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ORIGINAL ARTICLE

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The supply of matrix containing glucomannans to the innermost surface of S_2 layers is associated with changes in turgor pressure of differentiating tracheids in *Cryptomeria japonica*

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Abstract We investigated the relationship between turgor pressure and diurnal differences in secondary wall formation of differentiating tracheids. Saplings of *Cryptomeria japonica* were grown in a growth chamber with 12-h light:12-h dark cycles, and the tangential strain on the inner bark surface was measured as an indicator of the volumetric changes of differentiating cells. The innermost surface of developing secondary walls was then observed using field emission scanning electron microscopy at 1-h intervals after both light and dark periods. Dramatic changes in the aspects of the innermost surface of developing secondary walls occurred 3 h after the light was switched on and 4 h after the light was switched off. The amorphous material containing glucomannans became evident when the differentiating cells became fully turgid during the dark period. Conversely, cellulose microfibrils became clearly visible when the cell volume was low during the light period. These results suggest that the diurnal periodicity in the supply of hemicellulose-containing matrix to developing secondary walls is associated with the changes in turgor pressure of differentiating tracheids that result from the change in light conditions during the photoperiodic cycle.

Key words *Cryptomeria japonica* · Developing secondary wall · Diurnal difference · Tracheid · Turgor pressure

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Introduction

Diurnal differences in the innermost surface of developing secondary walls of differentiating tracheids have been observed by field emission scanning electron microscopy (FE–SEM) in *Cryptomeria japonica*.[1,2] Cellulose microfibrils are clearly perceptible during daytime, and an amorphous material containing glucomannans, which are the main components of softwood hemicelluloses,[3,4] is prevalent at night. Diurnal differences have also been observed in the saplings of *C. japonica* grown in growth chambers in which all conditions were constant except the photoperiodic cycle.[5]

The Golgi apparatus participates in the biosynthesis of hemicelluloses such as glucomannans, xylans, and xyloglucans.[6–11] Hemicelluloses synthesized in the Golgi apparatus are transported to the plasma membrane by the Golgi vesicles and then secreted to the innermost surface of cell walls by the fusion of these vesicles with the plasma membrane (exocytosis).[6,11] The exocytosis process in higher plants is associated with turgor pressure, i.e., the pressure of the protoplast against the cell wall.[12] Therefore, the secretion of cell wall components by exocytosis is enhanced at increased turgor pressure, in parallel with cell wall expansion.

During xylem cell development in woody plants, the interaction between the turgor pressure and cell wall properties determines the cell size.[13,14] The expansion of a plant cell occurs when the turgor pressure in the cell exceeds the yield point of the cell. The tangential strain changes on the inner bark surface can be used as an indicator of the volumetric changes of differentiating cells.[15] Our previous studies have demonstrated that diurnal differences in the innermost surface of developing secondary walls occur, corresponding to the diurnal changes in tangential strain.[1,2,5] These studies suggest that diurnal periodicity in the supply of cell wall components to developing secondary walls may be associated with diurnal changes in the turgor pressure of differentiating tracheids.

This study was designed to gain more insight into the relationship between turgor pressure and diurnal differ-

ences in the formation of the secondary wall of differentiating tracheids. We analyzed *C. japonica* saplings that were grown in a growth chamber with 12 h light:12 h dark cycles. The developing secondary walls were immunogold-labeled with antiglucomannan antiserum, and their innermost surface was observed under FE–SEM at 1-h intervals after both the light and dark periods. We measured the tangential strain on the inner bark surface and examined how the aspects of the innermost surface of developing secondary walls changed as the volume of the differentiating cells changed.

Materials and methods

Plant materials

Cloned saplings of 3-year-old *Cryptomeria japonica* D. Don. (mean height, 106 cm; mean diameter at 10 cm from ground, 1.5 cm) planted in pots were placed in a growth chamber (KG-50HLA; Koito Industries, Yokohama, Japan) at a controlled temperature (25° ± 1°C) and relative humidity (70% ± 7%) in mid-July 2002. The light cycle was set for 12 h of light (illuminance, 47 W/m^2) and 12 h of darkness (lights on at 12:00 h; lights off at 24:00 h). The saplings were irrigated with 200 ml of water at 10:00 h every day. To estimate the volumetric changes of differentiating cells, one of the saplings was subjected to a measurement of tangential strain on the inner bark surface.

About 3 weeks after the start of the experiment, samples were collected for FE–SEM observations. During the light period, one sapling was sampled each hour for 6 h from immediately after lights-on at 12:00 h until 18:00 h. During the dark period, specimens were collected each hour for 6 h from immediately after lights-off at 24:00 h until 6:00 h. Approximately 5-cm-long segments cut from the stems were fixed in 3% glutaraldehyde in 0.07 M phosphate buffer (pH 7.0) overnight at 4°C.

Measuring tangential strain on the inner bark surface

Measurements of the tangential strain on the inner bark surface were made at 10-min intervals according to the procedure of Hosoo et al.[5] The environmental conditions in the growth chamber were uniform throughout the study, and all saplings in the chamber grew under the same conditions. The changes in the tangential strain were applied to the analysis of the saplings used for FE–SEM observations.

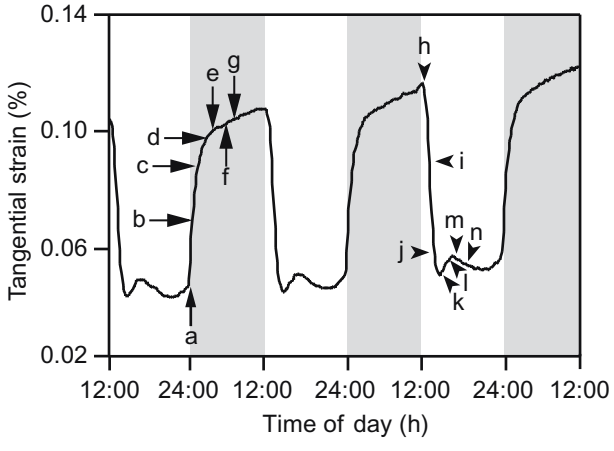
Scanning electron microscopy

Radial sections for FE–SEM observations were prepared as described by Hosoo et al.[5] Some sections were immunogold-labeled with antiglucomannan antiserum according to previous reports.[2,5] As a control, preimmune serum or antiserum previously incubated with glucomannans at 0.5 mg/ml was used for labeling. The preparation and specificity of the antisera used in this study are described elsewhere.[2] For the secondary antibody, 15-nm-gold-labeled goat antirabbit IgG (Auro Probe EM GAR G15; Amersham, Little Chalfont, UK) was used.

All sections were conductive-stained with 2% OsO_4 for 2 h, dehydrated through a graded ethanol series, and dried using the *t*-butyl alcohol freeze-drying method.[16] The dried sections were coated with approximately 3.5-nm-thick platinum–palladium using an ion sputter coater (E-1030; Hitachi, Tokyo, Japan) or 1- to 2-nm-thick osmium using an osmium coater (HPC-1S; Vacuum Device, Ibaragi, Japan) because a high contrast of gold particles is obtained.[17] The innermost surface of tracheid cell walls at the S_2-forming stage was observed using FE–SEM (S-4500; Hitachi) at an accelerating voltage of 1.5 kV and a working distance of 5 mm.

Quantification of immunogold labeling

Electron micrographs of the immunogold-labeled sections were scanned with an image scanner. The amounts of immunogold labeling in randomly selected areas were measured using KS 400 image-processing and analysis software (Carl Zeiss, Munich, Germany). To distinguish gold particles from background, binary images were obtained by segmentation based on the difference in luminance. Particles less than 15 nm in diameter and fibrillar objects derived from cellulose microfibrils were removed. Particles



Sampling time after lights-off		Sampling time after lights-on	
a	Immediately after	h	Immediately after
b	1 h after	i	1 h after
c	2 h after	j	2 h after
d	3 h after	k	3 h after
e	4 h after	l	4 h after
f	5 h after	m	5 h after
g	6 h after	n	6 h after

Fig. 1. Changes in the tangential strain on the inner bark surface of a sapling grown in a growth chamber. *Arrowheads* indicate sampling times after lights-on (light period). *Arrows* indicate sampling times after lights-off (dark period). *Shaded areas* show dark periods

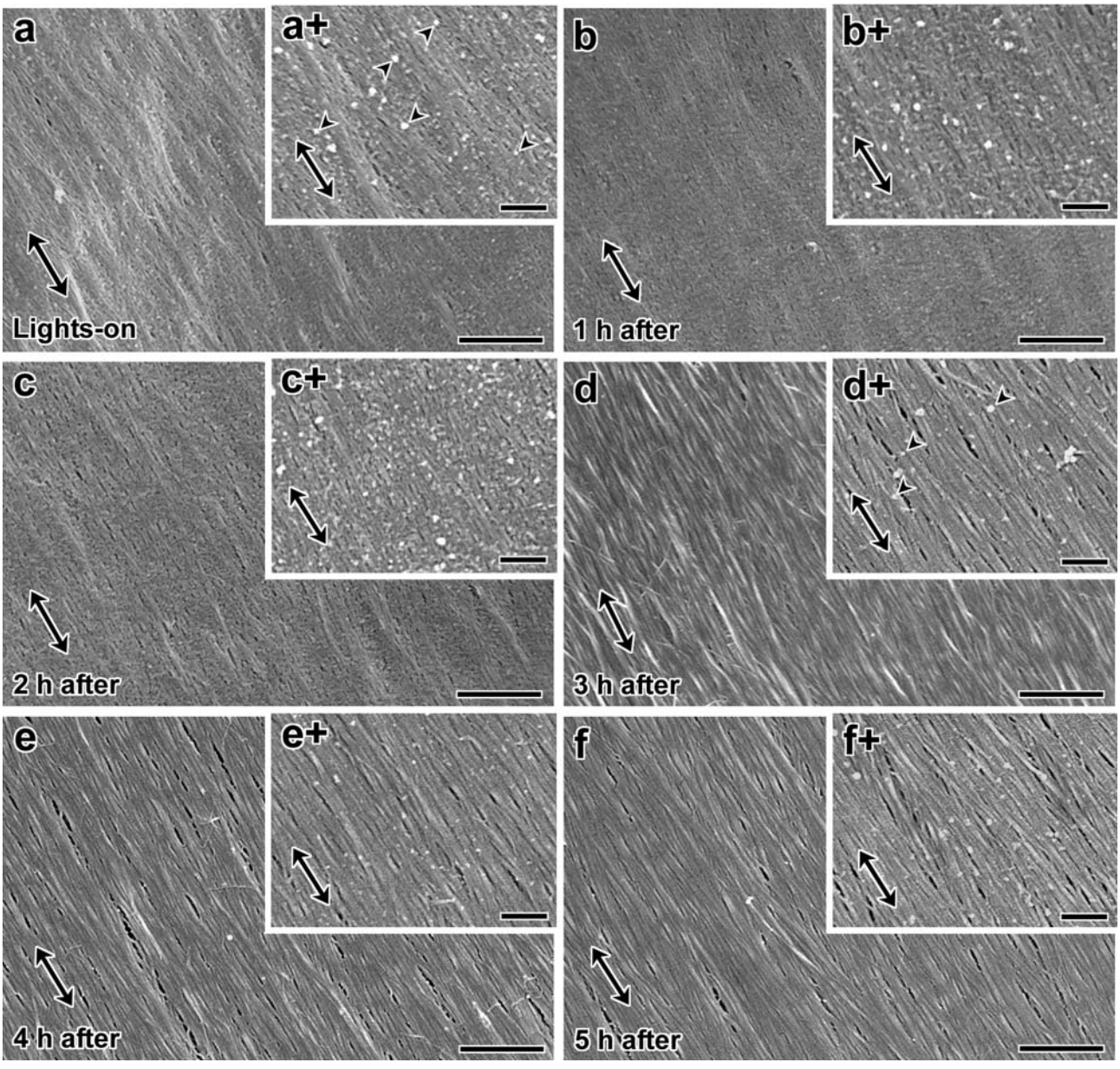


Fig. 2a–f. Innermost surface of developing secondary walls in specimens collected after lights-on. Main micrographs **a–f** show sections not labeled with antibodies (*bars* 500 nm); *insets* labeled **a+–f+** show sections with antiglucomannan antiserum (*bars* 200 nm). **a** Immediately after lights-on; **b** 1 h after lights-on; **c** 2 h after lights-on; **d** 3 h after lights-on; **e** 4 h after lights-on; **f** 5 h after lights-on. The longitudinal cell axes in the micrographs are vertical. *Double-headed arrows* show the orientation of cellulose microfibrils in each micrograph. *Arrowheads* indicate single or large aggregated gold particles representing antiglucomannan labels

15–25 nm in diameter were counted as gold particles coated with metal. Large gold particles formed by the aggregation of secondary antibodies were also counted as one particle. The labeling density was expressed as the mean number of gold particles per square micron. Approximately 15 different points on the innermost surface of developing secondary walls were analyzed.

Results and discussion

The tangential strain on the inner bark surface displayed diurnal changes as observed in our previous study,[5] decreasing immediately following lights-on, and increasing immediately after lights-off (Fig. 1). The changes in tangential strain are induced by changes in the water content and the volume of differentiating cells;[18–21] the strain is proportional to the volumetric changes in the cells. In the specimens collected

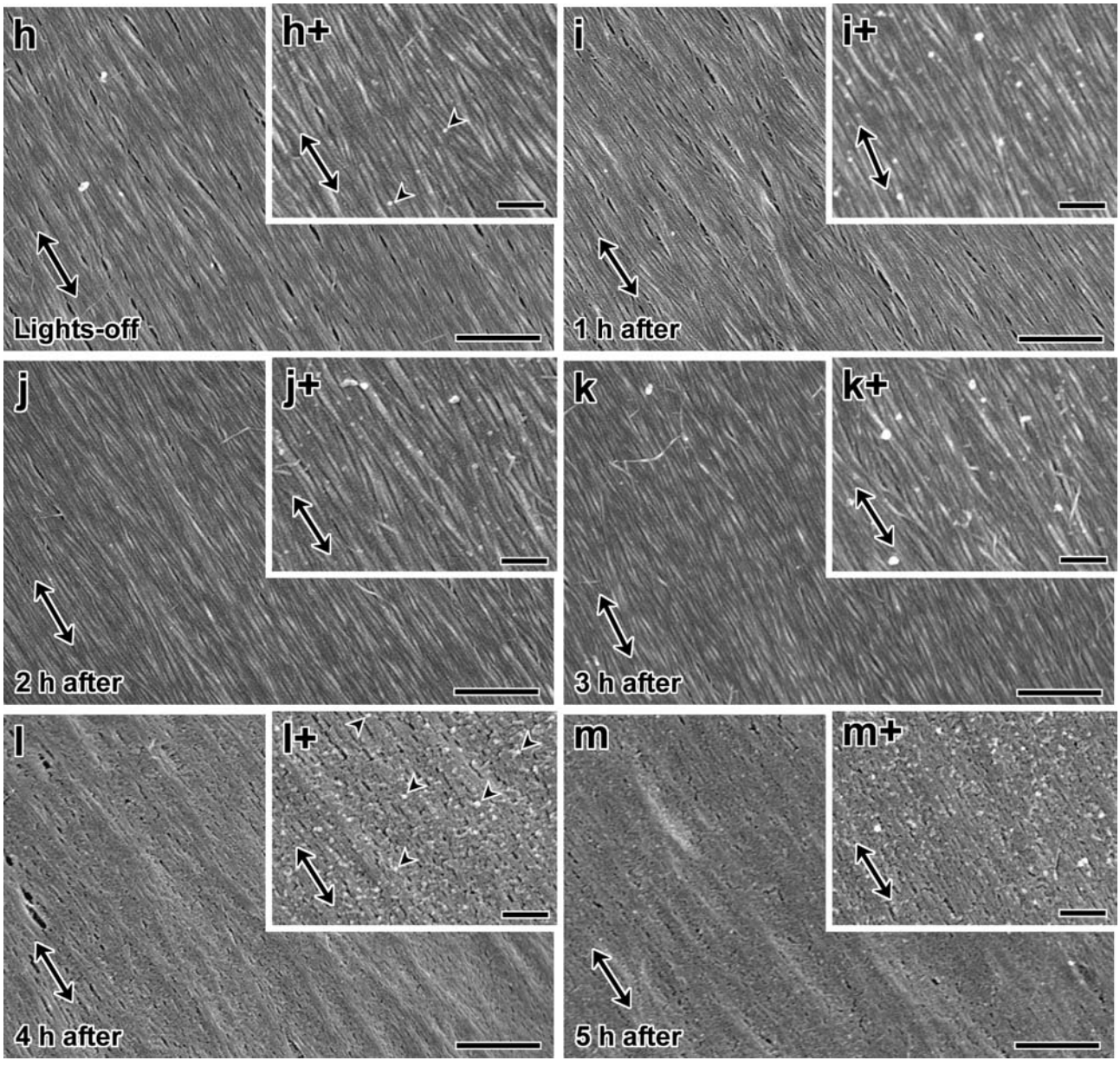


Fig. 3h–m. Innermost surface of developing secondary walls in the specimens collected after lights-off. Main micrographs **h**–**m** show sections not labeled with antibodies (*bars* 500 nm); *inset* labeled **h+**–**m+** show sections with antiglucomannan antiserum (*bars* 200 nm). **h** Immediately after lights-off; **i** 1 h after lights-off; **j** 2 h after lights-off; **k** 3 h after lights-off; **l** 4 h after lights-off; **m** 5 h after lights-off. The longitudinal cell axes in the micrographs are vertical. *Double-headed arrows* show the orientation of cellulose microfibrils in each micrograph. *Arrowheads* indicate single or large aggregated gold particles representing antiglucomannan labels

until 2 h after lights-on, the volume of differentiating cells was rapidly decreasing with water loss by transpiration; in the specimens collected between 3 and 6 h after lights-on, cell volume was low. In the specimens collected until 3 h after lights-off, the differentiating cells were expanding as a result of imbibition by low transpiration; cells in the specimens collected between 4 and 6 h after lights-off were fully turgid because they contained a large amount of water.

In the specimens collected after the dark period, amorphous material was present on the innermost surface of developing secondary walls, and a large amount of immunogold labeling was observed as bright spherical particles in the material until 2 h after lights-on (Figs. 2a–c, 4a). In the specimens collected 3 h after lights-on, cellulose microfibrils were clearly visible and amorphous material was rarely seen (Fig. 2d). In the antiserum-labeled sections, some labeling was observed, but far less than in the specimens collected until 2 h after lights-on (Figs. 2d, 4a). From 4 h after lights-on, the aspects of the innermost surface of developing secondary walls rarely changed (Fig. 2e–f) and rarely differed from those observed in previous studies.[2,5]

In the specimens collected after the light period, cellulose microfibrils were clearly observed on the innermost

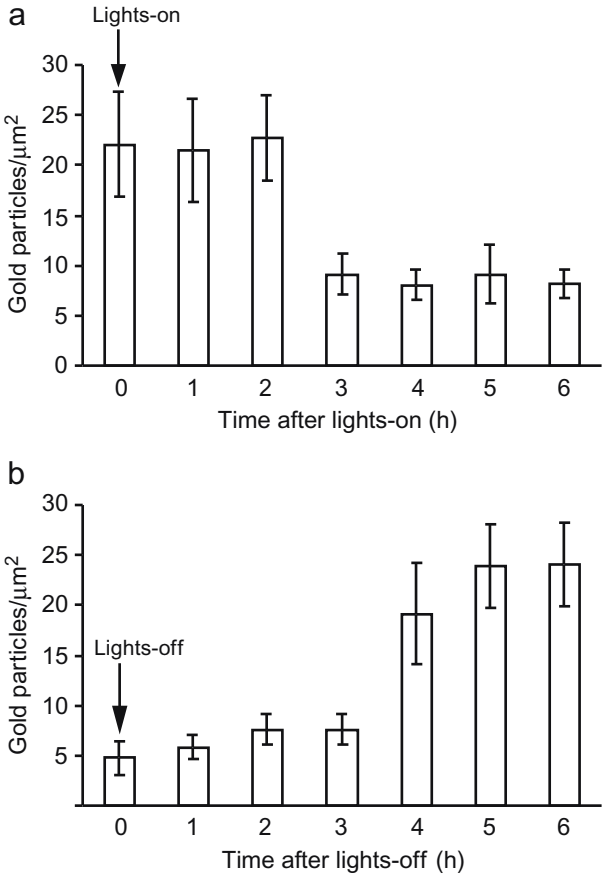


Fig. 4a,b. Number of gold particles per unit of innermost surface area in developing secondary walls. **a** Labeling density in the specimens collected after lights-on; **b** labeling density in the specimens collected after lights-off. Data given as mean ± SD. Approximately 15 different points were analyzed

surface of developing secondary walls, and there was little labeling with the antiserum until 3 h after lights-off (Figs. 3h–k, 4b). In the specimens collected 4 h after lights-off, amorphous material began to appear, and the density of labeled glucomannans in the innermost surface sharply increased (Figs. 3l, 4b). From 5 h after lights-off, amorphous material with much labeling was seen (Figs. 3m, 4b), and the characteristics of the material rarely differed from those observed in previous studies.[2,5] Labeling was rarely seen in any of the sections labeled with preimmune serum or antiserum previously incubated with glucomannans. Each photograph is representative of several photographs of the innermost surface of developing secondary walls obtained at specific times from FE–SEM observations.

Dramatic changes in the aspects of the innermost surface of developing secondary walls appeared and the density of labeled glucomannans sharply changed 3 h after the light was switched on and 4 h after the light was switched off. These changes indicate that there was a time lag of 3–4 h between the diurnal differences in the innermost surface of developing secondary walls and the change in light conditions during the photoperiodic cycle. The amorphous material containing glucomannans was seen while the differentiating cells were fully turgid, but it was rarely seen when the cell volume was low. These results suggest that the supply of the matrix containing hemicellulose to the innermost surface of developing secondary walls is affected by diurnal changes in the water status of differentiating cells, e.g., turgor pressure. The observed time lag may have resulted from the time taken for the differentiating tracheids to become fully turgid or to decrease their volumes enough after the change in light.

The central role of the Golgi apparatus in the production of cell wall polysaccharides is widely accepted. Samuels et al.[11] confirmed that glucomannans were synthesized in the Golgi apparatus and secreted into the cell wall thorough the plasma membrane by exocytosis in *Pinus contorta*. The exocytosis process is controlled by turgor pressure, and the secretion of new cell wall materials is enhanced by increasing turgor pressure.[12] In this study, the amorphous material containing glucomannans became evident and the labeling density sharply increased when the differentiating cells became fully turgid. This suggests that the amount of hemicelluloses secreted to the innermost surface of developing secondary walls is small immediately after lights-off because the turgor pressure of differentiating cells is low. It seems probable that the secretion becomes active at increased turgor pressure as a result of imbibition in the dark period and that the amorphous material becomes visible with the accumulation of the matrix containing hemicellulose.

The diurnal differences in the innermost surface of developing secondary walls occurred with time lags of 3–4 h after the light changed in the photoperiodic cycle. The time when the amorphous material containing glucomannans was seen corresponded to the time when the differentiating cells were fully turgid. The results of our study suggest that the diurnal periodicity in the supply of hemicellulose-containing matrix to developing secondary walls is associated with the diurnal changes in turgor pressure of differentiating tracheids resulting from changes in light conditions.

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