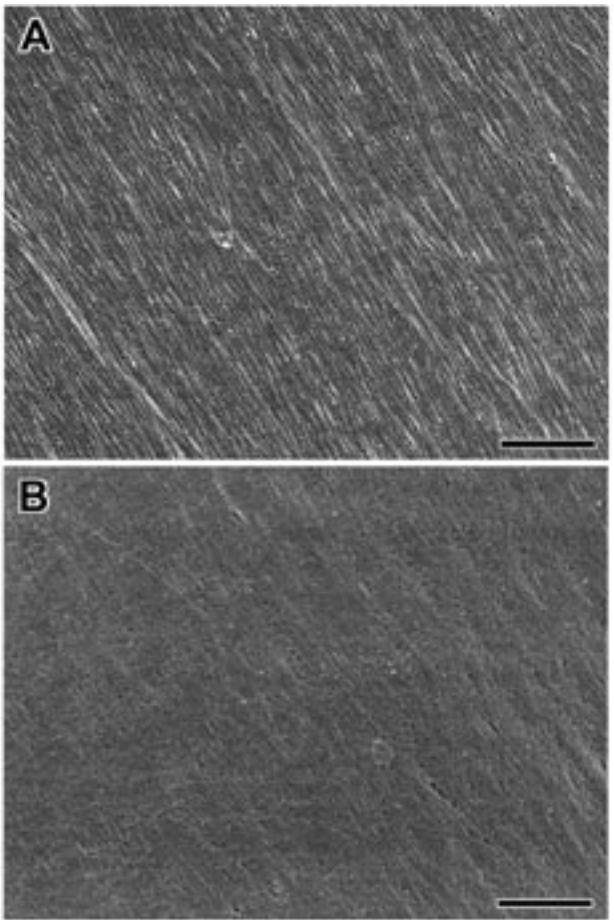

Fig. 1 Electron micrographs of the innermost surface of developing secondary walls (S_2 layers) in differentiating tracheids of *Cryptomeria japonica* D. Don grown in the field. The longitudinal cell axes in the micrographs are vertical. Bars = 500 nm. **A:** Samples collected at 14:00 h (daytime). **B:** Samples collected at 5: 00 h (night).

by observing the differentiating cell walls using TEM. The resolution of FE-SEM is adequate for detecting colloidal gold particles as small as 5-20 nm[19, 21-24].

Using FE-SEM, Hosoo et al.[12] observed diurnal differences in the innermost surface of developing secondary walls in differentiating tracheids of *Cryptomeria japonica* D. Don containing immunogold-labeled glucomannans. Cellulose microfibrils were clearly evident and the amount of labeling was small during the day (Fig. 2A). An amorphous material was observed and a large amount of labeling was found as bright spherical particles in the material at night (Fig. 2B). These results indicate that the amorphous material contains abundant levels of glucomannans. Glucomannans are the most abundant hemicellulose in softwoods and account for two-thirds of total hemicellulose[25-27]. Thus, it was confirmed that the amorphous material supplied to the innermost surface of developing secondary walls at night was matrix containing hemicellulose.

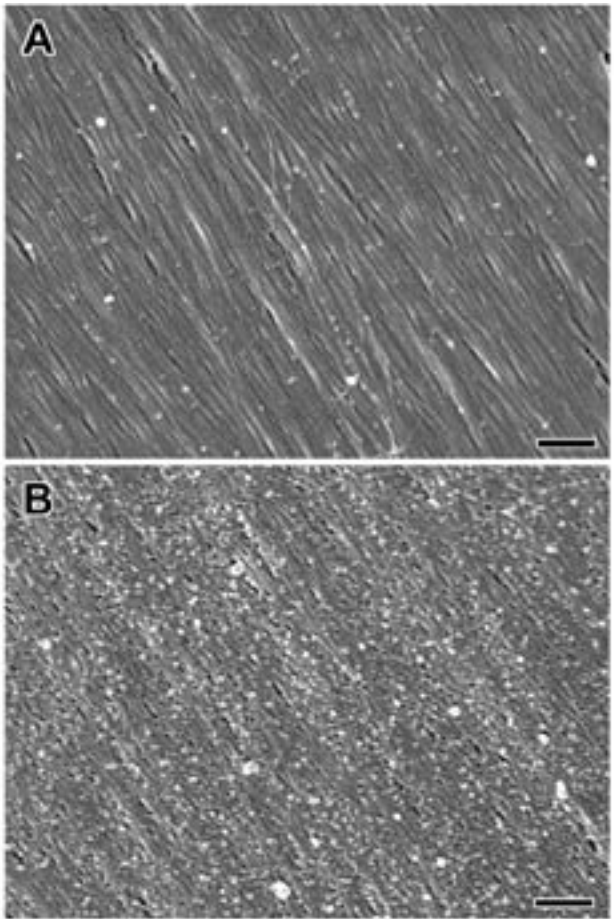

Fig. 2 Electron micrographs of the innermost surface of developing secondary walls (S_2 layers) immnogold-labeled with anti-glucomannan antiserum in differentiating tracheids of *Cryptomeria japonica* D. Don grown in the field. The longitudinal cell axes in the micrographs are vertical. Bars = 200 nm. **A:** Samples collected 14:00 h (daytime). **B:** Samples collected at 5: 00 h (night).

CHANGES IN THE TANGENTIAL STRAIN ON THE INNER BARK (VOLUMETRIC CHANGES OF DIFFERENTIATING CELLS)

The water status of a tree is reflected in changes in stem diameter[28, 29]. The diurnal fluctuation of stem diameter is caused mainly by changes in the water status of cells in the cambium and developing cells in the xylem and phloem[30-33]. At the level of plant cells, the extent of growth depends on the interaction between turgor pressure and the mechanical strength of the cell wall. Turgor pressure is the pressure of the protoplast against the cell wall and is proportional to the volumetric increase of a cell. The volumetric changes of differentiating cells can be estimated from changes in the tangential strain on the inner bark[34].

Diurnal fluctuation of the tangential strain on the inner bark is observed[11, 12, 34, 35]. Figure 3 shows the changes

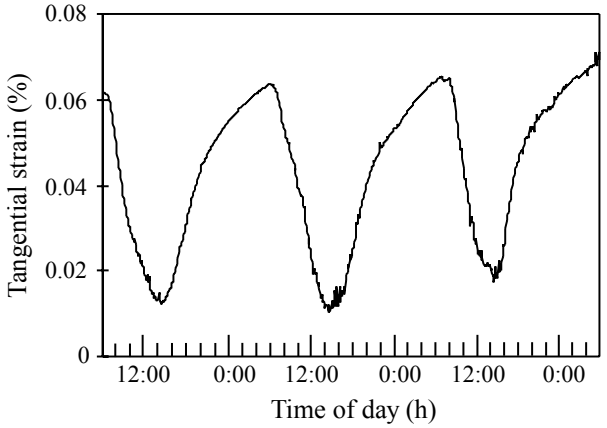

Fig. 3 Changes in tangential strain on the inner bark surface in *Cryptomeria japonica* D. Don. in the field at the middle of June 1998.

in tangential strain on the inner bark in the stem of *Cryptomeria japonica* D. Don. The tangential strain reaches a maximum just before daybreak and gradually decreases to a minimum during the day, due to transpiration from the stomata of leaves. After reaching its minimum, the strain increases at night when transpiration stops. These suggest that cellulose microfibrils are observed on the innermost surface of developing secondary walls during the day when the volume of differentiating cells is low as a result of water loss by transpiration, and that the matrix containing hemicellulose is observed at night when differentiating cells are turgid as a result of imbibition. The changes in the supply of hemicellulose matrix and cellulose microfibrils to the innermost surface of developing secondary walls might be correlated with diurnal changes in phenomena such as turgor pressure.

EFFECT OF A LIGHT-DARK CYCLE ON THE DIURNAL DIFFERENCES

Xylem development is influenced by external factors, such as water, temperature, light and nutrients. Differences from day to night in the innermost surface of developing secondary walls are observed in *Cryptomeria japonica* D. Don. grown in the field, *i.e.*, in the natural environment[11, 12]. In the natural environment, factors such as illuminance, temperatures and humidity change diurnally. Studies of *Cryptomeria japonica* D. Don. in the field cannot tell which of these factors is responsible for the diurnal periodicity in the supply of cell wall components to the innermost surface of developing secondary walls. In order to clarify this, it is necessary to observe these changes in an environment in which one factor changes diurnally and the others are kept constant.

Light is one of the most important environmental factors affecting plants. Light plays an important role in the processes

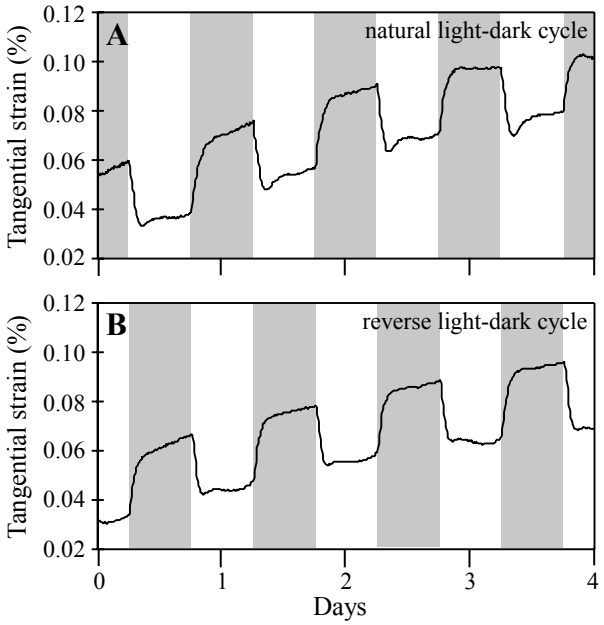

Fig. 4 Changes in tangential strain on the inner bark surface of *Cryptomeria japonica* D. Don. saplings in two growth chambers. Shaded areas indicate dark periods. **A:** A sapling grown in the natural light-dark cycle. **B:** A sapling grown in the reverse light-dark cycle to nature.

of growth and differentiation, such as germination, elongation growth, flower bud formation and aging. Plants always monitor the light environment and respond to changes in it appropriately. Hosoo et al.[36] studied the effect of a light-dark cycle on the diurnal differences seen in the innermost surface of developing secondary walls in differentiating tracheids. Saplings of *Cryptomeria japonica* D. Don. were grown in two growth chambers, in which temperature and relative humidity were kept constant and the light-dark phase of the photoperiodic cycle differed. One chamber reproduced the natural light-dark phase, while the other reversed it. The tangential strain on the inner bark was measured, and the innermost surface of developing secondary walls (S_2 layers) immunogold-labeled with anti-glucomannan antiserum was observed by FE-SEM. When the light and dark periods of the photoperiodic cycle were reversed, the volumetric changes in differentiating cells and the diurnal differences in the innermost surface of developing secondary walls were also reversed (Fig. 4, 5). Regardless of the sampling time, cellulose microfibrils were observed during the light period when the volume of differentiating cells is low, while the hemicellulose matrix was observed during the dark period when differentiating tracheids were turgid. This study clarified the diurnal changes in the volume of differentiating cells and aspects of the innermost surface of developing secondary walls, corresponding to the 24-h light-dark cycle.

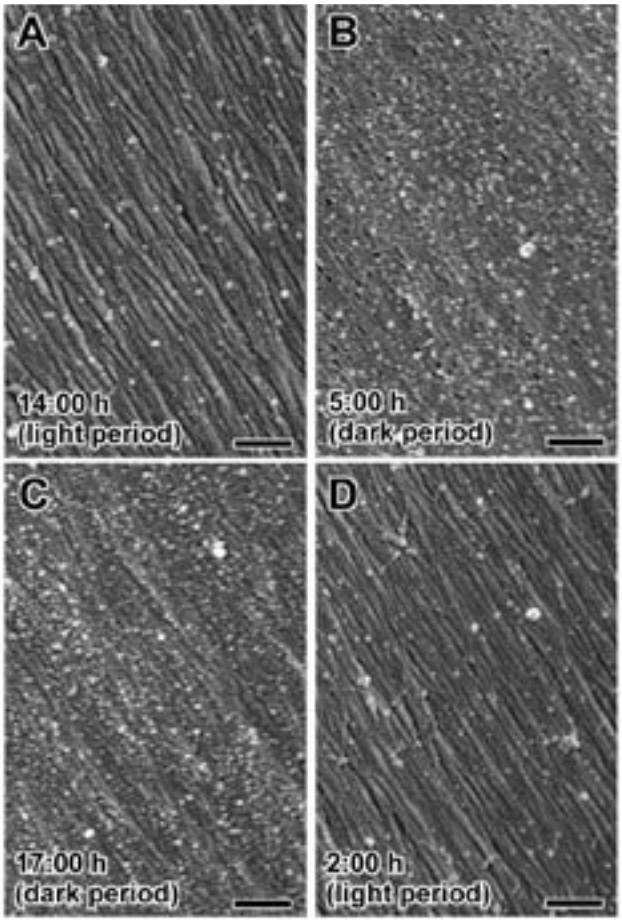

Fig. 5 Electron micrographs of the innermost surface of developing secondary walls (S_2 layers) immnogold-labeled with anti-glucomannan antiserum in *Cryptomeria japonica* D. Don. saplings. The longitudinal cell axes in the micrographs are vertical. Bars = 200 nm. **A:** At 14:00 h (light period) in a sapling grown in the natural light-dark cycle. **B:** At 5:00 h (dark period) in a sapling grown in the natural light-dark cycle. **C:** At 17:00 h (dark period) in a sapling grown in the reverse light-dark cycle to nature. **D:** At 2:00 h (light period) from a sapling grown in the reverse light-dark cycle to nature.

CONCLUSION

Woody plants are perennial plants that undergo radial growth via cambial activity. The cambium produces xylem cells toward the inside and phloem cells toward the outside. Since there is much more xylem than phloem, xylem occupies a large part of the trunk. Since xylem supports huge tree bodies by adding mechanical strength to the stem, tree growth is controlled by xylem cell wall formation. Elucidating the process of cell wall formation is important for a more detailed understanding of tree growth.

The diurnal periodicity in the supply of cell wall components to the innermost surface of developing

secondary walls was found in conifer tracheids. However, further studies are necessary to fully understand the diurnal periodicity in xylem cell wall formation. For example, which is the main signal to start the diurnal periodicity, changes in the water status such as turgor pressure or the light condition is not clear. Participation of the turgor pressure in cell wall formation also remains uncertain. Detailed knowledge of the mechanism of diurnal periodicity in cell wall formation is proposed to be related to the understanding of tree growth at the level of the cell wall.

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REFERENCES

1. Brown, R.M. Jr., Saxena, I.M., Kudlicka, K. (1996) Cellulose biosynthesis in higher plants. Trends Plant Sci. 1, 149-156.
2. Park, P., Ohno, T., Kato-Kikuchi, H., Miki, H. (1987) Alkaline bismuth stain as a tracer for Golgi vesiclesof plant cells. Stain Technol. 62, 253-256.
3. Samuels, A.L., Rensing, K.H., Douglas, C.J., Mansfield, S.D., Dharmawardhana, D.P., Ellis, B.E. (2002) Cellular machinery of wood production; differentiation of secondary xylem in *Pinus contrta* var. latifolia. Planta 216, 72-82.
4. Northcote, D.H., Davey, R., Lay, J. (1989) Use of antisera to localize callose, xylan and arabinogalactan in the cell-plate, primary and secondary walls of plant cells. Planta 178, 353-366.
5. Inomata, F., Takabe, K., Saiki, H. (1992) Cell wall formation of tracheid as revealed by rapid-freeze and substitution method. J. Electron Microsc. 41, 369-374.
6. Terashima, N., Fukushima, K., He, L.F., Takabe, K. (1993) Comprehensive model of the lignified plant cell wall. In: Forage cell wall structure and digestibility. Eds: Jung, H.G., Buxton, D.R., Hatfield, R.D., Raiph, J. American Society of Agronomy, Madison. pp. 247-269.
7. Abe, H., Funada, R., Ohtani, J., Fukazawa, K. (1995) Changes in the arrangement of microtubules and mirofibrils in differentiating conifer tracheids during the expansion of cells. Ann. Bot. 75, 305-310.
8. Abe, H., Funada, R., Imaizumi, H., Ohtani, J., Fukazawa, K. (1995) Dynamic changes in the arrangement of cortical microtubules in conifer tracheids during differentiation. Planta 197, 418-421.
9. Prodhan, A.K.M.A., Funada, R., Ohtani, J., Abe, H., Fukazawa, K. (1995) Orientaion of microtubules and microfibrils in developing tension wood fibers of Japanese ash (*Fraxinus mandshrica var. japonica*). Planta 196, 577-585.
10. Prodhan, A.K.M.A., Ohtani, J., Funada, R., Abe, H., Fukazawa, K. (1995) Ultrastructual investigation of tension wood fibre in *Fraxinus mandshrica* Rupr. var. *japonica* Maxim, Ann. Bot. 75, 311-317.
11. Yoshida, M., Hosoo, Y., Okuyama, T. (2000) Periodicity as a factor in the generation of isotropic compressive growth stressbetween microfibrils in cell wall formation during a twenty-four hour period. Holzforschung 54, 469-473.
12. Hosoo, Y., Yoshida, M., Imai, T., Okuyama, T. (2002) Diurnal difference in the amount of immunogold-labeled glucomannans detected with field emission scanning electron microscopy at the innermost surface of developing secondary walls of differentiating conifer tracheids. Planta 215, 1006-1012.
13. Takabe, K., Fujita, M., Harada, H., Saiki, H. (1981) Lignification process of Japanese black pine (*Pinus thunbergii* Parl.) tracheids. Mokuzai Gakkaishi 27, 813-820.
14. Terashima, N., Fukushima, K., Sano, Y. (1988) Heterogeneity in formation of lignin 10. Visualization of lignification process in differentiating xylem of pine by microautoradiography. Holzforschung 42, 347-350.
15. Donaldson, L.A. (1991) Seasonal changes in lignin distribution during tracheids development in *Pinus radiata*. Wood Sci. Technol. 25, 15-24.
16. Fukushima, K., Terashima, N. (1991) Heterogeneity in formation of lignin 14. Formation and structure of lignin in differentiating xylem of *Ginkgo biloba*. Holzforschung 45, 87-94.
17. Migne, C., Prensier, G., Grenet, E. (1994) Immunogold labeling of xylans and arabinoxylans in the plant cell walls of maize stem. Biol. Cell 81, 267-276.
18. Awano, T., Takabe, K., Fujita, M. (1998) Localization of glucronoxylans in Japanese beech visualized by immunogold labeling. Protoplasma 202, 213-222.
19. Awano, T., Takabe, K., Fujita, M., Daniel, G. (2000) Deposition of glucuronoxylans on the secondary cell wall of Japanese beech as observed by immuno-scanning electron microscopy. Protoplasma 212, 72-79.
20. Maeda, Y., Awano, T., Takabe, K., Fujita, M. (2000) Immunolocalization of glucomannans in the cell wall of differentiating tracheids in *Chamaecyparis obtusa*. Protoplasma 213, 148-156.
21. Pawley, J. Albrecht, R. (1988) Imaging colloidal gold labels in LVSEM. Scanning 10, 184-189.
22. Takata, K and Akimoto, Y. (1988) Colloidal gold label observed with a high resolution backscattered electron imaging in mouse lymphocytes. J. Electron Microsc. 37, 346-350.
23. Osumi, M., Yamada, H., Kobori, H., Yaguchi, H. (1992) Observation of colloidal gold particles on the surface of yeast protoplasts with UHR-LVSEM. J. Electron Microsc. 41, 392-396.
24. Osawa, T., Yoshida, Y., Tsuzuki, F., Nozaka, M., Takashiro, M., Nozaka, Y. (1999) The advantage of osmium conductive metal coating for the detection of the colloidal gold-conjugated antibody by SEM. J. Electron

Microsc. 48, 665-669.
25. Hamilton, J.K and Thompson, N.S. (1959) A comparison of the carbohydrates of hardwoods and softwoods. Tappi 42, 752-760.
26. Meier, H., Wilkie, K.B.C. (1959) The distribution of polysaccharides in cell wall of tracheids of pine (*Pinus silvestris L.*). Holzforschung 13, 177-182.
27. Puls, J., Schiusei, J. (1993) Chemistry of hemiceluloses: relationship between hemicellulose structure and the enzymes required for hydrolysis. In: Hemicellulose and hemicellulases. Eds. Coughlin, M.P., Hazlewood, G.P. Portland Press, London.
28. Sheriff, D.W., Whitehead, D. (1984) Photosynthesis and wood structure in *Pinus radiata* D. Don during dehydration and immediately after rewatering. Plant Cell Environ. 7, 53-62.
29. Horzog, K.M., Hasler, R., Thum, R. (1995) Diurnal changes in the radius of a subalpine Norway spruce stem: Their relation to the sap flow and their use to estimation transpiration. Trees 10, 94-101.
30. Klepper, B., Browning, V.D., Taylor, H.M. (1971) Stem diameter in relation to plant water status. Ecology 45, 149-155.
31. Molz, F.J., Klepper, B. (1973) On the mechanism of water-stress- induced stem deformation. Agron J. 65, 469-473.
32. Zweifel, R., Item, H., Hasler, R. (2001) Link between diurnal stem radius changes and tree water relations. Tree Physiol. 21, 869-877.
33. Perämäki, M., Nikinmaa, E., Sevanto, S., Ilvesniemi, H., Siivola, E., Hari, P., Vesara, T. (2001) Tree stem diameter variations and transpiration in Scots pine: An analysis using a dynamic sap flow model. Tree Physiol. 21, 889-897.
34. Okuyama, T., Yoshida, M., Yamamoto, H. (1995) An estimation of the turgor pressure change as one of the factors of growth stress generation in cell walls. Mokuzai Gakkaishi 41, 1070-1078.
35. Abe, H., Nakai, T. (1999) Effect of the water status within a tree on tracheid morphogenesis in *Cryptomeria japonica* D. Don. Trees 14, 124-129.
36. Hosoo, Y., Yoshida, M., Imai, T., Okuyama, T. (2003) Diurnal differences in the innermost surface of the S2 layer in differentiating tracheids of *Cryptomeria japonica* corresponding to a light-dark cycle. Holzforschung 57, 567-573.

針葉樹仮道管細胞壁形成の日周期に関する微細構造的研究

細尾佳宏[1]*・今井貴規[2]・吉田正人[2]

（平成17年１月14日受付）

要　約

針葉樹仮道管の二次壁新生面を電界放出形走査電子顕微鏡（FE-SEM）で観察すると、昼と夜で違いが見られる。昼はセルロースミクロフィブリルが明確に観察され、夜は無定形物質が観察される。免疫金標識を用いた実験により、無定形物質がグルコマンナンを豊富に含み、この物質がヘミセルロースを含むマトリックスであることを示した。内樹皮の接線ひずみにも日変動が観察され、二次壁新生面における日周期的な変化は分化中細胞の体積の日変動に伴って起こる。さらなる研究により、その日周期的な変化が光に影響されることが分かった。明暗周期の昼と夜が反転すると、分化中細胞の体積変動や二次壁新生面における日周期的な変化も反転した。これらの結果は、24時間の明暗周期に伴う二次壁新生面への細胞壁成分の供給の日周期が存在することを提案するものである。

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キーワード：細胞壁、日周期、FE-SEM、免疫金標識、仮道管

[1] 新潟大学大学院自然科学研究科

[2] 名古屋大学大学院生命農学研究科

*代表著者：hosoo@gs.niigata-u.ac.jp