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ORIGINAL ARTICLE

Seasonal variations of tracheid formation and amount of auxin (IAA) and gibberellin A4 (GA4) in cambial-region tissues of mature sugi (*Cryptomeria japonica*) cultivar grown in a Nelder plot with different tree densities

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Abstract The role of phytohormones in tracheid formation remains unclear in conifers. In this study, to obtain information on the role of auxin (IAA) and gibberellin A4 (GA4), we examined seasonal variation in the amount of phytohormones in cambial-region tissues and tracheid formation of sugi cultivar planted in a Nelder plot with different tree densities. We demonstrated that the amount of IAA was positively correlated with the number of tracheids formed in early and mid-season, but not in late season, and had no relation to tracheid differentiation. Crown length and height at the crown base had a positive and negative effect, respectively, on IAA amounts in early and mid-season, but not in late season. Height-to-diameter ratio was negatively correlated with IAA amounts in early and mid-season, but not in late season. Sugi trees with wider spacing continued tracheid formation in late season with smaller amounts of IAA, although the trees with narrower spacing ceased tracheid formation with larger amounts of IAA. Cambial growth cessation in late season might be controlled not by IAA amount, but by short-day-induced insensitivity to IAA. GA4 had no relation with the indexes of growth traits or tracheid formation.

Keywords IAA · GA4 · Tree density · Tracheid formation

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Introduction

Sugi (*Cryptomeria japonica*) is an important afforestation tree species in Japan, mainly used as a structural component of wooden structures. A reliable supply/use of this domestic wood is essential for the revitalization of forestry in Japan [1]. To increase the reliability of quality wood sugi products, it necessary to gain a precise understanding of wood formation in the cambial-region tissues, because wood formation affects the quality and quantity of the wood. We previously examined variation in wood properties [2–4], focusing on the role of phytohormones in xylem formation in sugi trees [5, 6]. We found that crown length and distance from the base of the crown affects the amounts of auxin (IAA) in cambial-region tissues [5] and that young sugi trees forming juvenile wood had very large amounts of IAA in these tissues compared to trees forming mature wood [6]. However, the role of endogenous phytohormones in cambial-region tissues on tracheid formation and differentiation in sugi trees remains unclear.

In other afforestation coniferous tree species, studies have reported on aspects of the role of IAA in wood formation. However, the precise role of IAA in wood formation in these species also remains unclear. Based on studies that applied exogenous IAA to *Pinus sylvestris* [7, 8] and *Pinus resinosa* [9], it was assumed that IAA is actively synthesized in elongating shoot apices, and transported to the stem cambium, where it then stimulates tracheid production. In those studies, the amount of endogenous IAA in the cambial-region tissues varied seasonally, being higher in summer and lower in autumn and winter in *Pinus contorta* and *Pinus densiflora* [10, 11]. Based on the radial distribution pattern of IAA from the phloem to the developed xylem in *P. sylvestris*, IAA was assumed to control xylem differentiation as a "morphogen" [12]. On the other hand,

it was suggested that tracheid production was not directly related to endogenous IAA concentration in the cambium of pine trees [13]. IAA amounts did not change with latewood initiation in *P. sylvestris* [14]. Therefore, more accurate and precise study of the role of endogenous IAA in afforestation coniferous tree species are needed.

The effects of gibberellins on xylem formation have also been reported. Increasing gibberellin (GA) levels in hybrid aspen through the overexpression of a key gene in the GA biosynthesis pathway induced increased rates of xylogenesis and elongated xylem fibers in comparison with wild-type counterparts [15]. A study in which gibberellin A3 (GA3) and inhibitors of the synthesis of gibberellin were applied found that gibberellin plays an important role in the tension wood formation of *Acacia mangium* seedlings [16]. The quantitative study showed that gibberellin A1 (GA1) and gibberellin A4 (GA4) were located in the zone of expansion in the xylem cells of aspen [17]. In angiosperm trees, gibberellins might play an important role in xylem cell differentiation. However, there have been very few studies on the role of endogenous gibberellins in tracheid differentiation in conifers.

In this study, we focused on the role of IAA and GA4 in wood formation in mature sugi trees. To obtain more accurate information on the role of phytohormones, we examined xylem formation in a sugi cultivar planted in a Nelder plot [18] with different tree densities. Nelder plots [18] were developed to analyze the impact of a continuous range of densities on the yield of agricultural crops over a smaller area than traditional designs to minimize the differences in yield related to variability within a site. Many researchers have utilized this design in forestry studies [19]. In addition, experimental plots with a sugi cultivar can be assumed to show a smaller effect of genetic variation on xylem formation than plots with sugi trees of unknown genotypes. As we previously reported [4], height-to-diameter ratios (H/D ratios) of sugi mature trees were beautifully controlled by tree density in the Nelder plots. Therefore, sugi cultivars grown in Nelder plots can be assumed to have wide range of radial growth and minimal effects of other factors (genetic variation and difference of soil conditions) that can affect xylem formation. As described previously, amounts of endogenous IAA varied seasonally [10, 11]. Using the pinning method [20], we try to examine the role of endogenous phytohormones separately in each season.

The objectives of the current study were to examine: (1) seasonal variation of xylem formation, and amount of IAA and GA4 in cambial-region tissues; (2) relationships between growth traits and amounts of phytohormones; and (3) relationships between amounts of phytohormones and indexes of xylem formation in stems of mature sugi cultivar trees (Tosaaka) grown in a Nelder plot with different tree densities.

Materials and methods

Sample trees and samples for evaluating tracheid formation and quantification of phytohormones

Specimens of a 38-year-old sugi cultivar (Tosaaka; Table 1) were used. The experimental plots for plantation spacing were established within a stand in the national forest in Kitago-cho, Miyazaki Prefecture, Japan. Three trees with no visible defects were selected per each initial density (trees/ha) in the plot. The plot used for this study was located in the southern part of Miyazaki Prefecture. Miyazaki city's average annual temperature and precipitation in the sampling year were 17.3 °C and 2590 mm, respectively. The altitude of the plot used for this study was 520 m. Diameter of the trees at breast height (DBH) and tree height was measured with a tape measure and ultrasonic hypsometer (Vertex III, Haglof, Inc.), respectively. Height at crown base was also measured with the ultrasonic hypsometer (Vertex III, Haglof, Inc.). The crown length was calculated from the height at the crown base and the tree height.

To measure the amount of IAA and GA4 in cambial-region tissues, samples (3 cm (T) × 4 cm (L) × 1 cm (R)) of cambial-region tissues sandwiched by the outer bark

Table 1 Initial tree densities and growth traits of sample trees

Symbol	Density (trees/ha)	Age	*n*	*H* (m)	DBH (cm)	HCB (m)	H/D ratio (%)
D	4823	38	3	17.0 (2.0)^{a}	22.1 (6.1)^{a}	11.5 (1.1)^{b}	79.7 (14.0)^{b}
E	3349	38	3	18.8 (1.4)^{a}	27.9 (4.0)^{ab}	11.8 (1.4)^{b}	68.0 (5.7)^{ab}
G	1615	38	3	17.5 (2.1)^{a}	25.8 (4.2)^{ab}	11.1 (1.1)^{b}	68.3 (6.9)^{ab}
H	1122	38	3	18.5 (3.4)^{a}	34.0 (11.8)^{ab}	9.3 (1.5)^{ab}	57.0 (12.0)^{ab}
J	541	38	3	20.3 (3.8)^{a}	47.5 (12.3)^{b}	5.4 (2.0)^{a}	43.4 (4.3)^{a}

The values represent averages in three trees; values in parentheses indicate standard deviations. Different characters show significant differences among density-zoning symbols ($p < 0.05$)

Symbol tree-density-zoning symbol, *Density* initial tree density, *n* number of sample trees, *H* tree height, *DBH* diameter at breast height, *HCB* height at crown base, *H/D ratio* height-to-diameter ratio

and the outermost wood were obtained from the sample trees, as listed in Table 1. The samples were obtained from a point 1.2 m above the ground in each sugi tree in June, August, October, and December 2011 (3 trees × 5 initial tree densities × 4 sampling months, for a total of 60 samples). Immediately after collection, samples were stored in a deep freezer (−80 °C) before extraction. Pin insertion into the cambium of sample trees was accomplished at each sampling date. The samples for the pinning method were cut from sample trees after the cessation of xylem formation to measure indexes for evaluating tracheid formation and differentiation.

Measurements of phytohormones in cambial-region tissue

IAA and GA4 in cambial-region tissues were identified and quantified by liquid chromatography/mass spectrometry (LC/MS). Samples were homogenized and extracted for 1 h by methanol with antioxidant medium (0.02 M diethyldithiocarbamic acid, Wako, Ltd.). Methanol extraction was repeated three times at 4 °C in darkness. To quantify the amount of IAA, 500 ng of deuterium IAA (D2-IAA, 97% content; Sigma Co., Ltd) and deuterium GA4 (D2-GA4, 90% content; Olchemim, Ltd) were added to the methanol for extraction as an internal standard. The extracts were evaporated, and residues were dissolved in 10-ml distilled water. The aqueous solutions were then adjusted to pH 2.5 with formic acid. Supernatants were obtained from the aqueous solutions by centrifugal separation, loaded onto reverse-phase cartridges (sep-pack cartridge, C18 500 mg, Waters), and eluted with 1-ml 80% methanol adjusted to pH 2.5. The effluents were subjected to LC/MS.

LC/MS analysis was carried out using a liquid chromatograph (Ultimate 3000, Thermo Fisher Scientific) coupled to a mass spectrometer (Q-exactive, Thermo Fisher Scientific) with an ion source operated in the ESI (electro-spray ionization) positive and negative mode for IAA and GA4, respectively. The column was an Atlantis T3 (100 × 2.1 mm, 3 μm, Waters), as the mobile phase, methanol containing 0.1% formic acid (v/v) and distilled water containing 0.1% formic acid (v/v) was used, and the flow rate was 0.2-ml/min for both IAA and GA4. For IAA, the linear gradient of methanol containing 0.1% formic acid (v/v) (B) in distilled water containing 0.1% formic acid (v/v) (A); 30% B to 70% B in 8 min, and 70% B in 2 min was used. For GA4, the linear gradient of methanol containing 0.1% formic acid (v/v) (B) in distilled water containing 0.1% formic acid (v/v) (A), 20% B to 90% B in 8 min, and 90% B in 2 min was used. Detection and quantification were carried out using Q-exactive operated in the positive (IAA) and negative (GA4) ion, targeted-SIM mode using calibration curve with deuterated IAA and GA4 as internal standards. Targeted *m*/*z* were 176.0705 (IAA), 178.0830 (D2-IAA), 331.1554 (GA4), and 333.1678 (D2-GA4). Identification of IAA and GA4 was accomplished based on retention time and *m*/*z* standard of phytohormones (mass tolerance: 3 ppm). As described in a previous report [5], amounts of IAA and GA4 in the cambial-region tissues are shown as amounts (ng) per cambium area (L × T cm^2) (ng/cm^2).

Measurements of indexes evaluating tracheid formation

To meet objectives (1) and (3), we evaluated cambial activity for tracheid formation in each season and indexes related to tracheid differentiation. We examined the number of tracheids formed in each season, initiation of xylem formation—June, June–August, August–October, and October–December, according to the method described in our previous studies [21, 22] using the pinning method [20]. We also measured cell wall thickness, cell radial, and tangential diameter in the cross section, and cell wall ratio (%) of tracheids formed in each season as indexes for evaluating tracheid differentiation. Samples for evaluating tracheid differentiation were embedded in spurr resin (Polysciences, Inc.), and 7-μm-thick cross sections were then obtained and stained with toluidine blue. The tracheids formed in each season were identified using the pinning method. According to our previously reported method [22], cell wall ratio, cell wall thickness, and radial and tangential diameter of the tracheids were measured using image J [23]. The accuracy of the measurement was 0.3 μm/pixel, and the cross-sectional indexes were obtained by averaging 10 measurements of different positions in the cross section.

Statistical analysis

For analysis of obtained data, the SPSS statistical analysis software (ver. 16 with Regression and Advanced Models) was used. Using one-way ANOVA (analysis of variance) and multiple comparisons tests (Tukey's HSD test and Bonferroni test), we examined the significant differences in indexes evaluating tracheid formation and the amounts of phytohormones (Figs. 1, 2) among tree density zones.

Results

Growth traits and tracheid formation of sample trees

As shown in Table 1, there were significant differences in growth traits of sample trees, except for tree height, among tree-density zones (ANOVA, $p < 0.01$). We observed that the J trees (541 trees/ha) had larger DBH, smaller HCB (height at crown base) and lower H/D ratio than D trees (4823 trees/ha; multiple comparisons tests,

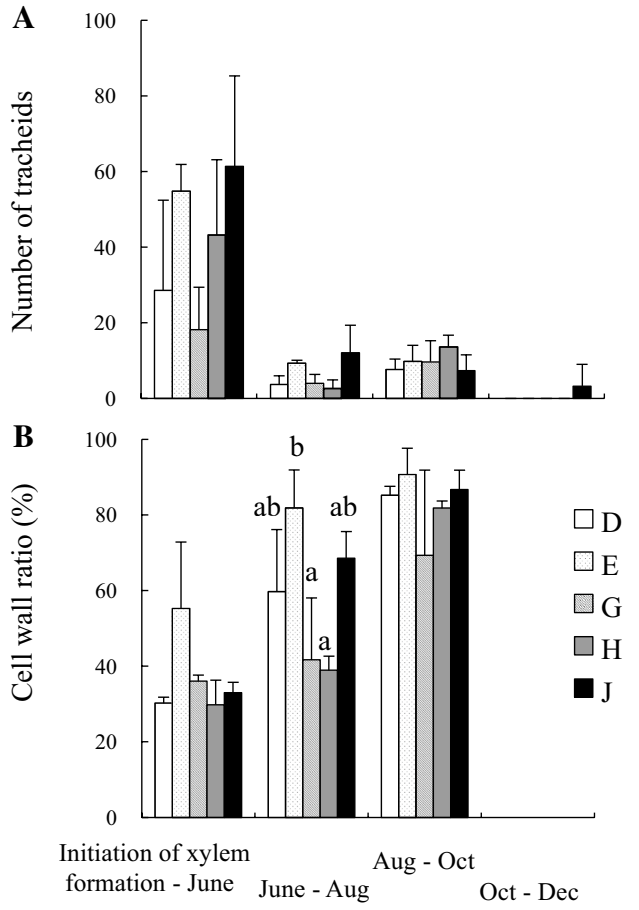

Fig. 1 Seasonal variation of xylem formation in sugi trees grown with different tree densities. *Different characters* show significant difference among density-zoning symbols ($p < 0.05$). *Error bars* indicate the standard deviations. Cell wall ratio of initiation of xylem formation—June was obtained from the xylem formed near pin marking in June. **A** Number of tracheids, **B** cell wall ratio

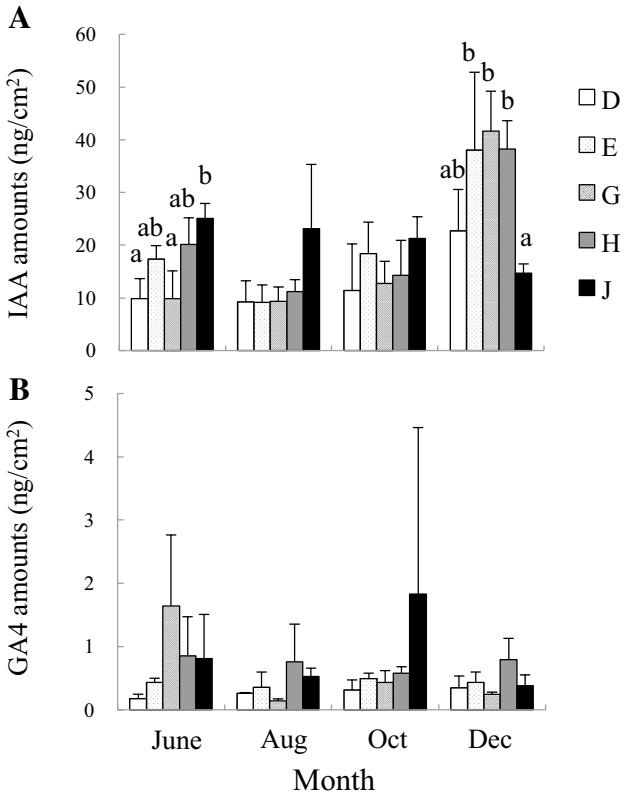

Fig. 2 Seasonal variation in measured amounts of IAA and GA4 in cambial-region tissues of sugi trees grown with different tree densities. *Different characters* show significant differences among tree-density-zoning symbols ($p < 0.05$). *Error bars* indicate standard deviations. **A** IAA amounts, **B** GA4 amounts

$p < 0.01$). However, effects of tree density on growth traits were not significant among E, G, and H trees (3349, 1615, and 1122 trees/ha, respectively). Based on the results in Table 1, it was assumed that decrease of tree density would inhibit the upward movement of the crown base and increase of crown length; therefore, increased amounts of crown may activate radial growth of trees.

Figure 1A shows seasonal variation of tracheid formation. A larger number of tracheids were formed at the initiation of xylem formation—June than in other seasons in all tree-density zones. J trees showed a larger average number of tracheids than trees in the other tree-density zones at the initiation of xylem formation—June and June–August, although the difference was not significant in each season. J trees continued tracheid formation in October–December, while trees in the other tree-density zones ceased tracheid formation during this season.

As shown in Fig. 1B, there were significant differences in the cell wall ratio of tracheids among tree-density zones in June–August, although significant differences were not recognized in other seasons (ANOVA, $p < 0.01$; multiple comparisons tests, $p < 0.01$). The same results were obtained for radial diameter and cell wall thickness of tracheids (data not shown). Based on Mork's definition, earlywood has a cell wall ratio <50%, and latewood has a cell wall ratio ≥50% [24]. As shown in Fig. 1B, G, and H trees were assumed to form earlywood tracheids in June–August, although other trees in tree-density zones formed latewood in the same season. J trees had larger average number of xylem cells and smaller average latewood percentage in annual rings than trees of the other density zones, although the differences in these indexes were not significant (data not shown).

Amount of phytohormones in cambial-region tissues of sample trees

As shown in Fig. 2a, there were significant differences in the amount of IAA in cambial-region tissues among

tree-density zones in June and December. J trees had significantly larger amounts of IAA than D and G trees in June (ANOVA, $p<0.01$; multiple comparisons tests, $p<0.01$). J trees also had larger average amounts of IAA than trees of other tree-density zones in August and October, although these differences were not significant. In contrast, J trees had significantly smaller amounts of IAA than E, G, and H trees in December (ANOVA, $p<0.05$; multiple comparisons tests, $p<0.05$). As previously described, J trees only formed tracheids during October–December (Fig. 1a).

Average amounts of GA4 in the cambial-region tissues of each tree-density zone varied from 0.15 to 1.83 ng/cm^2, and were very small in comparison with the amounts of IAA (Fig. 2b). There was no significant difference in the amount of GA4 among tree-density zones in any season.

Relationships between growth traits and amount of phytohormones

We previously reported the positive effects of crown length and the negative effects of distance from crown base on the amounts of IAA in the cambial-region tissues of the trunk [5]. The results in this study were in good accord with those of our previous reports (Table 2). In this study, height at crown base was used as an index of distance from the crown base, because all samples were cut at the same longitudinal position (1.2-m above ground). The crown length was closely related to the amount of IAA in June ($p<0.01$), moderately related in August, October ($p<0.05$), and not related in December. The height at crown base was closely related to the amount of IAA in June and August ($p<0.01$), and not related in October and December. H/D ratio also was related to amount of IAA in June and October ($p<0.01$), moderately related in August ($p<0.05$), and not related in December. No growth traits were related to the amount of GA4 except for height at crown base in October. It was recognized that the relationships obtained in our previous study (5) might be related to the seasons with cambial growth activity.

Relationships between amount of phytohormones and tracheid formation

We attempted to elucidate the role of phytohormones in tracheid formation. As shown in Fig. 3a and c, IAA amounts in June were closely related to number of tracheids formed in initiation of xylem formation—June ($r=0.75$, $p<0.01$). IAA amounts in August were moderately related to number of tracheids formed June–August ($r=0.55$, $p<0.05$). However, the amount of IAA in October and December were not related to number of tracheids formed in August–October or October–December, respectively. There was no significant correlation between the amount of GA4 and number of tracheids formed in any season (Fig. 3b). As shown in Table 3, amount of IAA and GA4 had no significant effect on differentiation of tracheids formed in any season, except for the effect in October of amount of IAA on cell wall ratio.

Discussion

In this study, we demonstrated that the main role of IAA in tracheid formation was promotion of cell division, not the control of differentiation related to transition from earlywood to latewood, based on the relationships between amounts of endogenous IAA and the indexes of tracheid formation (Fig. 3; Table 3). This promotion of cell division and lack of relation to differentiation were consistent with a previous study on pine trees [7] and our study on sugi trees [22], respectively. However, it was assumed that amount of IAA in cambial-region tissues would not be found to control cambial growth cessation and dormancy due to cambial growth cessation in the late season with relatively large amounts of IAA in cambial-region tissues (Figs. 1a, 2a). As previously described, IAA was actively synthesized in elongating shoot apices and transported to the stem cambium, where it then stimulated tracheid production. Recent studies revealed that auxin binding to the coreceptor results

Table 2 Coefficients of correlations between growth traits and amount of phytohormones

	Amount of IAA				Amount of GA4			
	June	August	October	December	June	August	October	December
H	0.38	0.07	0.62*	−0.07	−0.31	0.06	−0.26	0.36
DBH	0.69**	0.39	0.64*	−0.29	−0.05	0.17	0.05	0.37
H/D ratio	−0.83**	−0.55*	−0.70**	0.15	−0.11	−0.20	−0.34	−0.44
CL	0.74**	0.54*	0.57*	−0.40	−0.12	0.29	0.26	0.36
HCB	−0.70**	−0.70**	−0.23	0.51	−0.09	−0.36	−0.61*	−0.11

Coefficient of correlations were obtained from 15 sample trees. *H, DBH, H/D ratio*, and *HCB* were the same, as shown in Table 1

CL crown length

**$p<0.01$, *$p<0.05$

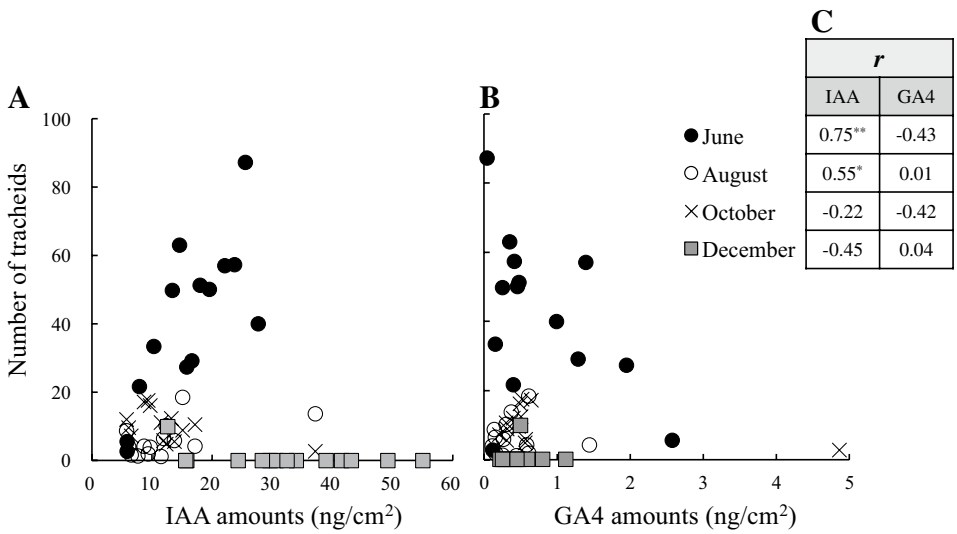

Fig. 3 Relationship between phyohormones and number of tracheids. The number of tracheids in June, August, October, and December was the number of tracheids formed at initiation of xylem formation—June, June–August, August–October, and October–December, respectively. **a** IAA amounts, **b** GA4 amounts, **c** coefficient of correlations between phyohormones and number of tracheids in each season, *r* coefficients of correlations, $^{**}p < 0.01$, $^{*}p < 0.05$

Table 3 Coefficients of correlations between amount of phytohormones and indexes of xylem formation

	Amount of IAA			Amount of GA4		
	June	August	October	June	August	October
Cell wall ratio	−0.15	0.26	0.60*	−0.11	−0.10	0.01
Cell wall thickness	0.27	0.41	0.54	−0.25	−0.17	0.30
R diameter	0.27	0.04	−0.24	−0.23	0.09	0.43
T diameter	0.53	0.20	−0.09	−0.40	0.06	-0.27

Indexes of xylem formation in June, August, October, and December were the values measured on xylems formed at initiation of xylem formation—June, June–August, August–October, and October–December, respectively

$^{*}p < 0.05$

in degradation of the Aux/IAAs through the proteasome and derepression of ARF-based transcription of target genes [25]. Therefore, IAA related to ARF-based transcription of target genes was metabolized. IAA amounts in cambial-region tissues are assumed to be the result of synthesis in the crown, transportation, and metabolism in cambial-region tissues.

As previously reported, crown length and the distance from the crown base had positive and negative effects, respectively, on the amount of IAA in the stem [5]. We hypothesized that crown length is related to the synthesis of IAA, and the distance from the crown base is related to the transportation and metabolism of IAA. In this study, crown length and height at crown base were beautifully controlled using a sugi cultivar grown in a Nelder plot (Table 1). The correlations previously reported [5] were also observed in early and mid-season, although not in late season in this study (Table 2). As shown in Table 2, the correlation between height at the crown base and IAA amount dropped in October. This lack of a relation in October might have been induced by the difference of sensitivity to IAA among tree-density zones. J trees, trees with wider spacing, still had cambial growth activity in October–December (Fig. 1a), with significantly smaller amounts of IAA (Fig. 2a); meanwhile, other trees with narrower spacing showed decreased cambial growth activity with larger amounts of IAA.

It was also reported that the day-length-regulated induction of cambial growth cessation and dormancy involves changes in IAA responses rather than IAA amounts in hybrid aspen [26]. Our previous study also showed the insensitivity of cambium to applied phytohormones in late season in sugi trees [22]. The wider spacing of J trees might inhibit the effects of short-day-induced cambial growth cessation and dormancy. In October–December, the cambium of J trees with sensitivity to IAA might metabolize the endogenous IAA for tracheid formation, resulting in significantly smaller amounts of IAA. Meanwhile, trees in other tree-density zones might not metabolize IAA, resulting in significantly larger amounts of IAA, because of short-day-induced insensitivity of cambium to IAA. Previous studies on pine trees showed relatively smaller amounts of IAA in late season compared to other seasons [10, 11]. However, as shown in Fig. 2a, IAA amounts in December

were relatively large compared to levels in other seasons. In this study, the reason for the relatively large amounts of IAA in late season remains unclear. The synthesis of IAA in the crown in late season in this study might be related to the environmental conditions of the plot (average annual temperature and precipitation: 17.3 °C and 2590 mm, respectively).

As shown in Table 2, the H/D ratio was negatively correlated with IAA amounts in the stem in June, August, and October. We reported that H/D ratio had a significantly positive effect on stem stiffness in sugi trees [4]. In addition, we reported that younger sugi trees forming juvenile wood with smaller stem stiffness had much larger amounts of IAA than older sugi trees forming mature wood with larger stem stiffness [6]. Therefore, large IAA amounts might have negative effects on stem stiffness in sugi trees.

In this study, the role of GA4 in tracheid formation remained unclear. No direct evidence was obtained to show that GA4 is related to tracheid formation and differentiation. Further studies involving controlled environmental factors might be needed to elucidate the role of GA4 in tracheid formation.

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