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ORIGINAL ARTICLE

Distribution of lignin and lignin precursors in differentiating xylem of Japanese cypress and poplar

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Abstract Lignin is an integral component of the cell wall of vascular plants. The mechanism of supply of lignin precursors from the cytosol into the cell wall of differentiating xylem has not yet been elucidated. The present study showed that a certain amount of coniferyl alcohol glucoside (coniferin) occurred in the differentiating xylem of Japanese cypress (*Chamaecyparis obtusa*), as previously reported in gymnosperms. Coniferin content peaked in the early stages of secondary wall formation and decreased during lignification. In contrast to gymnosperms, coniferin content was limited in the differentiating xylem of poplar (*Populus sieboldii* × *Populus grandidentata*). Moreover, coniferyl alcohol was not detected in all specimens. In the differentiating xylem of poplar, a higher amount of sinapyl alcohol occurred than glucoside (syringin). However, the phloem contained syringin and not sinapyl alcohol. The sinapyl alcohol content in the xylem peaked in the cells with ceasing cell wall formation, and decreased gradually towards the boundary of the annual ring, where the lignin content kept increasing. Sinapyl alcohol in the differentiating xylem of poplar may be used for the lignification of the xylem.

Keywords Coniferin · Sinapyl alcohol · Syringin · Coniferyl alcohol · Thioglycolic acid lignin

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Introduction

Lignin, an essential part of the vascular plant cell wall, is formed by polymerization of its precursors, which are classified into three types, namely, *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units. Lignin distribution and the composition of H, G, and S vary among plant species, tissues, cell types, and cell wall layers [1]. Gymnosperms and angiosperms contain G lignin, and G and S lignins, respectively, as the major lignin. Among the various precursors of lignin in the phenylpropanoid pathway, monolignols (*p*-coumaryl alcohol, coniferyl alcohol (CAlc), and sinapyl alcohol (SAlc)) are polymerized by the oxidative dehydrogenation process. Enzymes involved in biosynthesis and oxidation of monolignols have been investigated in detail [2–7].

Another important process in lignin biosynthesis is the transportation of lignin precursors from the cytosol to the cell wall. In the rosette leaves of *Arabidopsis* (*Arabidopsis thaliana*), ABC-like transporters mediated the translocation of monolignol and monolignol glucosides [8]. An *Arabidopsis* ABC transporter involved in the transport of *p*-coumaryl alcohol has been characterized by Alejandro et al. [9]. The ABC transporter is expressed in the vascular bundle of stems and has a weaker activity for SAlc transportation than for CAlc. Recently, Tsuyama et al. [10] demonstrated that the transport of coniferyl alcohol glucoside (coniferin) is a major translocation mechanism in differentiating xylem undergoing vigorous lignification in angiosperms and gymnosperms. The process is dependent on a proton gradient created by V-ATPase.

Coniferin is found in the cambial sap of many tree species [4], but is absent in the differentiating xylem cells in the S_3 layer formation stage [10, 11]. The distribution of coniferin in the radial direction was determined using

Pinus thunbergii [12] and *Ginkgo biloba* [13]. An immunohistochemical study of coniferin β-glucosidase in the differentiating xylem of lodgepole pine (*Pinus contorta*) confirmed that the enzyme is localized in secondary walls of tracheids [14]. Coniferin could be exported to the cell wall and hydrolyzed by coniferin β-glucosidase, thereby releasing coniferyl alcohol for lignification in gymnosperms [14–17]. Coniferin could be supplied after cell death by passive diffusion, or during cell activity by some transport mechanism as proposed by Tsuyama et al. [10].

On the other hand, little information on the content of S lignin precursors in the differentiating xylem has been reported. Terazawa et al. [18] reported that few species contain syringin in living tissues. The radial distribution of syringin has been investigated using *Magnolia kobus* [19]. Its distribution and seasonal variation suggested that syringin does not serve as a lignin precursor. Although the occurrence of SAlc has been reported in *Populus nigra* [18], *Populus tremuloides* [20], and *Arabidopsis* [21, 22], its distribution in the differentiating xylem has not been elucidated.

In this study, the distributions of lignin and lignin precursors (coniferin, syringin, CAlc, and SAlc) have been shown in Japanese cypress (*Chamaecyparis obtusa*) and poplar (*Populus sieboldii* × *P. grandidentata*). Coniferin was found in the differentiating xylem of Japanese cypress while sinapyl alcohol was found in poplar. These results would provide insight on the mechanism of lignification in differentiating xylem.

Materials and methods

Chemicals

Chemicals used in this study were purchased from Nakalai Tesque (Kyoto, Japan) and Tokyo Chemical Industry (Tokyo, Japan). Coniferin and syringin were provided by Professor Emeritus N. Terashima of Nagoya University, Japan.

Extraction of lignin precursors

Blocks [ca. 10(T) × 15(R) × 25(L) mm] consisting of outer bark tissues and a few annual rings were collected at breast height from a 40-year-old Japanese cypress (*Chamaecyparis obtusa*) and poplar (*Populus sieboldii* × *P. grandidentata*) tree in the middle of June and early July, respectively. The obtained blocks were frozen in liquid N_2 until the experiments were initiated.

The frozen specimen block containing bark, cambium, and differentiating and mature xylem was fixed on the freezing stage of a sliding microtome, and 100-μm-thick serial tangential sections were obtained from the bark to the matured xylem. In case of Japanese cypress, each section was put into a 1.5-mL vial. In the case of poplar, three sections cut from three blocks were grouped together and put into a vial (Fig. 1a). The vials were weighed before and after collecting the sections to calculate the weight of fresh sections. The mode of extraction and analysis are described in Fig. 1b. Approximately 1.2 mL of MeOH was added immediately after initial weighing and extracted overnight. The extract was collected and lignin precursors were determined. The sections were treated with acetone overnight post extraction with MeOH. After discarding the supernatant, the sections were dried and then weighed. The estimated water content was calculated by the following formula:

$$C_w = \frac{W_f - W_d}{W_d} \times 100 \quad (1)$$

where C_w is estimated water content (%), W_f is weight of fresh section, and W_d is weight of dry section after extraction. The lignin content of each whole section of Japanese cypress was quantified. The three sections of poplar in each tube were cut and mixed where one part of the sample was used to quantify lignin, and another was used for characterization.

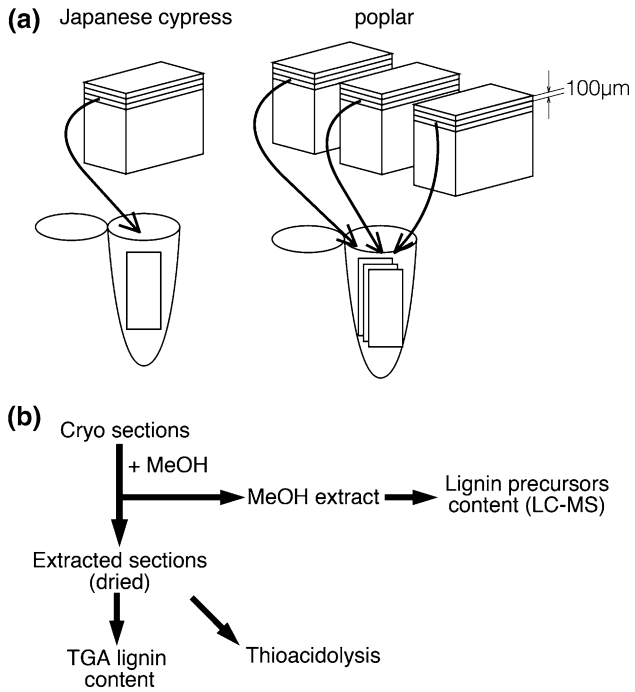


Fig. 1 Schematic illustrations of the extraction from tangential sections. **a** One section of Japanese cypress or three sections from three blocks of poplar were used for each extraction of monolignols and monolignol glucosides. Sections were sequentially numbered from the ones that could not maintain its shape (section number 0 in Japanese cypress, section number 0 and 1 in poplar). **b** Schematic representation of extraction and analyses

To confirm the existence of SAlc in poplar xylem, a 7-year-old poplar (*P. carolinensis* × *P. nigra*) was also used, in addition to the 40-year-old poplar (*P. sieboldii* × *P. grandidentata*). After de-barking the logs, the differentiating xylem was scraped off with razor blades. Differentiating xylem tissues were immediately frozen in liquid nitrogen and stored at −80 °C until required.

The scraped xylem of poplar (ca. 2 g) was extracted with 2 mL MeOH on ice. The extract was collected and content of lignin precursors was determined by LC–MS.

Quantification of lignin

The lignin content of the section was determined by the thioglycolic acid (TGA) method [23]. Briefly, samples were reacted with 1 mL 3 N HCl and 0.1 mL TGA in 2-mL tubes with screw caps at 80 °C for 3 h. After centrifugation at 16,000×*g* for 10 min and washing of the pellet with 1 mL distilled water, it was re-suspended in 1 mL 1 N NaOH and shaken vertically at 120 rpm overnight. After centrifuging in the above condition, 1 mL of supernatant was collected in a 1.5-mL tube and 0.2 mL of concentrated HCl was added. TGA lignin was precipitated by keeping the tube at 4 °C for 4 h. After centrifuging in the above condition, the pellet was dissolved in 1 mL of 1 N NaOH. Absorbance of the dissolved solution at 280 nm was measured to determine the amount of TGA lignin. The absorbance values were plotted to prepare the standard curves of the TGA lignin from pine (*Pinus densiflora*) (2–20 mg wood) and poplar (*Populus sieboldii* × *P. grandidentata*) (1–20 mg wood).

Characterization of lignin

Extractive-free samples were used to determine the monolignol composition of lignin by microscale thioacidolysis method [24], with tetracosane (6 µL of 5 mg/mL in CH_2Cl_2) as the internal standard. Gas chromatography (GC) analyses were performed on a GC-14A (Shimadzu, Kyoto, Japan) equipped with an HP-1 column (30 m × 0.25 mm i.d.; Agilent Technologies, CA, USA). The sample volume injected was 1.5 µL. The injector and detector temperatures were 250 and 280 °C, respectively. The initial oven temperature was 180 °C (held for 1 min) and then ramped at a rate of 2 °C per min to 280 °C and held for 5 min.

LC–MS analysis

Methanol extract was centrifuged at 20,000×*g* for 20 min, after which the supernatant was dried and redissolved in 0.3 mL 50 % MeOH for LC–MS. Samples were injected by means of a SIL-20A autosampler (Shimadzu, Kyoto, Japan) onto a reversed-phase ODS-3 column (150 × 2.4 mm i.d., 4 µm; GL Sciences, Tokyo, Japan). A gradient separation (LC-20AD HPLC pump; Shimadzu) was run using the following conditions: flow of 0.2 mL per min, column temperature of 30 °C, 30 % (v/v) MeOH (time: 0 min), 50 % (v/v) MeOH (time: 20–40 min), and 80 % (v/v) MeOH (time: 50–90 min). Detection was monitored with an SPD-20A detector (Shimadzu) at 265 nm, and mass determination was achieved by ESI in positive ion polarity (LCMS-2020; Shimadzu). The MS conditions were as follows: DL temperature of 250 °C, heat block temperature of 200 °C, nebulizer gas flow set at 1.5 L/min, and drying gas flow set at 15.0 mL/min. Lignin precursors were identified based on the data of authentic compounds. The retention time and mass of each authentic compound used for identification were as follows: coniferin (17.5 min, $m/z = 365$), syringin (19.3 min, $m/z = 395$), CAlc (29.5 min, $m/z = 381$), SAlc (29.0 min, $m/z = 263$).

Histochemical analyses

The differentiating xylem of Japanese cypress and poplar were fixed in 2.5 % glutaraldehyde and 0.07 M phosphate buffer (pH 7.2), and 40-µm-thick transverse sections were prepared. For Wiesner reaction, the sections were incubated with 2 % (w/v) phloroglucinol in 95 % (v/v) ethanol for 1 min, followed by addition of 6 N HCl. For Mäule reaction, sections were treated with 1 % (w/v) $KMnO_4$ solution for 5 min. After a brief wash in distilled water, the sections were incubated in 2 N HCl for 5 min, and then washed with distilled water. Sections were mounted onto glass slides and treated with concentrated NH_3 solution.

Results and discussion

The coniferin distribution in conifers has been reported in previous papers [11–13]. However, there are little data on the relationship between the amount of lignin precursors and lignin content because of the difficulty in accurately quantifying lignin content in small samples. The TGA method was suitable for determining the lignin contents in the samples that contained large amounts of polyphenol and proteins [25], as well as in the smaller samples [26]. Therefore, we adopted a small-scale TGA method [23] to quantify lignin in a small section obtained from differentiating xylem and phloem, and achieved to determine lignin precursors and lignin content in the identical section.

Lignin and lignin precursor distribution in Japanese cypress

Approximately 100-µm-thick serial tangential cryosections were cut between phloem and mature xylem of Japanese

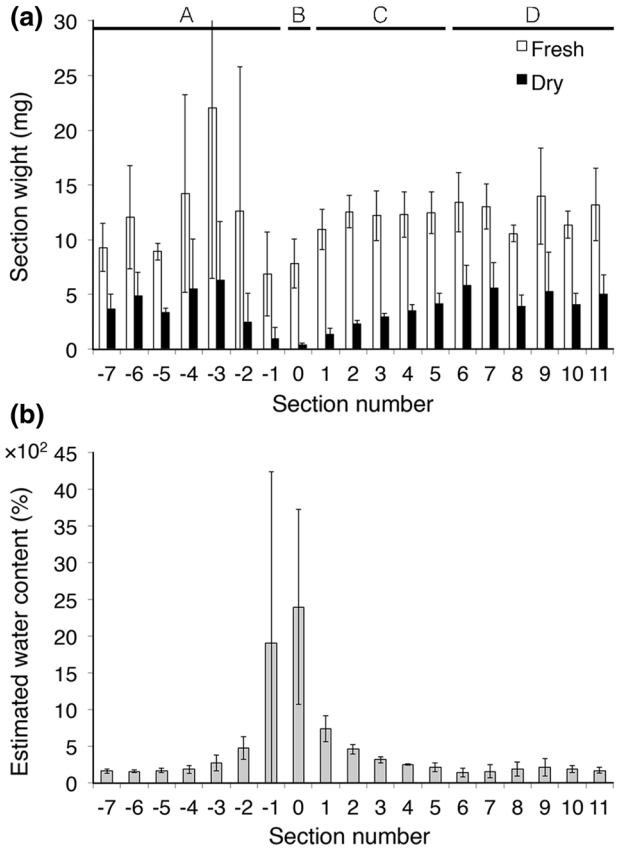


Fig. 2 **a** Fresh and dry weight of tangential sections obtained from blocks of Japanese cypress wood. **b** Estimated water content in tangential sections obtained from blocks of Japanese cypress wood. *Region A* phloem, *B* cambium and cell expansion zone, *C* differentiating xylem, *D* xylem formed in the previous year. Data are represented as means of three extractions ± SD

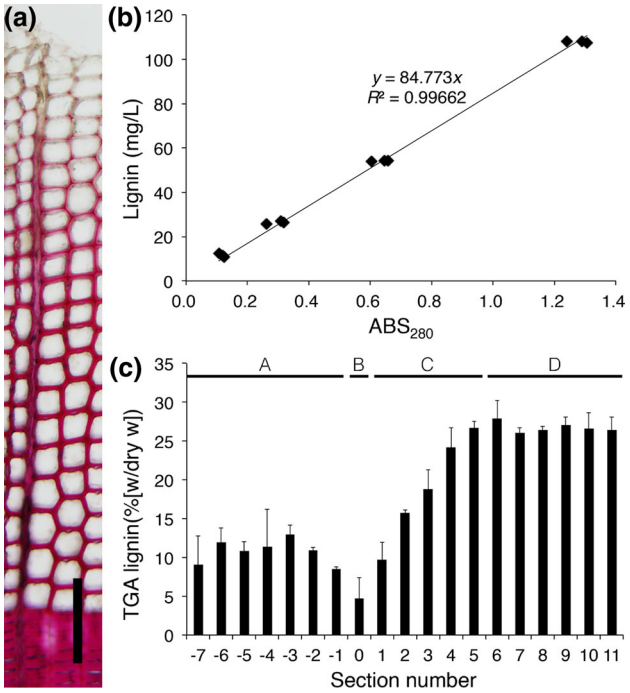


Fig. 3 Distribution of lignin in the radial direction of Japanese cypress. **a** Cross-section of Japanese cypress differentiating xylem subjected to Wiesner reaction; *bar* = 100 µm. **b** Calibration curve for thioglycolic acid lignin derived from pinewood. Lignin concentration was calculated from Klason (acid insoluble) lignin content in mature xylem of pine. **c** Distribution of lignin in the radial direction of Japanese cypress determined by thioglycolic acid method. *Region A* phloem, *B* cambium and cell expansion zone, *C* differentiating xylem, *D* xylem formed in the previous year. Data are represented as means of three extractions ± SD

cypress blocks. A section (section 0) that could not maintain its shape was also contained in each block, which was used to sequence the data obtained from each section of the different blocks. The dry weight of the sections gradually decreased towards section 0 and then gradually increased towards section 6, where it remained constant (Fig. 2a). Estimated water content was maximal in section 0, decreased as xylem formation proceeded, and remained constant around section 5 (Fig. 2b). Cross-sections subjected to the Wiesner staining showed the manner of lignification. The annual ring was within the sections at a distance of 500–700 µm from the de-barking position (Fig. 3a); the boundary of the annual ring was around section 6. The above results indicated that section 0 contained the cells in cambium and cell expansion zone, and that cell wall formation in xylem ceased around section 5.

To determine the lignin content of each section, we prepared the standard curve of TGA lignin for gymnosperm samples. The standard curve obtained from pinewood using Klason lignin as standard lignin content showed good linearity (Fig. 3b). Lignin content determined by the TGA method was minimal in section 0; lignin content increased as xylem formation proceeded, and was almost constant around section 5 (Fig. 3c).

Coniferin content was determined by LC–MS. Figure 4a represents the mass spectra at the retention time for coniferin. We confirmed that the preeminently accountable ion in Japanese cypress extract was $m/z = 365$ at the retention time of coniferin, which was identical to the authentic coniferin; thus the substance at the retention time could be coniferin. The UV spectrum was used to determine the coniferin content because of the better-fitted standard curve than that derived from the mass spectrum. Coniferin content was increased towards section 1 followed by a decrease, and maintained consistent around section 7 (Fig. 4b). Syringin, CAlc, and SAlc were not detected in the specimens.

Coniferin content was seen at the maximum in the early stage of secondary wall formation (Fig. 4), as reported by Fukushima et al. [12] and Aoki et al. [13]. However, the coniferin content decreased when the cell wall formation proceeded (Figs. 2 and 4), indicating that coniferin

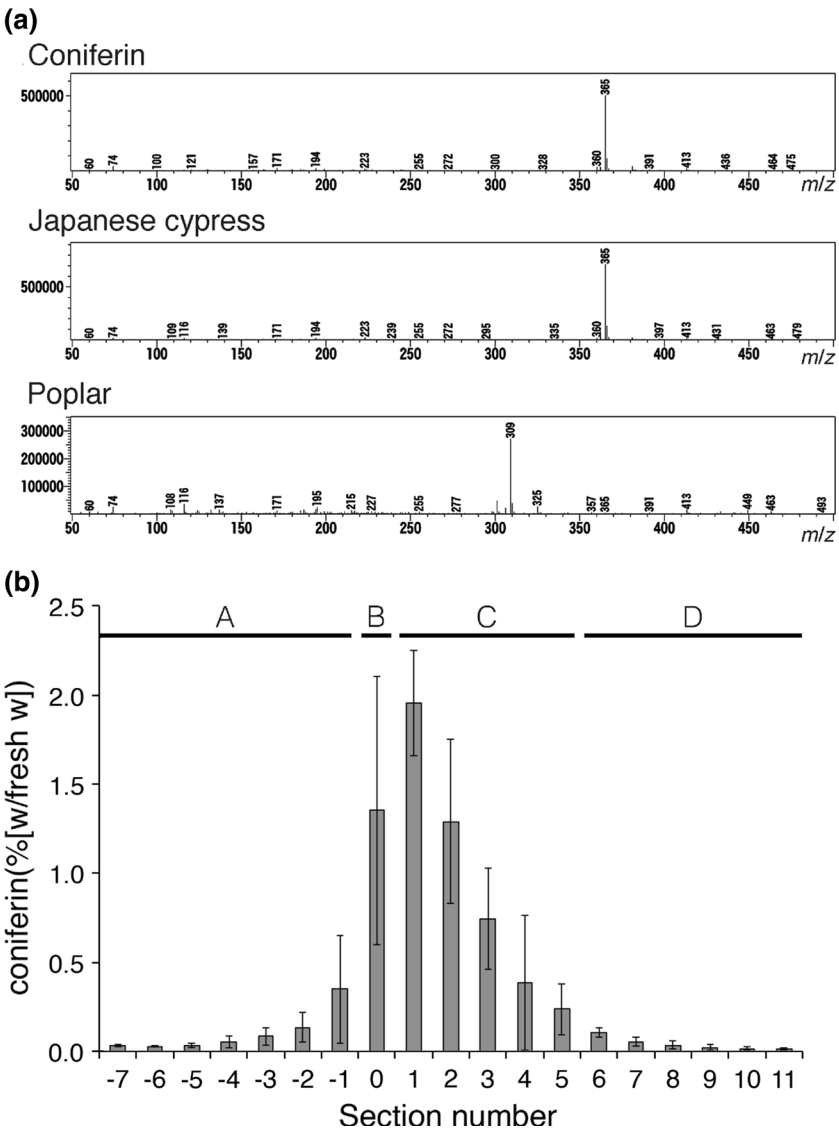


Fig. 4 **a** Positive mass spectra obtained at the retention time of coniferin from authentic coniferin (*top*), Japanese cypress extract (*middle*), poplar extract (*bottom*). **b** Distribution of coniferin in the radial direction of Japanese cypress determined by UV area of HPLC. *Region A* phloem, *B* cambium and cell expansion zone, *C* differentiating xylem, *D* xylem formed in the previous year. Data are represented as means of three extractions ± SD

transport is controlled by cell activity rather than passive diffusion after cell death. The distribution of coniferin in Japanese cypress was consistent with the requirement of lignin precursors for lignification (Figs. 3 and 4). Previous studies reported that coniferin β-glucosidase is localized in the differentiating xylem of lodgepole pine, especially in the secondary wall of tracheids [14, 16]. Coniferin could be transported to the cell wall [10] and used for lignification of the secondary wall where coniferin β-glucosidase is localized [17].

Distribution of lignin and lignin precursors in poplar

When serial tangential cryosections were obtained from the phloem of poplar blocks, it was found that two sections could not maintain their shape in each block. These sections (numbers 0 and 1) were used to sequence the data of obtained sections from different blocks. Dry weight was minimal in section 0; it increased as xylem formation proceeded, and was almost constant around section 9 (Fig. 5a). The estimated water content peaked in section 0, decreased with the formation of xylem, and then remained constant around section 9 (Fig. 5b), suggesting that xylem cells, except parenchyma, died around section 9. The cross-section subjected to Mäure reaction showed the accumulation of syringyl lignin (Fig. 6a). This section also showed that the annual ring was within the sections at a distance of approximately 1,700 μm from the de-barking position (Fig. 6a); the boundary of the annual ring was contained around section 17. The above results indicated that section 0 contained the cells in cambium and the expansion zone, and cell wall formation in xylem ceased around section 9.

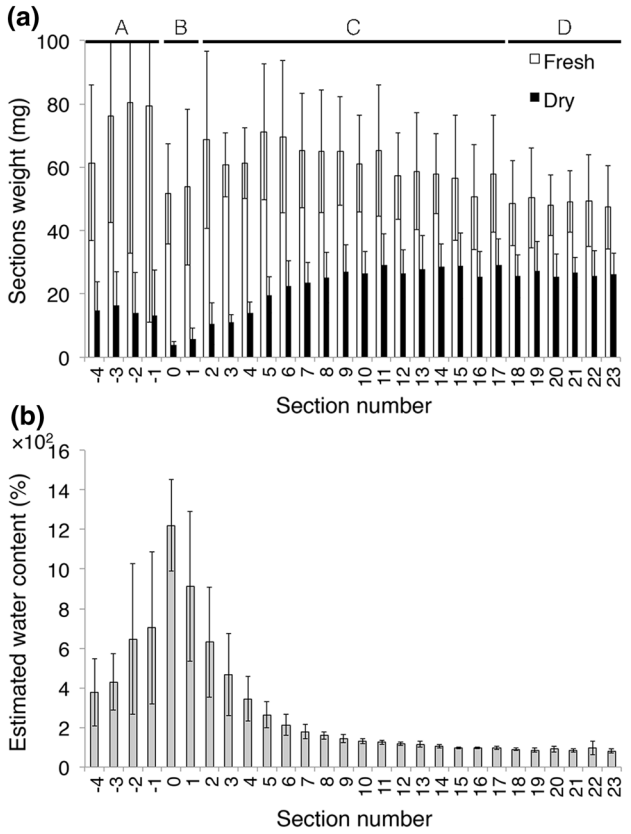


Fig. 5 **a** Fresh and dry weight of tangential sections obtained from blocks of poplar wood. **b** Estimated water content in tangential sections obtained from blocks of poplar wood. *Region A* phloem, *B* cambium and cell expansion zone, *C* xylem formed in the present year, *D* xylem formed in the previous year. Data are represented as means of three extractions ± SD

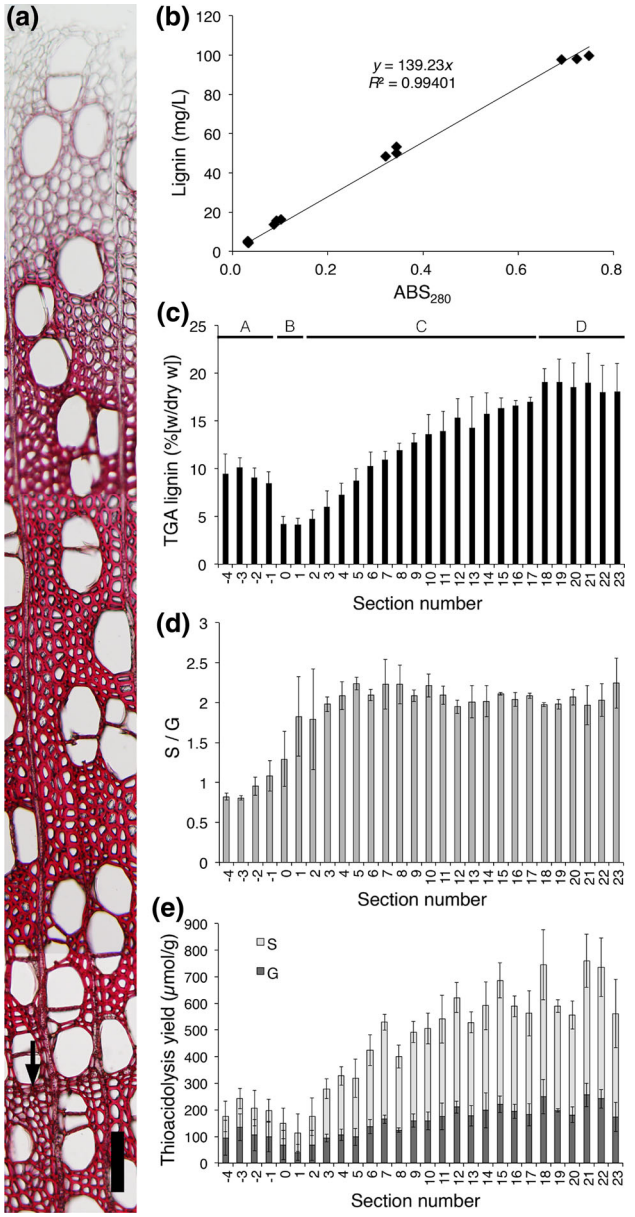
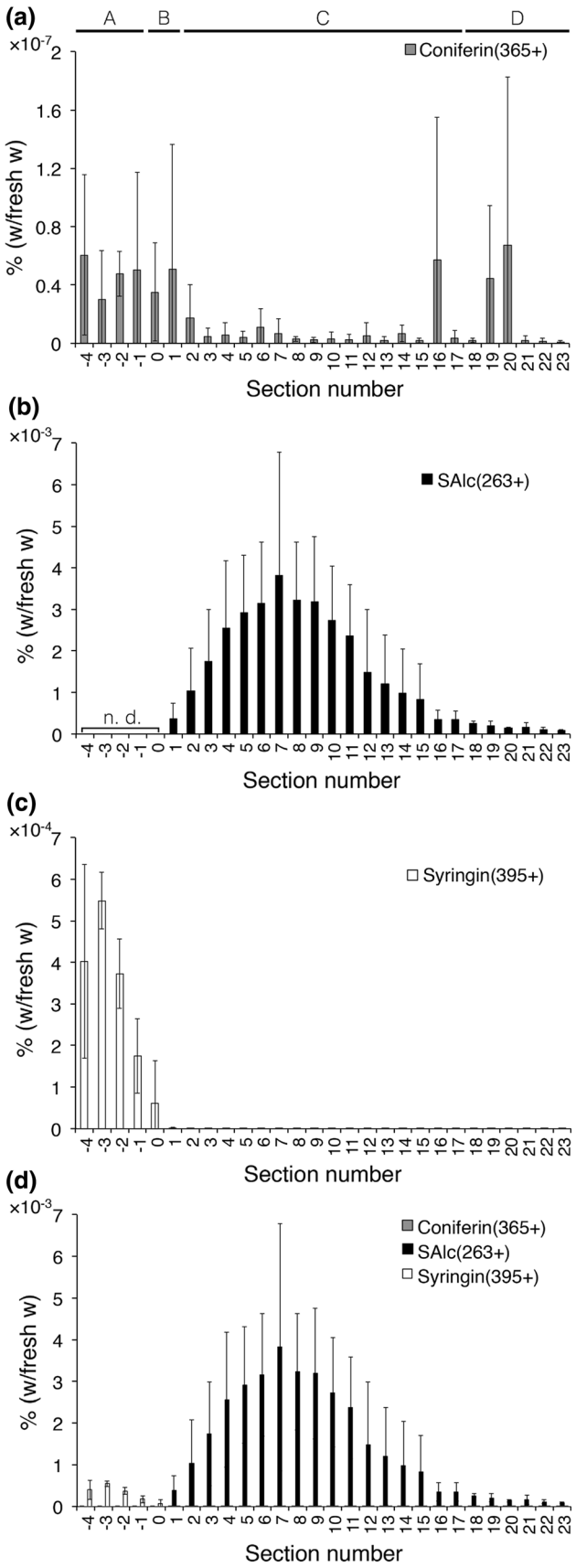


Fig. 6 Distribution of lignin in the radial direction of poplar. **a** Cross-section of poplar differentiating xylem subjected to Mäure reaction. *Arrow*, boundary of annual ring; *bar* = 100 μm. **b** Calibration curve for thioglycolic acid lignin derived from poplar wood formed in previous years. Lignin concentration was calculated from Klason (acid insoluble) lignin content in mature xylem of poplar. **c** Distribution of lignin in the radial direction of poplar determined by thioglycolic acid method. **d** Distribution of S/G ratio in the radial direction of poplar determined by thioacidolysis. **e** Syringyl and guaiacyl monomer yield by thioacidolysis. *Region A* phloem, *B* cambium and cell expansion zone, *C* xylem formed in the present year, *D* xylem formed in the previous year. Data are represented as means of three extractions ± SD

The standard curve of TGA lignin for angiosperm samples obtained from poplar (*Populus sieboldii* × *P. grandidentata*) wood using Klason lignin as the standard lignin content showed good linearity even with small samples (1–20 mg; Fig. 6b). Lignin content determined by TGA method was minimal around section 0, and increased in accordance with xylem maturation. Xylem formed in the previous year contained more lignin than differentiated xylem formed in the year of study (Fig. 6c). Thioacidolysis was also used to determine the composition of the lignin monomers involved in β-*O*-4 linkage. The S/G ratio of the thioacidolysis monomer was almost constant in the xylem, except near the cambium (Fig. 6d). It was apparent that the monomer yield by thioacidolysis increased in accordance with xylem maturation (Fig. 6e), which is consistent with the TGA lignin content.

The contents of lignin precursors in poplar specimens were determined by LC–MS (Fig. 7). In contrast to Japanese cypress, coniferin was not a major substance at the retention time of coniferin in the poplar specimen (Fig. 4a); mass spectrum was used to determine the content. Mass of SAlc ($m/z = 263$) and CAlc ($m/z = 381$) can not be explained by only addition of Na^+, which is similar



◀**Fig. 7** Distribution of lignin precursors in the radial direction of poplar. **a** Coniferin content determined by mass spectra ($m/z = 365$). **b** Sinapyl alcohol (SAlc) content determined by mass spectra ($m/z = 263$). **c** Syringin content determined by mass spectra ($m/z = 395$). **d** Combined graph of (**a**–**c**). *Region A* phloem, *B* cambium and cell expansion zone, *C* xylem formed in the present year, *D* xylem formed in the previous year. *n.d.* Not detected. Data are represented as means of three extractions ± SD

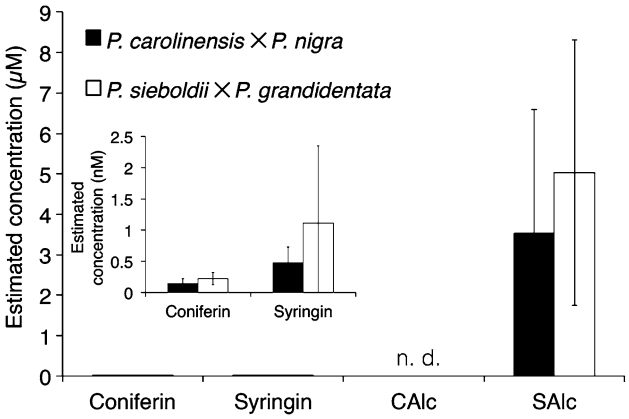


Fig. 8 Estimated content of lignin precursors in scraped differentiating xylem of two poplar species. The inset shows estimated content of coniferin and syringin. Scraped xylem (ca. 2 g) was extracted with 2 mL MeOH. Content of lignin precursors in the extract was determined by MS area. *n.d.* Not detected. Data are means of three extractions ± SD

to other studies [21, 27]. In the poplar specimen, a limited amount of coniferin was contained (Fig. 7a) whereas a certain amount of SAlc and syringin was contained in the xylem and phloem, respectively (Fig. 7b–d). The content of SAlc increased in accordance with xylem formation and peaked at section 7, followed by a gradual decrease after cessation of cell wall formation (Fig. 7b, d).

Analysis of MeOH extract from other poplar species also demonstrated the existence of more SAlc than CAlc, coniferin, or syringin in the differentiating xylem (Fig. 8). Although the spectrum of UV absorbance suggested that the extract from *P. sieboldii* × *P. grandidentata* contained certain amount of coniferin (ca. 20 μM), mass spectra proved that the major substance at the retention time was not coniferin. The estimated coniferin concentration of *P. sieboldii* × *P. grandidentata* as determined by mass spectra was very limited (ca. 0.2 nM), which was similar to that of *P. carolinensis* × *P. nigra*. Coniferyl alcohol could not be detected in poplar specimens (Figs. 8, 9). Because poplar specimen also contained G lignin (Fig. 6), its precursors are likely to have been transported immediately after synthesis. Recently, coniferin transport depending on V-ATPase has been found in the differentiating xylem of poplar [10]. Further studies on coniferin β-glucosidase in

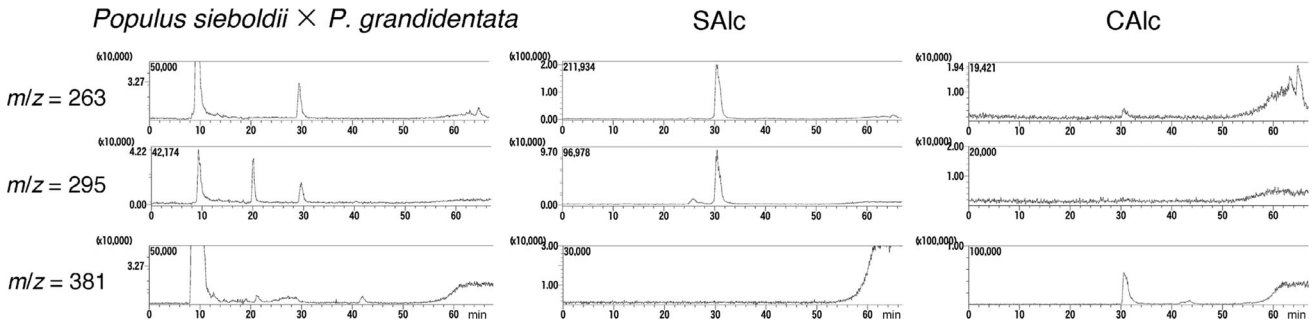


Fig. 9 LC–MS profiles showing that differentiating xylem of poplar contained certain amount of SAlc but little of CAlc. Top *m/z* = 263, *middle* *m/z* = 295, *bottom* *m/z* = 381. *Left* MeOH extract from scraped xylem of *Populus sieboldii* × *P. grandidentata*, *middle* authentic SAlc, *right* authentic CAlc. Presence of SAlc was also confirmed by another MS (*m/z* = 295)

differentiating xylems of poplar would help to elucidate the role of coniferin in lignification.

The presence of SAlc (Figs. 7, 8, 9) is consistent with previous reports on other poplar species [18, 20]. In contrast to precursors of G lignin, SAlc consumption is likely to be slower in comparison with its synthesis in the differentiating xylem of poplar (Figs. 7, 8). SAlc content in xylem peaked in cells with ceasing cell wall formation, and decreased gradually towards the boundary of the annual ring, where the lignin content was still gradually increasing (Figs. 5, 6, 7). Additional lignification has been indicated to occur after the cell death of vessels [28]. SAlc contained in the differentiating xylem of poplar could be used for the lignification of xylem even after cell death of vessels and fibers. The present study shows the distribution of SAlc in the differentiating xylem of poplar; however, whether SAlc is localized in the fiber or ray parenchyma is still not distinguishable. Further research on the localization of SAlc in differentiating xylem would help to explain the mechanism of transport of S lignin precursors for lignification.

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