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Immunohistochemical localization of agatharesinol, a heartwood norlignan, in *Cryptomeria japonica*

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Abstract

Localization of a heartwood norlignan, agatharesinol, in Sugi (Japanese cedar, *Cryptomeria japonica* D. Don, Taxodiaceae) was investigated by immunohistochemistry. Immuno light microscopy showed that the contents of ray parenchyma cells were immunostained in heartwood but not in sapwood. The staining of the heartwood tissue was competitively inhibited by agatharesinol but not by other Sugi heartwood extractives, and was, furthermore, markedly reduced by pre-extraction of the tissue with MeOH. These results indicated that the staining can be ascribed to the immunolabeling of agatharesinol in situ. The accumulations over the inner surface of some tracheid cell walls adjacent to the ray parenchyma cells were also immunolabeled, while the contents in axial parenchyma cells were not. In conclusion, agatharesinol was localized in the ray parenchyma cells in Sugi heartwood, and differences between the chemical structure of the contents of ray and axial parenchyma cells were also suggested.

Keywords: *Cryptomeria japonica*; Taxodiaceae; Sugi; Immunohistochemistry; Heartwood extractives; Norlignan; Agatharesinol; Ray parenchyma cell

1. Introduction

Most tree species have a dark colored zone called heartwood in the central parts of their trunks. Heartwood is distinguished from the surrounding pale zone called sapwood by some features such as, in addition to the color, larger amounts of peculiar extractives, lower moisture content and death of parenchyma cells.

The formation of heartwood extractives has been studied from both biochemical and cytological perspectives. In cytological studies, reagents that react with chemically unsaturated structures, such as potassium permanganate or osmium tetraoxide, and UV-visible microscopic spectrometry, are used to examine heartwood extractives. Nobuchi and Harada (1985) reported that in the transition zone (Hillis, 1987), the inner intermediate wood between sapwood and heartwood, of Sugi, cytoplasmic matrices in ray parenchyma cells and accumulations over the inner surfaces of tracheid cell walls adjacent to ray parenchyma cells were strongly stained with osmium tetraoxide. This suggests that the heartwood extractives were biosynthesized in the ray parenchyma cells and then exuded into adjacent tracheary elements where they accumulate. Kuroda and Shimaji (1983) reported that in Sugi the contents of ray and axial parenchyma cells both had absorption in the UV range, but differed in absorption in the visible range, indicating that these two types of parenchyma cells produce different kinds of phenolic substances. Many papers reported the participation of parenchyma cells in the formation of heartwood extractives (Wardrop and Cronshaw, 1962; Fengel, 1970; Parameswaran and Bauch, 1975; Kwon et al., 2001). However, determination of the precise chemical structure of these observed substances was not possible, and dye-reagents or microscopic spectrometry provided no

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definite proof that those substances were heartwood extractives.

In immunological studies, a particular compound can be surveyed and characterized according to the selectivity of an antigen–antibody affinity. By applying immunolabeling to microscopic sections of plant tissues, various kinds of plant polymers have been localized in situ, and their formation was examined (Joseleau and Ruel, 1997; Nakashima et al., 1997; Awano et al., 1998).

Immunolabeling would be useful for the investigation of heartwood extractives. However, extractives are not ordinarily antigenic due to their low M_r. In order to cope with this problem, a hapten-carrier conjugate (artificial antigen) was synthesized in which a Sugi heartwood norlignan, namely agatharesinol, was covalently bound to bovine serum albumin via an interposing *p*-aminohippuric acid. By immunizing a rabbit against this artificial antigen, an antiserum was obtained that reacted most strongly with agatharesinol and recognized norlignans almost selectively among Sugi heartwood extractives (Nagasaki et al., 2001).

In this paper, the antiserum was applied to Sugi tissue sections to investigate the localization of agatharesinol. In situ immunolabeling of agatharesinol was verified by immunohistochemical control experiments. The site of agatharesinol, biosynthesis, transfer and accumulation are discussed from a histochemical viewpoint.

2. Results and discussion

Immunolabeling is one of the most powerful methods used to visualize the localization of a particular molecule in situ. In order to study localization of agatharesinol by immunohistochemistry, an anti-agatharesinol antiserum prepared previously (Nagasaki et al., 2001) was applied to microscopic sections of Sugi tissue. With the staining procedure using an alkaline phosphatase (AP) labeled secondary antibody and a 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate system (see Experimental), the immunostaining produces a blue color. The contents of ray parenchyma cells were well immunostained, and the lumen-sides of the cell walls were also stained in the heartwood sections (Fig. 1a and b). No immunostaining was found in the heartwood sections treated with the pre-immune serum (Fig. 1c and d). These results indicated that the staining was attributed to the antigen–antibody reaction on the tissue sections.

The contents of the ray parenchyma cells were not immunostained in sapwood, although the staining was seen on the cell walls (Fig. 1e and f). The staining in the ray parenchyma cells was remarkably reduced in the heartwood tissue sections pre-extracted with MeOH, although the staining was still seen on the cell walls (Fig. 1g). The immunostained substances in the ray parenchyma cells are, therefore, peculiar to the heartwood and extractable with MeOH. This fits the definition of heartwood extractives.

As characterized previously by competitive inhibition-ELISA (enzyme-linked immunosorbent assay) using Sugi heartwood extracts and several phenylpropanoids as competitors, our antiserum recognizes norlignans preferentially, with the highest reactivity against agatharesinol among Sugi heartwood extractives (Nagasaki et al., 2001). In addition, the staining was never found in heartwood tissue sections treated with antiserum that was pre-incubated with agatharesinol (Fig. 1h), while ferruginol, another major heartwood extractive of Sugi classified as diterpene (Nobuchi et al., 1985), did not inhibit the staining (Fig. 1i). The inhibition by agatharesinol is due to the fact that anti-agatharesinol antibodies had already reacted with agatharesinol during the preincubation, resulting in the loss of the antibodies available for the following reaction on the tissue sections. Therefore, the immunostaining of the heartwood tissue appears to be caused by the antigen–antibody reaction, where the anti-agatharesinol antibody is participating. These results suggest that the staining in the ray parenchyma cells was ascribed to the immunolabeling of agatharesinol in situ.

To support the suggestion above, radial changes in both the content of agatharesinol and the density of labeling in the ray parenchyma cells were compared (Fig. 2). Both the content of agatharesinol and the density of labeling had their lowest values in sapwood, and began to increase from the transition zone to the heartwood. They had maximum values in the outer heartwood, and then decreased in the inner part of the heartwood. Thus, the radial changes in the staining in the ray parenchyma cells showed a similar tendency with that of the content of agatharesinol. These results support the immunolabeling of agatharesinol in the ray parenchyma cells. The localization of agatharesinol in the ray parenchyma cells agrees with previous reports where the ray parenchyma cells were proposed as possible sites for the formation of heartwood extractives (Wardrop and Cronshaw, 1962; Parameswaran and Bauch, 1975; Nobuchi and Harada, 1985; Gang et al., 1998).

The staining on the lumen-side of the cell walls is not ascribed to the labeling of the so-called heartwood norlignan because stainable substances which are not extractable with MeOH also exist in sapwood. Two explanations could answer the question, "What is the staining on the cell walls ascribed to?" The first possibility is that the antiserum reacted with norlignans in the cell walls that were not extractable under these conditions. Previous studies suggested that norlignans were bound to lignin in the cell walls, resulting in the difficulty of extracting them (Kai and Teratani, 1977).

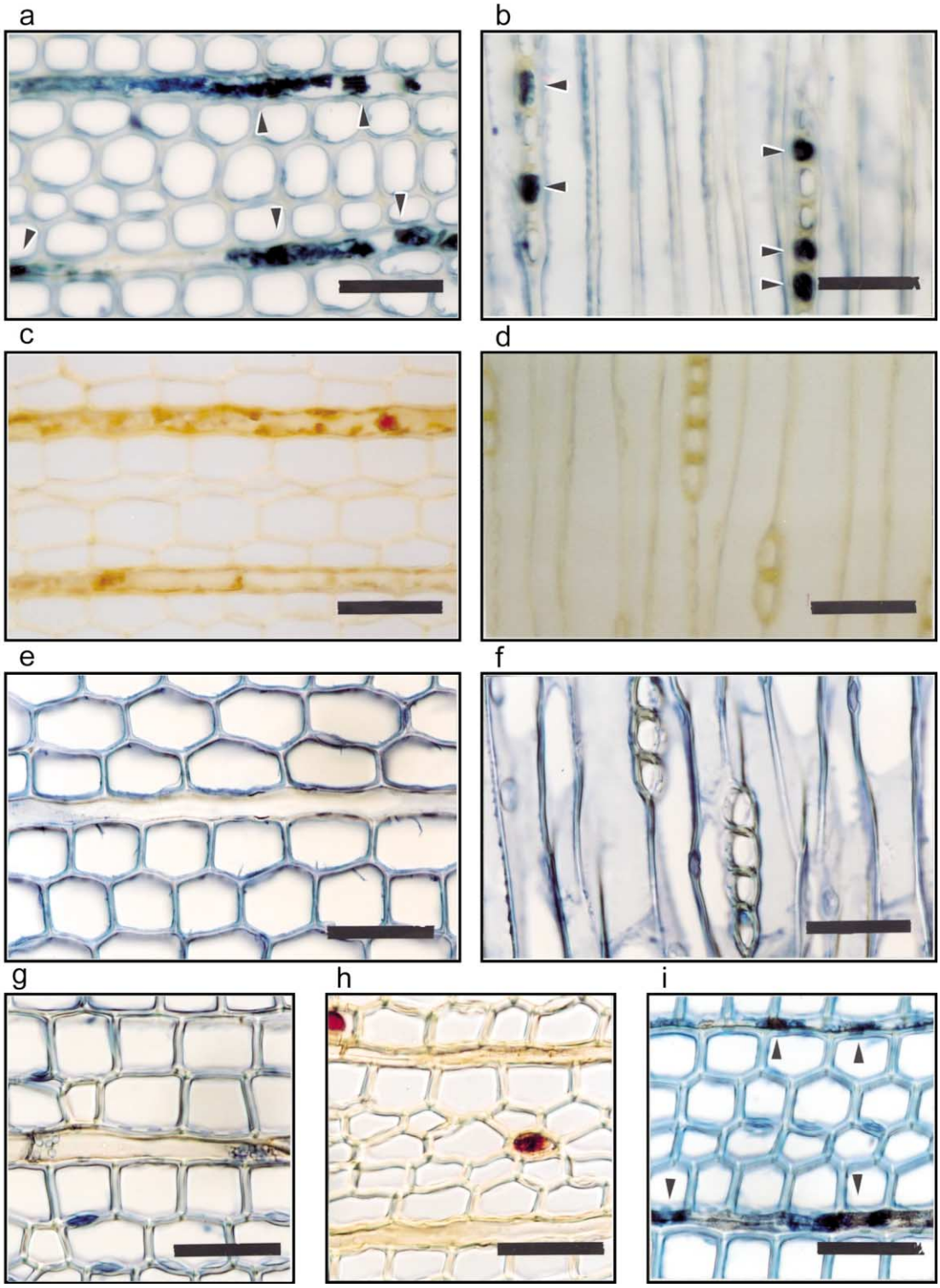

Fig. 1. Immuno light microscopic photographs of Sugi tissues. (a) Cross-section of heartwood tissue treated with anti-agatharesinol antiserum. (b) Tangential section of heartwood tissue treated with anti-agatharesinol antiserum. (c) Cross-section of heartwood tissue treated with pre-immune serum. (d) Tangential section of heartwood tissue treated with pre-immune serum. (e) Cross-section of sapwood tissue treated with anti-agatharesinol antiserum. (f) Tangential section of sapwood tissue treated with anti-agatharesinol antiserum. (g) Cross section of MeOH-pre-extracted heartwood tissue treated with anti-agatharesinol antiserum. (h) Cross-section of heartwood tissue treated with anti-agatharesinol antiserum pre-incubated with agatharesinol. (i) Cross-section of heartwood tissue treated with anti-agatharesinol antiserum preincubated with ferruginol. Bar: 50 μm. The contents of ray parenchyma cells of the heartwood were stained by anti-agatharesinol antiserum (arrow head).

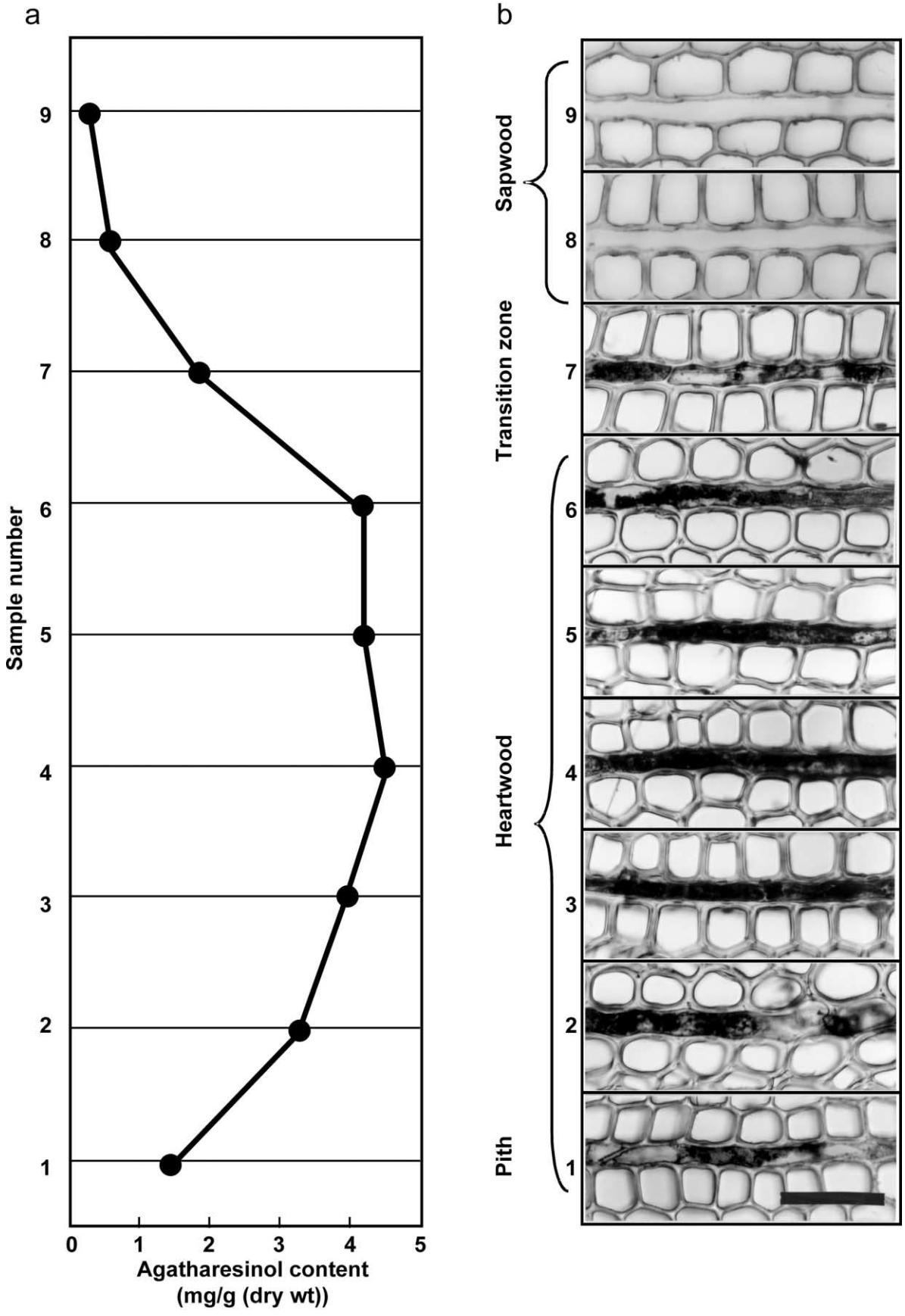

Fig. 2. Radial changes of the content of agatharesinol and the density of immunostaining. Bar: 50 μm.

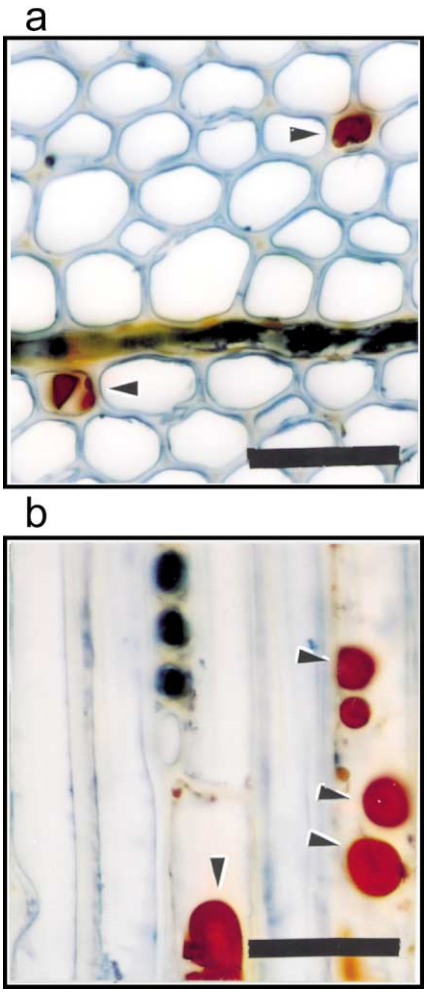

Fig. 3. Immuno light microscopic photographs of Sugi heartwood sections showing brown colored materials (arrow head) in axial parenchyma cells. (a) Cross section; (b) tangential section. Bar: 50 μm.

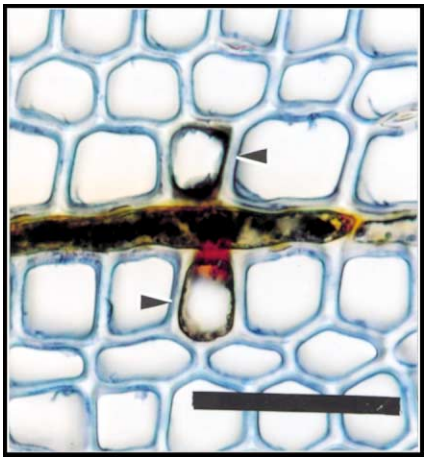

Fig. 4. Immuno light microscopic photograph of Sugi heartwood section showing accumulations in tracheid cells adjacent to ray parenchyma cell (arrow head). Bar: 50 μm.

Alternatively, the lumen-side of the cell walls may be coated with some lignans (Hillis, 1987). The second possible explanation is that the antiserum reacted with lignin in the cell walls since both agatharesinol and lignin have phenylpropanoid structural units. Although our antiserum has much lower reactivity with a lignin model compound, namely guaiacylglycerol-β-guaiacyl ether, than with agatharesinol as evaluated previously by competitive inhibition-ELISA (Nagasaki et al., 2001), a much more abundant amount of lignin than that of agatharesinol might compensate for the lower reactivity of the antiserum with lignin. However, this possibility could be weakened by the fact that the middle lamella, where lignin content is much higher than the secondary wall, is less immunostained. Another possible explanation is that some other constituent of the cell walls, which was not identified in this study, reacted with the antiserum.

No labeling was seen on the brown colored materials, so-called oil droplets (Nobuchi and Harada, 1985), in axial parenchyma cells (Fig. 3), although they could be easily assumed to be heartwood extractives due to their color and exclusive existence in the heartwood. Using UV-visible microscopic spectrometry, Miyamoto et al. (1989) reported that these materials had absorption in the UV range, indicating that they were heartwood phenolics. Therefore, it was suggested that ray and axial parenchyma cells could biosynthesize and accumulate different kinds of heartwood extractives. It has been reported that both axial and ray parenchyma cells are involved in heartwood formation (Kwon et al., 2001), and in Sugi these two types of parenchyma cells participate in heartwood formation with different functions (Kuroda and Shimaji, 1983; Nobuchi and Harada, 1985).

Some tracheid cells adjacent to the ray parenchyma cells contained immunolabeled accumulations over the inner surface of their cell walls (Fig. 4). This result is in agreement with the previous reports that heartwood extractives biosynthesized in the ray parenchyma cells were released from these cells and then infused into neighboring tracheid cells (Nobuchi and Harada, 1985; Gang et al., 1998).

3. Conclusions

Agatharesinol was localized in the ray parenchyma cells in Sugi heartwood, and its transfer from the ray parenchyma cells to the tracheid cells, as well as the accumulation over the inner surface of the cell walls were demonstrated by the immunohistochemical method. It was suggested that ray and axial parenchyma cells could biosynthesize and accumulate different kinds of heartwood extractives. To our knowledge, this is the first report of localization of a particular heartwood extractive.

4. Experimental

4.1. General experimental procedures

Microscopic sections were observed under an Olympus BX50 microscope equipped with an Olympus PM-30 camera. Microphotographs were taken on FUJICOLOR SUPERIA 100 film. GC was performed using a G L Sciences gas chromatograph (GC 353) equipped with a TC-1 capillary column (60 m×0.25 mm i.d.) and a FID. Anti-rabbit IgG alkaline phosphatase (AP) conjugate and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate system for immunolabeling were purchased from SIGMA.

4.2. Plant material

Sugi (*Cryptomeria japonica*) grown at Nagoya University (Chikusa, Nagoya, Japan) was used in this study. Xylem cores that include bark to pith, were taken with an increment borer (7 mm diameter) at a point 30 cm above the ground, and were fractionated for each 1.5 cm from the pith to the inner sapwood. The materials were immediately frozen in liquid nitrogen and stored in a freezer at –20 °C until use.

4.3. Immunolabeling

All the procedures described below were performed at room temp. Microscopic tissue sections (20 μm thick) were obtained from the xylem tissues using a sliding microtome. The sections were washed with phosphate buffered saline (PBS, pH 7.4), and then fixed with 3% glutaraldehyde in 0.05 M phosphate buffer (pH 6.98) for 30 min. After washing with PBST [0.1% (v/v) Tween 20 in PBS] three times, the sections were treated with PBST-milk [3% (w/v) skim milk in PBST] for 30 min to avoid nonspecific antibody binding. After washing with PBST three times, the anti-agatharesinol antiserum (primary antibody) diluted 1:200 in PBST was applied to the sections. After 2 h, the sections were washed thoroughly with PBST, and then anti-rabbit IgG AP conjugate (secondary antibody) diluted 1:300 in PBST was applied to the sections. After 1 h, the sections were washed thoroughly with PBST, and then rinsed with Tris buffered saline (100 mM Tris–HCl, pH 7.5, 150 mM NaCl) containing 0.1% (v/v) Tween 20. Color development was performed using a BCIP/NBT liquid substrate system according to the instruction manual (Sigma).

4.4. Immunolabeling controls

The preimmune serum and the antiserum preincubated with agatharesinol (10^{-2} M) (competitive inhibition) were employed as primary antibodies for immunolabeling under the same conditions and diluted as described in Section 4.3. In addition, heartwood tissue sections pre-extracted with MeOH were used for immunolabeling performed under the same procedure described in Section 4.3.

4.5. Quantitative analysis of agatharesinol

The fractionated tissues were extracted with MeOH thoroughly. The extracts were trimethysilylated and analyzed by GC. The oven temperature was held at 280, and N_2 at 2.0 ml min^{-1} was used as a mobile phase. The amount of agatharesinol was calculated from the GC peak areas, using an authentic sample (Takahashi, 1981) for calibration.

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