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NOTE

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# Determination of the distribution and reaction of polysaccharides in wood cell walls by the isotope tracer technique VIII: Selective radiolabeling of xylan in mature cell walls of magnolia (*Magnolia kobus* DC.) and visualization of its distribution by microautoradiography*

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**Abstract** To radiolabel xylan in mature cell walls selectively, magnolia (*Magnolia kobus* DC.) was administered with *myo*-inositol-[2-$^3$H] and allowed to metabolize for 1 month. The radiolabeled xylem tissue was then submitted to sulfuric acid hydrolysis and nitrobenzene oxidation. A large amount of radioactivity was found mainly in xylose, although slight activities were detected in glucose and in vanillin and syringaldehyde. The labeled tissue was submitted to a preparation of holocellulose followed by treatment with 24% potassium hydroxide (KOH). Radioactivity was distributed mainly in the KOH-soluble part of the holocellulose. These results indicate that most radioactivity was incorporated into xylan in the cell walls. The distribution of the incorporated radioactivity in the xylem tissue was visualized by microautoradiography. Radioactivities were distributed in the xylem more than 400 μm from the cambium; and an inner layer of a secondary wall had formed at the labeled xylem. Consequently, selective radiolabeling of xylan was visualized in mature cell walls.

**Key words** Xylan · Selective radiolabeling · Microautoradiography · *Myo*-inositol · *Magnolia kobus* DC.

## Introduction

The distribution of noncellulosic polysaccharides in tree cell walls and their behavior during various reactions such as chemical analysis or pulping are still open to investigation. The radiotracer method is effective not only for detecting cell wall components in cell walls but also for tracing their topochemical reactions.[1–6] We have been developing methods for selective radiolabeling of polysaccharides in tree cell walls by employing suitable precursors radiolabeled at specific positions and by regulating the metabolism of the precursors with enzyme inhibitors.[4,7–9]

We have reported previously that xylan was radiolabeled almost selectively when *myo*-inositol-[2-$^3$H] was administered to a growing stem of magnolia followed by allowing the plant to metabolize for 24 h.[4] The distribution of the radioactivity in the labeled xylem tissue was visualized by microautoradiography. It was shown that radioactivity was incorporated into immature differentiating cell walls, and the deposition of xylan was most active during formation of the secondary wall.[4]

To investigate the topochemical reactions of cell wall polysaccharides by a radiotracer method, mature cell walls should be radiolabeled. Magnolia was allowed to grow for 5 months after administration of *myo*-inositol-[2-$^3$H].[5] The selectivity in xylan labeling was estimated by determining the radioactivities of the sulfuric acid hydrolysates and nitrobenzene oxidation products of the labeled xylem tissue. The chemical analyses showed that radioactivity was incorporated mainly into xylan. Some cells were newly formed during the growing period, and the radiolabeled cell walls were supposed to mature. Xylan could be radiolabeled selectively in mature cell walls.[5] The mature wood tissue in which xylan was radiolabeled selectively was treated with kraft pulping, and the dissolution and redeposition of xylan during the pulping were demonstrated clearly by a radiotracer method.[5]

The xylan labeling in the mature cell walls has been estimated by chemical analyses, but the distribution of the radioactivity in the xylem tissue has not yet been visualized.

In this study, to radiolabel xylan in mature cell walls magnolia was administered *myo*-inositol-[2-$^3$H] and then allowed to grow further for 1 month. The selectivity of xylan labeling was estimated by chemical analyses. The purpose of this work was to examine by microautoradiography whether radioactivity was incorporated into mature cell walls. The results were compared with those of an experiment that allowed the plants to metabolize for 24 h after administration of the precursor.

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## Materials and methods

### Plant material and administration of precursor

A 2-year-old shoot was obtained in June from a magnolia (*Magnolia kobus* DC.) grown on the campus of Nagoya University. A 10 cm long portion with leaves divided from the shoot and a 3-year-old magnolia grown in a pot were used respectively for short-term (24 h) and long-term (1 month) metabolism after the administration of radiolabeled precursor. A small V-shaped groove (2 × 4 mm) was made with a razor blade in the circumferential direction on both the cut shoot and the stem of the 3-year-old tree. A solution of *myo*-inositol-[2-$^3$H] (1.85 MBq) (ARC, St. Louis, MO, USA) was added dropwise to the groove. After the precursor solution was absorbed completely, each groove was covered with wax. The shoot was placed in a vial containing distilled water and allowed to metabolize for 24 h. The plant in the pot was allowed to grow further for about 1 month. After the metabolizing periods, a drop of 3% glutaraldehyde was added to the groove, and a small block of xylem tissue near the groove was cut and fixed again with 3% glutaraldehyde in a refrigerator. The block was dehydrated through a graded ethanol series by the conventional method and embedded in epoxy resin according to the method of Terashima et al.[10] Another portion of the newly formed xylem near the groove was milled to pass a 40 mesh and extracted with benzene-ethanol (2:1, v/v) and hot water, successively. The extractive free wood meal was used for chemical analyses.

### Sulfuric acid hydrolysis

The extractive free wood meal was treated with 72% sulfuric acid at 30°C for 1 h followed by dilution with distilled water to 3% and heating at 121°C for 1 h. The hydrolysate was neutralized with saturated barium hydroxide and centrifuged. The supernatant was concentrated to 2–3 ml and passed through short columns of anion-exchange resin (Dowex 1 × 8; acetate form) and cation-exchange resin (Dowex 50; $H^+$ form) successively. The eluate was evaporated to dryness, and the residue was dissolved in distilled water. An aliquot of the solution was submitted to analysis by high-performance liquid chromatography (HPLC) on a Shimadzu HPLC system equipped with a refractive index detector and a Unisil Q $NH_2$ column ($\phi$4.6 × 250 mm; GL Science) and 80% acetonitrile as an eluant. The fractions containing xylose and glucose were collected separately and added to Bray's cocktail. Their radioactivities were determined by a liquid scintillation counter (Aloka LSC-5100).

### Nitrobenzene oxidation

The wood meal, 2N sodium hydroxide, and nitrobenzene were heated at 165°C for 2.5 h with shaking in a stainless steel bomb. The reaction mixture was centrifuged, and the supernatant was extracted with ether. The aqueous layer was acidified and extracted again with ether. The ether fraction was evaporated to dryness, and the residue was dissolved in dioxane containing 3,5-dihydroxybenzoic acid as an internal standard. An aliquot of the solution was submitted to HPLC analysis according to the method of He and Terashima.[11] The fractions containing vanillin and syringaldehyde were collected separately for determination of their radioactivities by liquid scintillation counting.

### Fractionation of cell wall components

Extractive-free wood meal was treated with sodium chlorite in an acidic solution at 70°C to prepare holocellulose (Wise method). A part of the soluble portion was deposited on filter paper. The holocellulose was treated with 24% potassium hydroxide (KOH) at room temperature overnight under a nitrogen atmosphere. A part of the soluble portion also was deposited on filter paper, and the residual part was washed with distilled water until the washings became neutral. The filter papers and the residue were dried in a desiccator and then subjected to combustion by a sample oxidizer (Aloka ASC 113) to give $^3H_2O$. The radioactivities of the $^3H_2O$ were determined by liquid scintillation counting.

### Microautoradiography

Transverse sections (2 μm thick) were cut from the embedded xylem tissue on a Reichert Jung Supercut 2050 microtome equipped with a glass knife. The sections were mounted on glass slides and covered with Konica NR-M2 autoradiographic emulsion. The glass slides were stored in a refrigerator for 3–6 months. They were developed with Rendol and fixed with Renfix. The sections were stained with toluidine blue O, and photomicrographs were obtained with a Zeiss photomicroscope III. Semiquantitative determinations of the incorporated radioactivities were made by counting the number of silver grains on the microautoradiograms. A polarizing microscope (Olympus BX 50) was used for the observations of $S_1$ (outer layer of the secondary wall) and $S_3$ (inner layer of the secondary wall) formation.

## Results and discussion

### Estimation of the selectivity of radiolabeling

We have reported previously that radioactivity was incorporated mainly into xylan when *myo*-inositol-[2-$^3$H] was administered to a growing stem of magnolia followed by allowing the plant to metabolize for 24 h.[4] Xylan was radiolabeled almost selectively also in the xylem of magnolia grown for 5 months after administration of $^3$H-labeled *myo*-inositol.[5] In the present study, magnolia plants were allowed to metabolize for 24 h and 1 month after administration of $^3$H-*myo*-inositol.

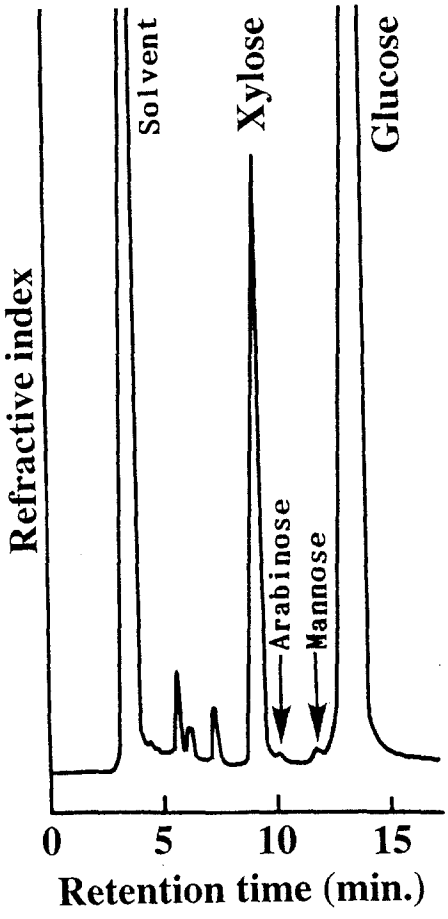


**Fig. 1.** High-performance liquid chromatogram of the sulfuric acid hydrolysate derived from magnolia xylem

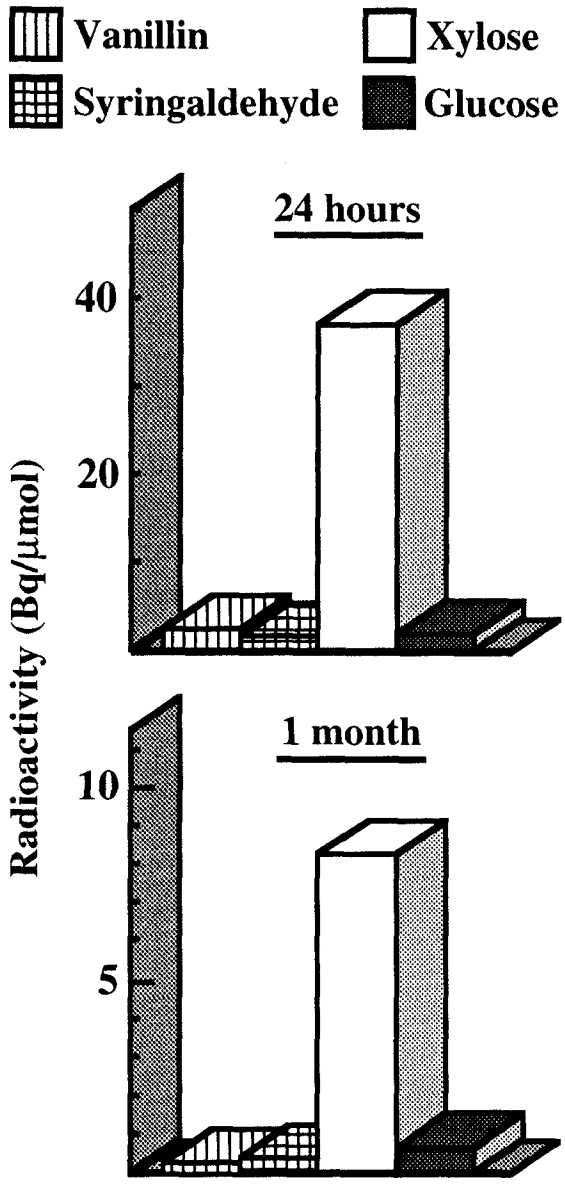


**Fig. 2.** Radioactivities of the nitrobenzene oxidation products and sulfuric acid hydrolysates derived from the magnolia xylem allowed to metabolize for 24 h and 1 month after the administration of *myo*-inositol-[2-$^3$H]

The radiolabeled wood tissue was submitted to chemical analyses to estimate the incorporation of radioactivity into cell wall components. Figure 1 shows the HPLC reading of the sulfuric acid hydrolysate derived from newly formed xylem of magnolia. Hydrolysis of magnolia xylem gave enough xylose and glucose to determine their radioactivities, and only trace amount of arabinose and mannose were detected in the hydrolysate by HPLC analysis. These results indicate that cellulose and xylan are the main polysaccharides constituting the cell wall of magnolia; hence the incorporation of radioactivity into cell wall polysaccharides in magnolia could be estimated by determining the radioactivities of glucose and xylose. Nitrobenzene oxidation of magnolia xylem gave enough vanillin and syringaldehyde for the radioassay, and the incorporation of radioactivity into lignin was estimated by determining the radioactivities of the oxidation products.

Figure 2 shows the specific radioactivities of vanillin, syringaldehyde, xylose, and glucose obtained from magnolia xylem administered *myo*-inositol-[2-$^3$H] and then grown for 24 h and 1 month. The incorporation of radioactivity from *myo*-inositol-[2-$^3$H] into the cell wall components coincided well with that reported previously.[4,5] For both metabolizing periods, a large amount of radioactivity was found mainly in xylose, and slight radioactivities were detected also in vanillin, syringaldehyde, and glucose, indicating that radioactivity was incorporated mainly into xylan, although slight activities were incorporated also into lignin and glucan.

To estimate the distribution of the radiolabel among cell-wall polymers, the labeled xylem tissue was submitted to the preparation of holocellulose followed by treatment with 24% KOH. The radioactivities of the soluble portion of the sodium chlorite acidic solution, the 24% KOH-soluble portion, and the insoluble portion of the holocellulose were then determined. HPLC analyses have shown that the hydrolysates of the 24% KOH-soluble portion of magnolia holocellulose consists of almost all xylose, and the insoluble portion consists of glucose (data not shown), indicating that xylan and cellulose fractionated into the soluble and in-

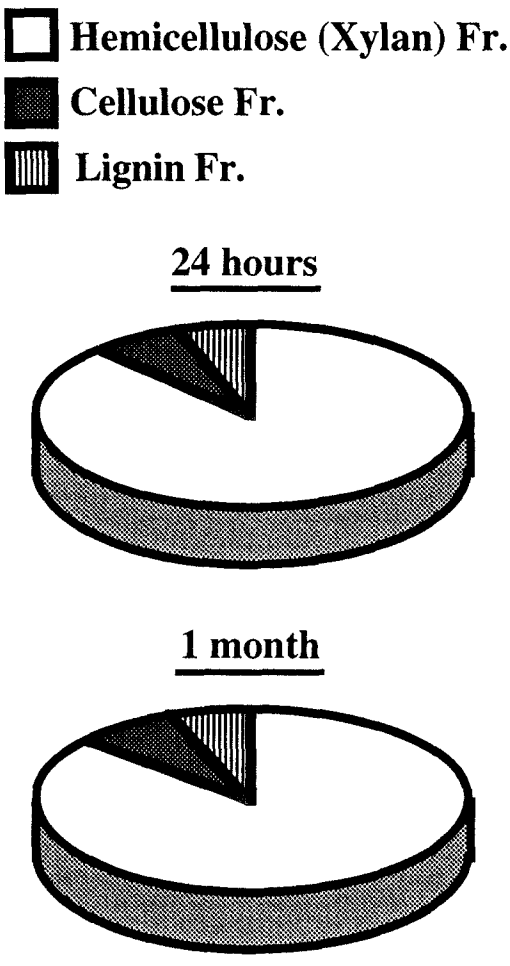


**Fig. 3.** Distribution of radioactivity among cell wall components in the magnolia xylem allowed to metabolized for 24h and 1 month after the administration of *myo*-inositol-[2-$^3$H]. *Hemicellulose (Xylan) Fr.*, 24% potassium hydroxide (KOH)-soluble part of holocellulose; *Cellulose Fr.*, 24% KOH-insoluble part of holocellulose; *Lignin Fr.*, sodium chlorite acidic solution-soluble part

soluble portions, respectively, by the 24% KOH treatment. Therefore, the 24% KOH-soluble and KOH-insoluble portions correspond to the hemicellulose (xylan) and cellulose fractions, respectively. The sodium chlorite acidic solution-soluble portion corresponds to the lignin fraction. Figure 3 shows the distribution of the radioactivity among the three fractions at 24h and 1 month. For both metabolizing periods more than 85% of the radioactivity was distributed in the 24% KOH-soluble part [i.e., the hemicellulose (xylan) fraction] and less than 15% of the activity in the other fractions (i.e., lignin and cellulose fractions). This result indicates that radioactivity was incorporated mainly into xylan as estimated by determining the radioactivities of nitrobenzene oxidation products and sulfuric acid hydrolysates (Fig. 2). It was demonstrated again that xylan was radiolabeled selectively in cell walls by feeding *myo*-inositol-[2-$^3$H] to magnolia.

Many researchers have reported that some polysaccharides (e.g., pectin and xyloglucan) were once deposited in cell walls and then depolymerized and remetabolized during the maturation of the cell walls.[12–15] If the depolymerization and remetabolism are applicable for xylan deposition, the selectivity of the labeling should lessen in the magnolia allowed to metabolize for a long period after administration of the precursor. In the present study, the decrease in selectivity did not occur, which indicates that xylan was not depolymerized or remetabolized and was deposited irreversibly during formation of the plant cell wall.

## Visualization of the distribution of xylan in the cell wall of magnolia by microautoradiography

We have previously visualized by microautoradiography the distribution of radioactivity in the differentiating xylem of magnolia administered *myo*-inositol-[2-$^3$H] and then allowed to metabolize for 24h. Those studies indicated that the deposition of xylan was most active during formation of the secondary wall.[4]

As shown by the previous microautoradiographic study, radioactivity was incorporated into differentiating immature cell walls when the plant was allowed to metabolize 24h after administration of the precursor. The immature materials are unsuitable for studies on the various reactions of polysaccharides in wood cell walls. To radiolabel xylan in mature cell walls, magnolia was administered *myo*-inositol-[2-$^3$H] and then allowed to grow further for 5 months.[5] Chemical analyses of the mature xylem tissue showed that xylan was radiolabeled almost selectively.[5] However, the distribution of the radioactivity in the xylem tissue has not yet been visualized.

Figures 4 and 5 show the microautoradiograms of the magnolia xylem administered *myo*-inositol-[2-$^3$H] and then allowed to metabolize for 24h and 1 month, respectively. Sliver grains were located on cell walls in both autoradiograms, and it was confirmed that the radioactivity was incorporated into cell walls and the administered $^3$H-*myo*-inositol was assimilated into the cell wall polysaccharide. Figure 6 shows the distribution of silver grains on the autoradiograms. The deposition and distribution of xylan was estimated semiquantitatively by counting the number of silver grains on the autoradiograms. In the case of metabolism for 24h after administration of the precursor, the incorporation of radioactivity coincided well with that reported previously.[4] Radioactivity was incorporated into the cell walls in differentiating xylem, and the incorporation was most significant at the stage following the start of $S_1$ formation, indicating that the deposition of xylan was most active during secondary wall formation, as shown in Figs. 4 and 6. In the microautoradiogram of the magnolia grown for 1 month after administration, silver grains were observed mainly on the cell walls in the xylem more than 400 μm from the cambium (Figs. 5, 6). The sparsely labeled cell walls between the cambium and the labeled cell walls are those formed during the 1-month growth period; and $S_3$ formation was observed at the labeled xylem. The selective radiolabeling of xylan in mature cell walls was well visualized by microautoradiography.

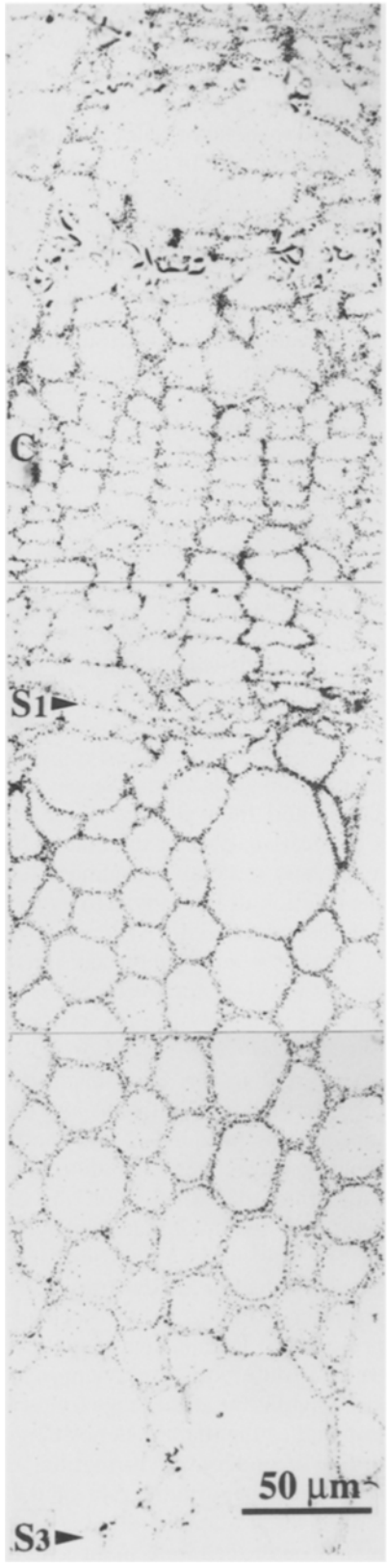


**Fig. 4.** Microautoradiogram of the differentiating xylem of magnolia administered with *myo*-inositol-[2-$^3$H] and allowed to metabolize for 24 h. *C*, cambium; $S_1$, start of formation of the outer layer of the secondary wall; $S_3$, start of formation of the inner layer of the secondary wall

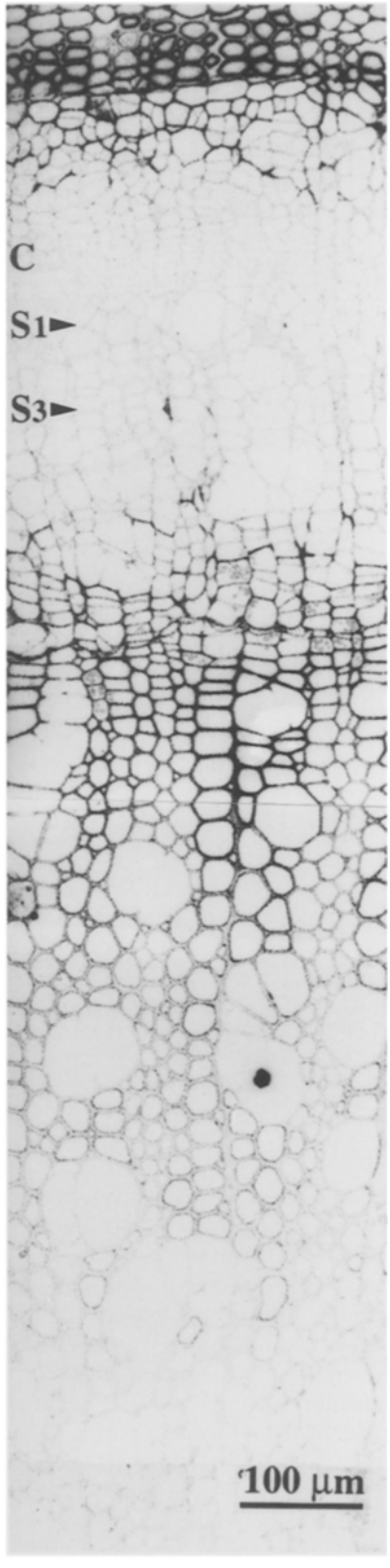


**Fig. 5.** Microautoradiogram of the magnolia xylem administered *myo*-inositol-[2-$^3$H] and allowed to metabolize for 1 month. Symbols are the same as in Fig. 4

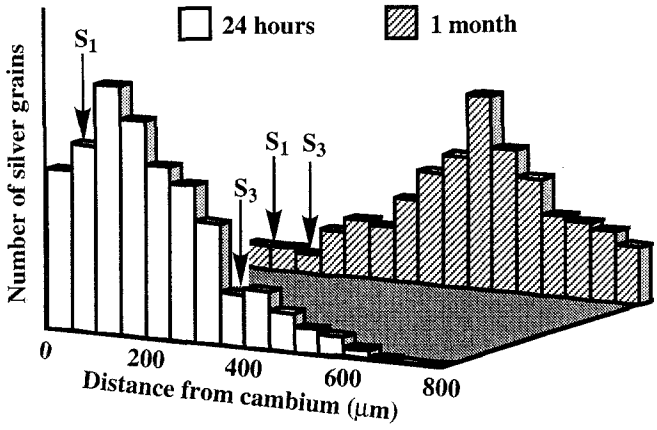


**Fig. 6.** Distribution of silver grains in the microautoradiograms of the magnolia xylem allowed to metabolized for 24h and 1 month after administration of *myo*-inositol-[2-$^3$H]. Symbols are the same as in Fig. 4

## Conclusion

Magnolia was administered *myo*-inositol-[2-$^3$H] and then allowed to grow for 1 month. Chemical analyses showed that radioactivity was incorporated mainly into xylan. Microautoradiography showed that mature cell walls were radiolabeled. The labeling method could be useful for studying the topochemistry of the cell wall polysaccharide during various reactions.

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