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Localization of glucuronoxylans in Japanese beech visualized by immunogold labelling

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Summary. An antiserum against glucuronoxylans (GXs) has been raised from a mouse. The dot-blot immunoassay and competitive inhibition test indicated that the antibodies could bind specifically to GXs. Therefore, the antiserum was used for immunogold labelling to investigate the localization of GXs in Japanese beech. Labelling of GXs was seen only in the secondary walls of xylem cells, but not in the primary walls or the middle lamella. GXs were evenly distributed in the secondary walls except for the outer part of the outer secondary-wall layer in which they were less abundant. The labelling density in each secondary-wall layer (S_1, S_2, and S_3) increased during cell wall formation. This result strongly suggests that the deposition of GXs occurs in a penetrative way.

Keywords: *Fagus crenata*; Glucuronoxylans; Immunogold-labelling; Secondary wall.

Introduction

Glucuronoxylans (GXs) are the main hemicellulosic component of the hardwood and account for 20–35% of the woody cell wall. Their importance has been recognized not only because of their abundance in wood but for their chemical properties. The localization of GXs in plant cell walls has been studied by microdissection coupled with chemical analysis (Meier 1961), affinity labelling with xylanase-gold complex (Vian et al. 1983, 1986, 1992), and immunogold labelling (Northcote et al. 1989, Migne 1994). Cell wall components could not be precisely localized by the microdissection method because it was impossible to separate the developing stages of the cell wall from the differentiating xylem. One of the other two in situ labelling techniques was therefore used to determine the distribution of xylan within the cell wall. The methods showed that GXs are present in the secondary wall of dicotyledons (Vian et al. 1983, 1986, 1992; Northcote et al. 1989).

The chemical properties of GXs were reviewed and their possible roles were postulated by Reis et al. (1994). They are composed of a linear backbone of β-(1,4)-linked-D-xylopyranose and irregularly substituted at O-2 position by 4-O-methyl-α-D-glucuronic acid (Fengel and Wegener 1989). GXs can tightly bind to cellulose microfibrils because of the hydrogen bonding between the xylan backbone and the glucan chain of cellulose microfibrils (Labavitch and Ray 1974, McNeil et al. 1975, Mora et al. 1986, Neville 1988, Joseleau et al. 1991). GXs give negative charges onto the surface of cellulose microfibrils owing to their 4-O-methyl-glucuronic acid groups. This separates each cellulose microfibril and enables them to assemble in cholesteric mesophase (helicoidal structure) (Vian et al. 1992). Another role of GXs is as a host structure for lignin precursors. Taylor et al. (1992) suggested that the secondary-wall assembly occurs as a self-perpetuating cascade in which xylan mediates the localization of lignin.

The aim of this paper is (1) to prepare an antiserum specific for glucuronoxylans, (2) to investigate GXs distribution in differentiating xylem of Japanese beech by immunogold labelling, and (3) to discuss the mode of GXs deposition during cell wall formation in the beech.

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Material and methods

Polysaccharides

Glucuronoxylan from Japanese beech (*Fagus crenata*) was generously donated by Dr. T. Watanabe (Wood Research Institute, Kyoto University, Japan).

Oligosaccharides

Oligosaccharides were prepared from glucuronoxylan by partial acid hydrolysis in 0.1 M H_2SO_4 at 100 °C for 2 h. The reaction mixture was neutralized with 0.1 M $Ba(OH)_2$ and centrifuged to remove the precipitate. Supernatant was lyophilized and dissolved in distilled water. The oligosaccharide solution was applied to a Bio-Gel P2 column and eluted with water. An aliquot of each fraction was spotted onto a TLC plate and developed by the following solvent: isopropanol : ethanol : H_2O = 7 : 1 : 2. Fractions containing oligosaccharides with a degree of polymerization between 3–6 were combined and lyophilized again.

Preparation of oligosaccharides-KLH conjugates

Oligosaccharides were coupled to keyhole limpets hemocyanin (KLH) by the reductive amination method according to Roy et al. (1983). Oligosaccharide (20 mg) and KLH (20 mg) were dissolved in 1 ml of 0.2 M borate buffer (pH. 9.0) containing 20 mg of cyanoborohydride. The reaction was carried out with continuous stirring at 50 °C for 5 days. The reaction mixture was neutralized to pH 4.0 with 80% acetic acid and dialyzed against distilled water for two days. The solution containing the oligosaccharides-KLH conjugate was then lyophilized and dissolved in 1 ml of distilled water. The solution of the conjugates was purified on a Bio-Gel P2 column to separate unreacted oligosaccharides. The fractions containing the conjugates were collected and lyophilized, and the conjugates were dissolved in phosphate-buffered saline (PBS; 1 mg/ml) and stored at –80 °C.

Immunization

8-week-old BALB/c female mice were immunized. Before the first immunization, preimmune sera were taken from each mouse. The initial injection was carried out intraperitoneally with the mixture of 100 µl of the conjugates and 100 µl of Freund's complete adjuvant. This intraperitoneal injection was repeated twice at 2-week intervals in the same way with Freund's incomplete adjuvant (FIA). Another injection with a mixture of 100 µl of glucuronoxylan solution (1 mg/ml) and 100 µl of Freund's incomplete adjuvant followed 2 weeks later and was repeated twice at 2-week intervals. Three days after the final injection, 300 µl of antiserum was taken from one of the immunized mice and used for the following immunoassays.

Dot-blot immunoassay

The antibodies were detected by dot-blot immunoassay with a positive-charged nylon membrane, Biodine B (Pall, East Hills, New York, U.S.A.), as immunoadsorbent. All the procedures described below were performed at room temperature. A sheet of Biodine B membrane was placed in a dot-blot manifold. Antigen solution containing glucuronoxylan was made up in 2 M $NaHCO_3$ at 1 mg/ml concentration. The membrane in the manifold was washed in 2 M $NaHCO_3$ and 100 µl of various diluted antigen solutions (1- to 64-fold) was applied to the wells (1.6–100 µg/dot). Solutions were aspirated with a handy vacuum pump, then all the wells were washed with 100 µl of PBS-T (PBS containing 0.1% (v/v) Tween 20) in the same manner. To avoid nonspecific antibody-binding, all wells were blocked with 3% (w/v) skim milk in PBS-T (100 µl). After the wells were washed four times with PBS-T, 100 µl of various diluted antisera (100- to 25600-fold) were applied to the wells without aspirating. At the end of 1 h incubation, the wells were washed thoroughly with PBS-T and then the membrane was removed from the manifold. The membrane was again washed with PBS-T and then incubated for 1 h with goat anti-mouse IgG(H+L)-alkali phosphatase conjugate (diluted 1 : 4000 in 3% skim milk in PBS-T; Gibco BRL, Gaithersburg, MD, U.S.A.). After the membrane was again washed with PBS-T, the bound antibodies were visualized by using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate toluidinium as substrate according to the instruction manual (Boehringer Mannheim, Mannheim, Federal Republic of Germany). Color development was stopped by incubation with 50 mM EDTA and the membrane was washed with deionized water.

Tissues

Japanese beech (*Fagus crenata*) grown in the Kyoto University Forest in Ashiu (Miyamacho, Kyoto, Japan) was used in this study. Small pieces containing differentiating xylem were cut from the stem and fixed in 3% glutaraldehyde in 1/15 M phosphate buffer at pH 7.2, overnight at 4 °C. The pieces were washed six times with the buffer, dehydrated through a graded ethanol series and embedded in epoxy resin.

Light microscopy (immunogold-silver staining)

For light microscopic observation, 1 µm thick sections were cut from the embedded block and mounted on glass slides. The following immunolabelling procedures were performed on these slides.

All the procedures described below were performed at room temperature unless otherwise noted. Sections were incubated for 15 min with 50 mM glycine in PBS. After three washes in PBS, they were incubated for 30 min with 3% skim milk in PBS-T to avoid nonspecific antibody binding. At the end of the incubation, sections were washed three times for 5 min each time in PBS-T, then incubated in the antiserum (diluted 1 : 100 in 3% skim milk in PBS-T) for 2 h at 37 °C. For the control experiment, the serial sections were incubated in either normal serum (1 : 100 dilution) taken before the first immunization or in the antiserum previously incubated with glucuronoxylan (1 mg/ml). After the incubation, sections were washed three times for 5 min each time in PBS-T and then incubated in Auro Probe GAM G15 (goat anti-mouse IgG 15 nm colloidal gold conjugate; Amersham, Buckinghamshire, U.K.) diluted 1 : 25 in PBS for 2 h at 37 °C. They were again washed three times for 5 min each in PBS-T and fixed with 2% glutaraldehyde in PBS for 5 min, after which they were washed with distilled water. To detect the labelling under a light microscope, the sections were treated in an Inten SE M Silver enhancement kit (Amersham) for 15–20 min. Reaction was stopped by washing with distilled water and observation was made under a light microscope without any dye-staining.

Electron microscopy (immunogold labelling)

Ultrathin sections were prepared sequentially from the embedded specimen which was used for the preparation of light microscopic sections, and mounted on 75-mesh nickel grids. The procedures described below were performed at room temperature unless otherwise noted. The sections on grids were immersed in 50 mM glycine

in PBS for 15 min. After three washes in PBS, they were incubated in blocking buffer (PBS containing 0.8% bovine serum albumin; 0.1% immunogold silver-staining quality gelatin (Amersham), 5% goat serum and 2 mM NaN_3) for 30 min, then washed three times for 5 min each in washing buffer (same as blocking buffer but without goat serum) and incubated in antiserum for 2 h at 37 °C. For the control experiment, serial sections were incubated in preimmune serum or antisera previously incubated with one of the following saccharides: xylose, xylobiose, xylotriose, glucuronoxylan, pectin, xyloglucan, or glucomannan. Sections were then washed in washing buffer three times for 5 min each and incubated in Auro Probe GAM G15 diluted 1 : 25 in blocking solution for 2 h at 37 °C. They were washed in washing buffer three additional times and fixed with 2% glutaraldehyde in PBS for 5 min, after which they were washed with distilled water, stained with 2% aqueous uranyl acetate and Reynolds's lead citrate and examined with a TEM (JEM-2000 ES; JEOL, Tokyo, Japan) at 100 kV.

Quantification of labellings

Electron micrographs were taken from one of the labelled sections and were printed at 10000-fold magnification. They were scanned by an image scanner and stored in Macintosh. The area of each cell wall layer and the number of gold particles were measured by NIH Image software, and then the number of particles per unit of cell wall area was calculated.

Results

Dot-blot immunoassay

The result of the dot-blot immunoassay is shown in Fig. 1. Color developments were proportional to the concentration of antigen or antiserum. This development was not observed when the dots were incubated with PBS, nor was any color development seen in the dots which had not absorbed GXs.

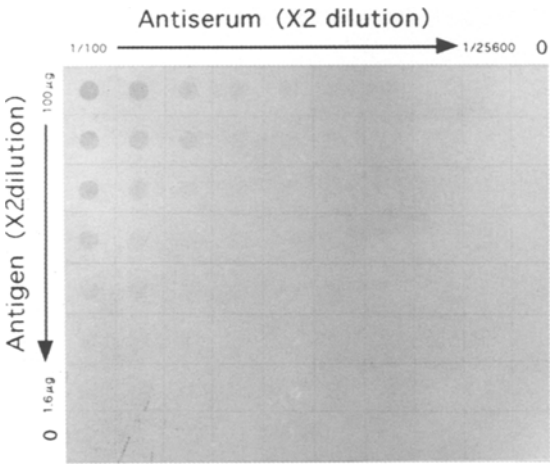


Fig. 1. Specificity of antiserum examined by dot-blot immunoassay. Color development gradually decreased according to the dilution of antiserum and/or antigen. No color development was observed in the dots which did not absorb GXs (bottom lane). When the antiserum incubation was omitted, color development was not observed (extreme right lane)

Table 1. Summary of the results of the competitive inhibition test

Competitor	Gold particles	Inhibition
No addition	++	no
GXs	–	full
Xylotriose	–	full
Xylobiose	–	full
Xylose	+	partial
Glucomannan	++	no
Xyloglucan	++	no
Pectin	++	no

Antiserum was incubated with one of the various competitors for 1 h and then used for immunogold labelling

Immunocytochemical controls

When the ultrathin sections were treated with the antiserum, many gold particles were seen in the secondary wall but not in the primary walls or the middle lamella (Fig. 2 A). However, gold particles were seldom found in the sections treated with the antiserum pre-incubated with glucuronoxylans (Fig. 2 B). When xylotriose or xylobiose was used as a competitor, the immunogold labelling was almost completely inhibited (Fig. 2 C). Fewer gold particles were observed when the antiserum was pre-incubated with xylose (Fig. 2 D). Pectin, xyloglucan, and glucomannan did not inhibit labelling (Fig. 2 E), and labelling was rarely found when the sections were treated with preimmune serum (Fig. 2 F). Results of the competitive inhibition test are shown in Table 1.

Light microscopy

Labelling was seen in the secondary wall of differentiating xylem (Fig. 3) and their intensity increased in the course of cell wall formation. The cell walls in the cambial zone and expansion zone were not labelled. At high magnification, no labelling was found in the lumen, compound middle lamella or pit chambers (Fig. 4).

Electron microscopy

Gold particles were found only in the secondary walls of xylem elements, i.e., vessel elements, fibers, axial parenchyma, and ray parenchyma. In matured fibers, vessel elements and axial parenchyma, these particles were evenly distributed throughout the secondary

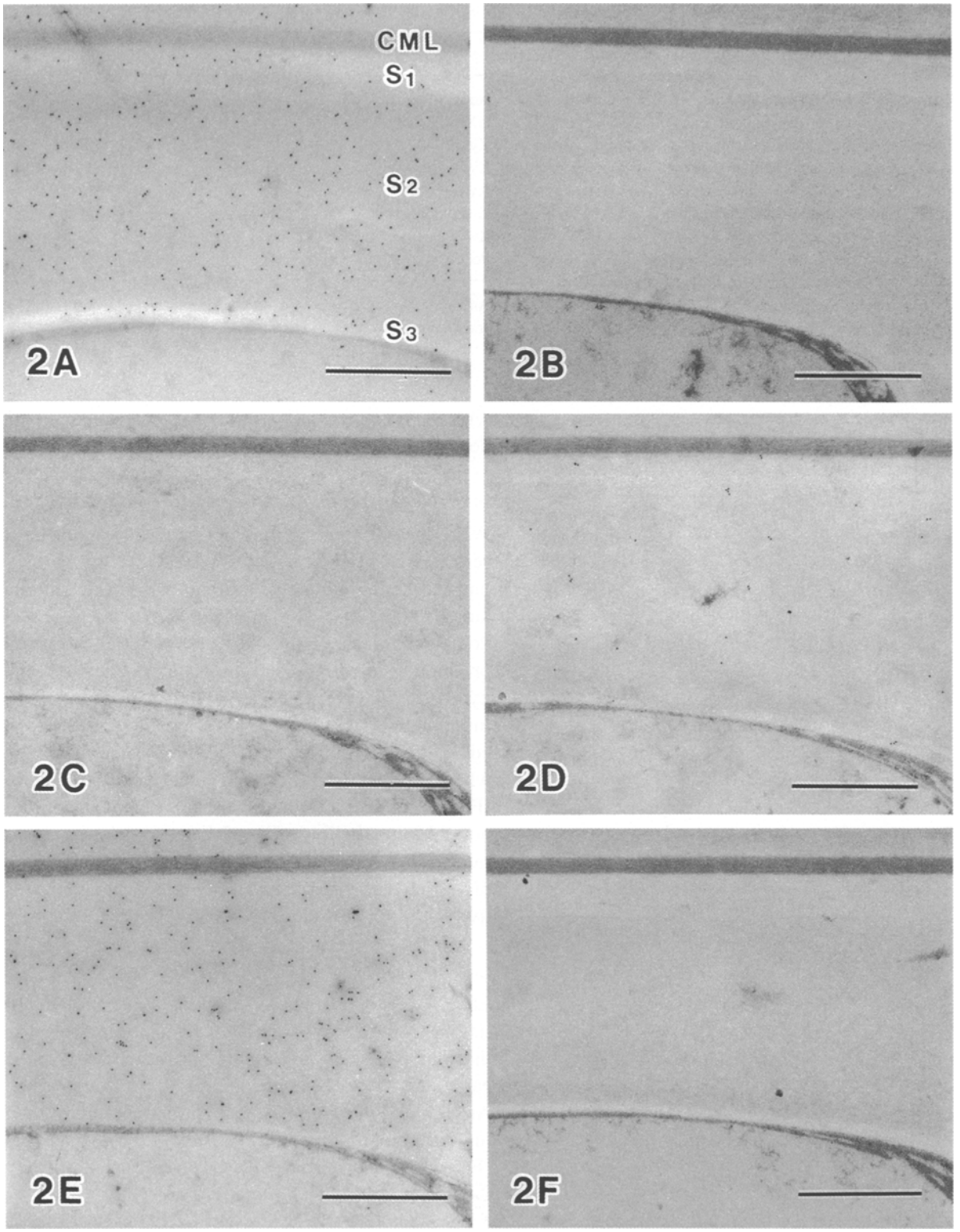


Fig. 2 A–F. Six serial ultrathin sections were immunolabelled with various sera: **A** antiserum against GXs, **B** antiserum against GXs previously incubated with GXs, **C** antiserum against GXs previously incubated with xylobiose, **D** antiserum against GXs previously incubated with xylose, **E** antiserum against GXs previously incubated with xyloglucan, **F** preimmune serum. GXs, xylobiose, and xylose inhibited the antiserum from binding the sections though xyloglucan did not. *CML* Compound middle lammella; S_1, S_2, S_3 outer, middle, and inner layer of secondary wall. Bars: 1 μm

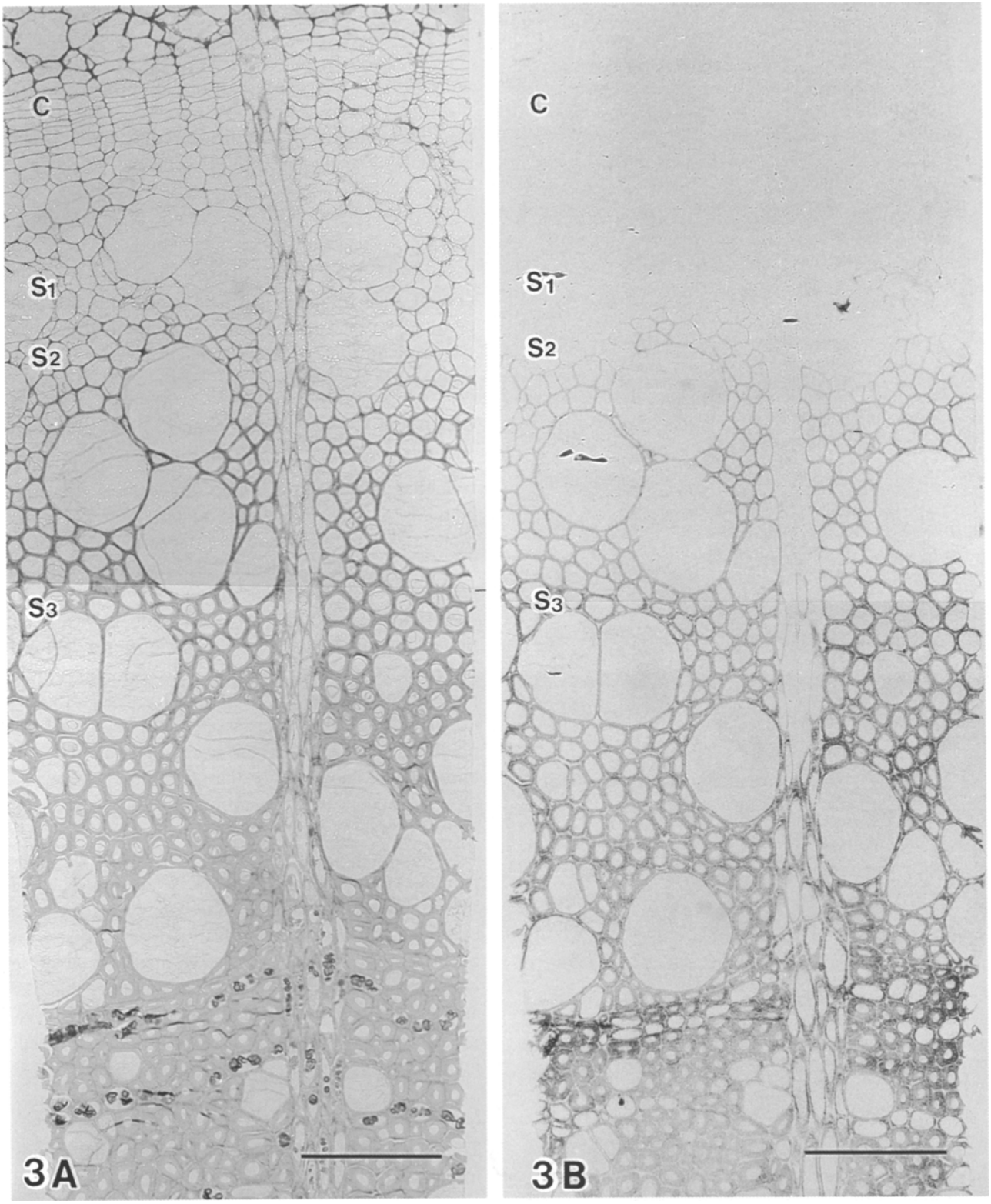


Fig. 3 A, B. Serial sections were cut, and some of them were stained with 1% safranin (**A**) and others labelled with antiserum (**B**). In **B**, labelling appears in the cell wall of xylem elements, i.e., vessel elements, fibers, axial parenchyma and ray parenchyma. *C* Cambial zone; S_1, S_2, and S_3 starting position of the corresponding cell wall layer formation. Bar. 100 μm

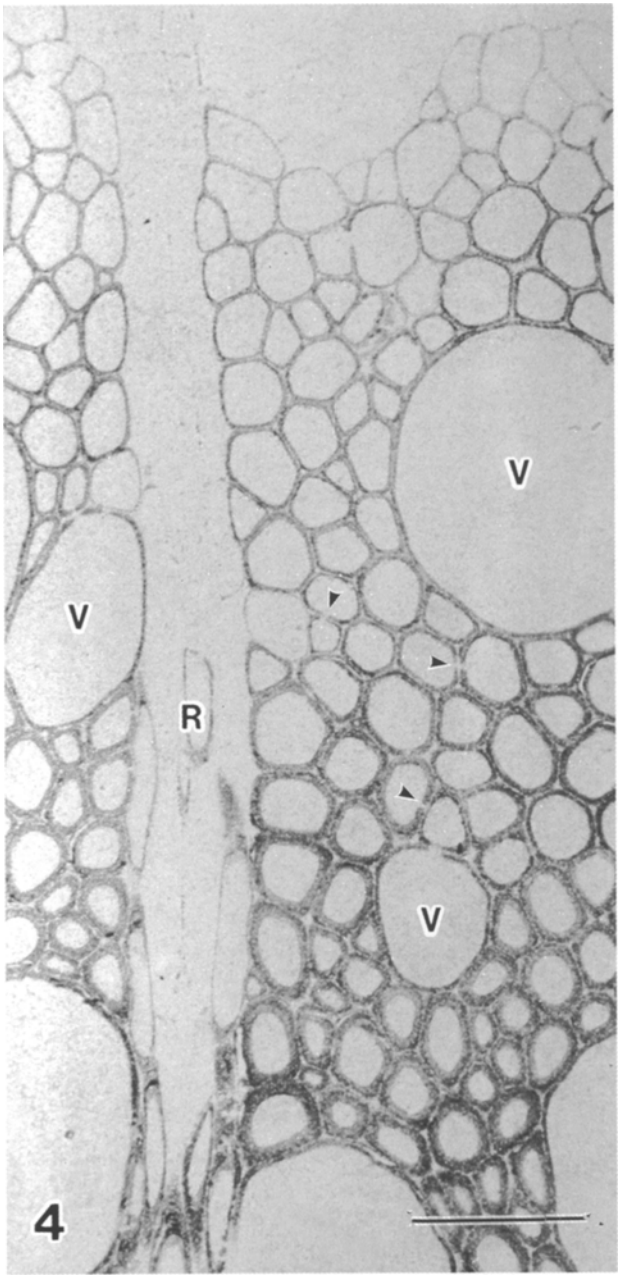


Fig. 4. Labelled sections were observed under a light microscope at high magnification. No labelling was found in the lumen, compound middle lamella, and pit chambers (arrowheads). Density of labelling increased in the course of cell wall formation. *R* Ray, *V* vessel element. Bar: 50 μm

wall, except for the outer part of the S_1 layer in which they were less abundant (Figs. 5 and 6). No labelling was found in the cell walls during expansion growth (Fig. 7). In the S_1-forming fibers, labelling was found in the inner part of the S_1 layer but not in the outer part (Fig. 8), while in the S_2-forming fibers, labelling was found in the inner part of the S_1 layer and in the S_2 layer. Labelling density of the S_2 layer was the same extent as that of the inner part of the S_1 layer (Fig. 9). Gold particles in the S_1 and S_2 layers were more abundant during the S_3 formation stage of fiber than those during the S_2 formation stage (Fig. 10). In the fibers which had completed the S_3 layer formation, labelling increased in all cell wall layers (Fig. 11).

Quantification of labelling

The variation in labelling density during cell wall formation is shown in Fig. 12. The number of labellings per cell surface unit in the S_1, S_2, and S_3 layers increased during cell wall formation.

Discussion

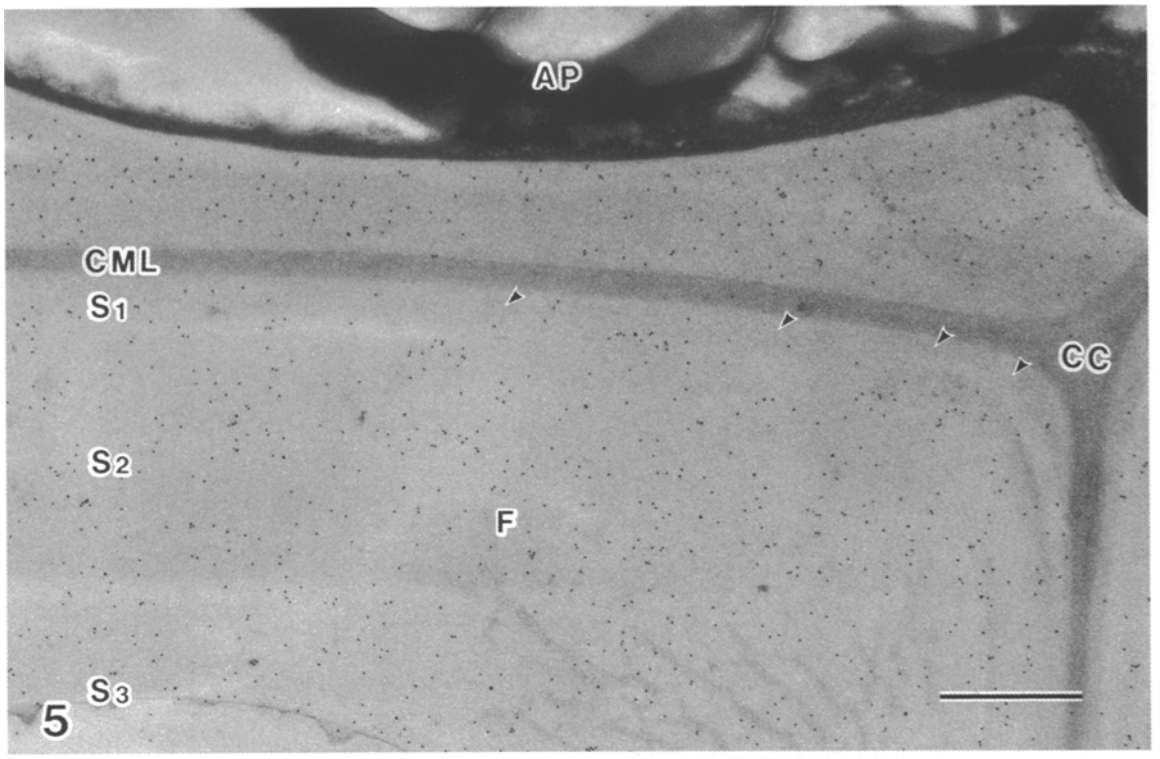
Characterization of antiserum

Immunogold labelling is one of the most powerful methods to visualize the localization of particular molecules when the reaction of the antibody is specific to the targeted molecule. Because only a small amount of antiserum was taken from a mouse, its specificity was examined by the competitive inhibition of immunolabelling. When the ultrathin sections of beech were labelled with the antiserum, many gold particles were found in the secondary walls. However, when the sections were treated with the antiserum previously mixed with an excess amount of glucuronoxylan, labelling was hardly seen. These results clearly indicate that the antibodies bind to the glucuronoxylans. Similar inhibitions were seen when β1-4-xylobiose or β1-4-xylotriose was used as a competitor, though many particles were found when xylose was used. When the antiserum was mixed with other cell wall polysaccharides, such as pectin, xyloglucan, or glucomannan as a competitor, labelling was also abundant. These results suggest that the antibodies mainly recognize the structure of β1-4-xylosil linkages but not the linkages of other cell wall polysaccharides.

Localization of glucuronoxylans in Japanese beech

The localization of GXs has been studied by various in situ labelling methods, e.g., the xylanase-gold method (Vian et al. 1983, 1986, 1992), xylanase and anti-xylanase antibodies (Taylor et al. 1993), and the immunogold method (Northcote 1989).

Most of these works, however, did not show the deposition of GXs during the cell wall formation in detail.



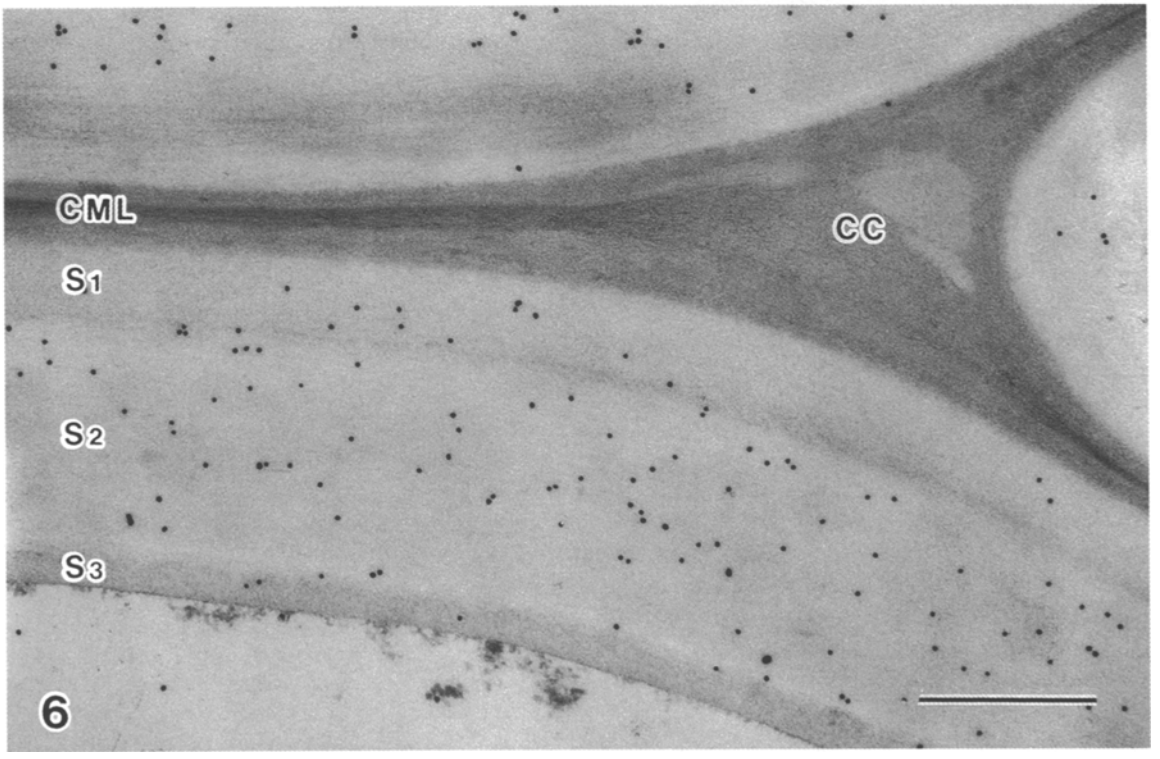


Fig. 5. Immunogold labelling was found only in the secondary wall of both fiber (*F*) and axial parenchyma (*AP*). Gold particles were rarely found in the compound middle lamella (*CML*), cell corner (*CC*), and lumen. Particles were evenly seen in the secondary wall except that few were seen in the outer part of the S_1 layer (arrowheads). Bar: 1 μm

Fig. 6. The secondary wall of vessel element was labelled with anti GXs antiserum. Immunogold labelling was again found only in the secondary wall of vessel elements. Gold particles were scarcely found in the compound middle lamella, cell corner, and lumen. Little labelling was observed in the outer part of the S_1 layer. Bar: 0.5 μm

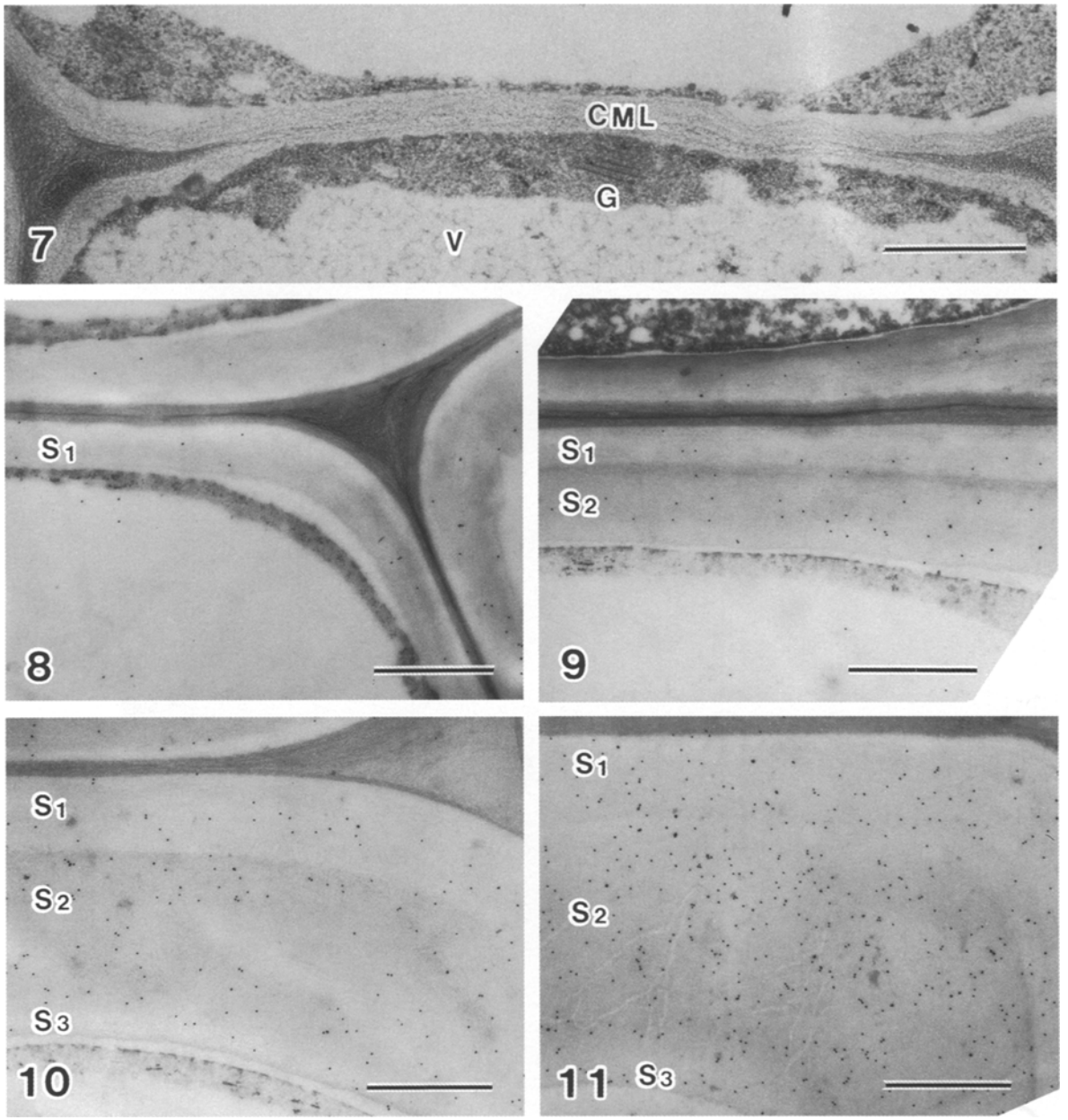


Fig. 7. Cells in cambium zone were labelled with anti GXs antiserum. Labellings were hardly found in the cell wall and cytoplasm. *G* Golgi apparatus; *V* vacuole. Bar: 0.5 μm

Fig. 8. The S_1-forming fiber was labelled with anti GXs antiserum. Labellings were found in the secondary wall of the S_1-forming fiber. The number of labellings in the S_1 layer was less than that of matured fibers (compare with Fig. 11). Bar: 0.5 μm

Fig. 9. The S_2-forming fiber was labelled with anti GXs antiserum. Gold particles were found in the inner part of the S_1 layer and in the S_2 layer, though few were found in the outer part of the S_1 layer. Labelling was not seen in the primary wall, middle lamella and cytoplasm. Note that the number of particles in both the S_1 and S_2 layers was less than that of matured fibers (compare with Fig. 11). Bar: 0.5 μm

Fig. 10. The S_3-forming fiber was labelled with anti GXs antiserum. Labellings were found in overall secondary wall except for the outer part of the S_1 layer. Labelling was rarely seen in the primary wall, compound middle lamella, and cytoplasm. Note that the number of particles in both the S_1 and S_2 layers was less than that in the matured fibers (compare with Fig. 11). Bar: 0.5 μm

Fig. 11. The S_3-forming fiber was labelled with anti GXs antiserum. Labelling was found in overall secondary wall except for the outer part of the S_1 layer. Labelling was rarely seen in the compound middle lamella. Note that the number of particles in both the S_1 and S_2 layers was greater than that of the S_3-forming fibers (compare with Fig. 10). Bar: 0.5 μm

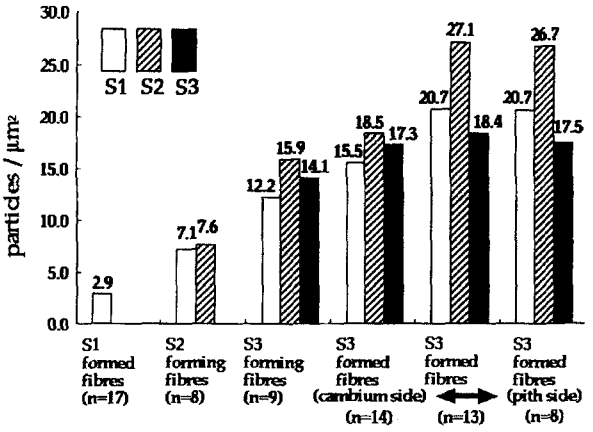


Fig. 12. The number of gold particles per unit of cell wall area was calculated from electron micrographs taken from one of the labelled sections. The labelling density in the S_1, S_2, and S_3 layers gradually increased during the cell wall formation. Interestingly, that in the S_1 and S_2 layer increased continuously after the deposition of cellulose microfibrils in the S_3 layer. Numbers in parentheses indicate the number of cells calculated

In this study, therefore, GXs deposition was observed from cambial cells to differentiated cells with light and electron microscopes by the immunogold labelling method.

Labelling was seen in the secondary wall of xylem cells, that is, vessel elements, wood fibers, axial parenchyma, and ray parenchyma, but was not found in the compound middle lamella, pit chambers, or cell lumen. The cell walls in the cambial zone and cell expansion zone were not labelled. Therefore, GXs were distributed only in the secondary wall.

Electron microscopic observation also showed labelling was only in the secondary wall of xylem elements. In matured fibers, vessel elements, and axial parenchyma, gold particles were found throughout the secondary wall, though few particles were observed in the outer part of the S_1 layer. There are two possible explanations for the less abundant labelling in this region: the antigenic sites in this region may have been masked by phenolic components such as lignins, or GXs may actually be less abundant. The former possibility is probably less likely, because few particles were found in the S_1 layer of differentiating fibers which were not lignified. Therefore, masking of antigenic sites would not occur in this specimen. GXs deposition would start in the middle of the S_1 layer formation stage.

The results in this paper differ from those of the studies using the enzyme-gold method by Vian et al. (1983, 1986, 1992). They found that the labelling of GXs was preferentially localized in the transition zone S_1–S_2. This difference may be due to the difference of specificity between antibodies and xylanase. On the basis of chemical extraction, Vian et al. (1986) suggested that there are two kinds of GXs: one encrusting the overall wall is strongly bound to cellulose microfibrils and the other is embedded in the microfibrils of the transition zone. The antiserum used in this study could bind to both types of GXs, while the xylanase-gold complex was able to recognize only the latter.

The mode of GXs deposition

Labelling density increased during cell wall formation. For example, in the fibers which had just completed the S_3 layer formation, the labelling densities in the S_1 and S_2 layers were 12.2 and 15.5 (particles per μm^2), respectively. Interestingly, the densities in these layers increased to 20.7 and 26.7, respectively, in the fibers which located farther from the cambium. This means that GXs secreted into the inner surface of the developing cell wall were able to penetrate through the cell wall layers. Two possible mechanisms of GXs deposition could be hypothesized. One is that GXs are synthesized in the Golgi apparatus and secreted in the periplasm via secretory vesicles. They bind to the surface of cellulose microfibrils with hydrogen bonding. The microfibrils coated with GXs are then separated by negative charge given by uronic acid groups. When the binding sites of cellulose microfibrils are saturated, GXs penetrate into the space between microfibrils. The other possibility is that there are two molecular types of GXs, one has high affinity to cellulose microfibrils and the other has not. This difference could be caused by their chemical structure, such as the degree of substitution by acetyl groups. GXs with high affinity to cellulose microfibrils bind to microfibrils and separate them. Then, GXs with lower affinity to cellulose microfibrils penetrate into the inter-microfibrillar spaces. In either case, there may be two types of GXs behavior, i.e., binding to cellulose microfibrils and filling the space between microfibrils.

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