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NOTE

Effects of application of *trans*-zeatin on tracheid differentiation in mature sugi (*Cryptomeria japonica*) trees

Yoshio Kijidani[1] · Shinya Koga[2] · Hiroki Sakagami[2] · Hiroshi Matsunaga[3]

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Abstract The mechanism of differentiation of tracheid (earlywood or latewood) should be elucidated to improve the wood properties of sugi trees (*Cryptomeria japonica*). Water deficit affects tracheid differentiation in conifers. However, the signals, which transmit the information of water contents in the soil to the differentiating tracheid, remain unknown. Plant responses with deficits of macronutrients or water showed some differences but also similarities, mostly involving hormonal long-distance signaling. In *Arabidopsis*, *trans*-zeatin (tZ)-type cytokinins play a role as a root-to-shoot acropetal signal. In this study, we report the effects of applying tZ alone or in combination with other phytohormones on tracheid differentiation in mature sugi trees forming latewood. The application of tZ induced the formation of earlywood-type tracheids with significantly smaller cell wall ratios and larger microfibril angles than those of controls in July, August and September. The application of indole acetic acid (IAA) in combination with tZ inhibited the effects of applied tZ, although gibberellic acid (GA3) did not. In October and November, application of tZ could not affect the xylem formation or dormancy of cambium. We hypothesized that tZ might play a role in the differentiation of earlywood tracheid.

✉ Yoshio Kijidani
kijiyo@cc.miyazaki-u.ac.jp

[1] Division of Forest Science, Faculty of Agriculture, Miyazaki University, Miyazaki 889-2192, Japan

[2] Department of Agro-environmental Sciences, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan

[3] Department of Wood Improvement, Forestry and Forest Products Research Institute, Tsukuba 305-8687, Japan

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Introduction

Softwood is one of the most important renewable resources in the world and is mainly used in the structural components of wooden structures. Tracheid is the main component of the annual rings of softwoods, and latewood tracheid has a narrower radial diameter, thicker cell walls and fewer bordered pits than earlywood tracheid [1]. Variation of mechanical properties in annual rings showed minimum values in earlywood and maximum in latewood in many conifers [2]. Therefore, tracheid differentiation (earlywood or latewood) assumed to have close relation with the wood properties of conifers. Sugi (*Cryptomeria japonica*, Japanese cedar) is one of the important coniferous plantation species in Japan. This domestic wood is mainly used for structural applications. The latewood percentage of sugi was relatively small and decreased with increase of ring width, although latewood percentage of slash pine was relatively large and constant with increase of ring width [3]. Slash pine is one of the popular species of southern pine with superior growth rate and mechanical properties to sugi. The mechanism of differentiation of tracheid (earlywood or latewood) should be elucidated to improve the wood properties of sugi trees.

The effect of water deficit on differentiation of tracheid was recognized in conifers. Sugi trees irrigated every day formed tracheids with large radial diameter, although sugi trees irrigated every 3 days formed tracheid with small radial diameter [4]. The effect of water deficit reported in sugi trees was also recognized in Norway spruce. Drought events could induce false-ring formation in Norway spruce

[5, 6]. From these studies, it was assumed that there was a close relationship between water contents in the soil and the differentiation of tracheids in conifers. However, the signals, which transmit the information of water contents in the soil to the differentiating tracheid, remain unknown.

Based on quantitative and application studies of phytohormones, indole acetic acid (IAA) has been believed to be one of important regulators for tracheid formation. It was reported that trees with higher growth rates had greater endogenous IAA amounts [7], and the application of exogenous IAA to disbudded segments increased the endogenous IAA amounts and the number of lignified tracheids in a dose-dependent manner [8]. The application of auxin to a young red pine (*Pinus resinosa*) producing latewood tracheids induced the formation of earlywood [9]. However, based on the other studies of tracheid differentiation, other internal factors besides the decrease in the IAA amounts may control the differentiation of tracheids. Tracheid production in response to the application of IAA decreased with cambial age in *Pinus sylvestris* [10]. The transition from earlywood to latewood occurred concurrently with the decrease in IAA amounts in cambial region tissues; however, it occurred at different IAA amounts for different stem positions and different trees in *Pinus densiflora* [11]. The IAA amounts did not change with latewood initiation in *P. sylvestris* [12].

The effects of other phytohormones [cytokinins and gibberellins (GAs)] on xylem formation have also been reported as follows. Positive effects were obtained from the application of 6 benzylaminopurine to the stems of intact trees [13], although no effects were observed to result from the application of cytokinins to isolated stem segments [14, 15]. Increasing the 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 to wild-type counterparts [16]. Based on the study of application of GA3 and inhibitors of the synthesis of gibberellin, it was reported that gibberellin plays an important role in tension wood formation of *Acacia mangium* seedlings [17]. The quantitative study showed that GA1 and GA4 were located in the zone of expansion of xylem cells of aspen [18]. However, the roles of cytokinins and gibberellins in tracheid differentiation of conifers remain unknown.

Recently, cytokinin biosynthesis, compartmentalization and translocation in *Arabidopsis* were examined precisely, and potential roles were reported for cytokinins as local and long-distance signals [19]. Macronutrients (nitrate, sulfate and phosphate) regulate the gene expression of a key enzyme of cytokinin biosynthesis [20, 21]. Cytokinin application represses the macronutrient transporter gene [22, 23]. It was suggested that cytokinins play a critical role in balancing the acquisition and distribution of macronutrients [19]. Xylem sap predominantly contains *trans*-zeatin (tZ)-type cytokinins, and phloem sap predominantly contains N^6-(Δ^2-isopentenyl) adenine (iP)-type and *cis*-zeatin (cZ)-type cytokinins in *Arabidopsis* [19]. It is assumed that roots are major sites of tZ production, and that *trans*-zeatin riboside (tZR) plays a role as a root-to-shoot acropetal signal [19]. Water deficit increased cytokinin oxidase and implied cytokinin degradation in *Zea mays* [24]. Plant responses with deficits of macronutrients or water showed some differences but also similarities, mostly involving hormonal long-distance signaling [25]. As previously described, water deficit induced formation of tracheid with small radial diameter in sugi trees [4]. If the results for tZ in *Arabidopsis* and *Zea mays* held true in sugi trees, deficit of water and macronutrient may affect degradation and inhibition of cytokinin synthesis, and the decreased amounts of cytokinins may affect tracheid differentiation in sugi trees. We hypothesize that tZ-type cytokinins may be the signal which transmits the information of water and macronutrients contents in the soil to the differentiating tracheid in sugi trees.

We focused on the potential role of tZ in earlywood formation in sugi trees in this study, based on the results for tZ-type cytokinins in *Arabidopsis* and *Zea mays* and for water deficit in conifers. However, the effects of tZ alone or in combination with other phytohormones on the differentiation of tracheid (earlywood or latewood) in mature intact sugi trees have not been examined precisely. We planed the experiments of phytohormones application to intact trees forming latewood, because we hypothesized the potential role of tZ in earlywood formation and expected the significant effects of tZ in intact trees forming latewood. In addition, the effects of application of tZ may vary during the application period. We also planed the application experiments with different application period.

The objectives of the current study were to examine: (1) the effect of applied tZ alone or tZ in combination with other phytohormones on tracheid differentiation in June–July when latewood formation had just initiated; (2) the seasonal variations of the effects of the application of tZ alone on tracheid differentiation in stems of mature sugi trees (*C. japonica*).

Materials and methods

Sample trees and application of phytohormones

In the experiments using isolated stem segments, wounding may affects the amounts and type of endogenous phytohormones in the isolated stem segments. In this study, we applied the phytohormones to the stem surface of intact trees. Twenty-eight-year-old sugi trees (unknown sugi

cultivars) were used for this study. These trees had been planted in a stand for timber production established in the experimental forest of Miyazaki University (initial density 3000 trees/ha). Several thinning had been carried out at this stand. The altitude of the stand used in the current study ranged from 130 to 150 m. Ten sugi trees with similar growth traits (average diameter at breast height (DBH): 18.7 cm) were selected as sample trees from this stand. The average annual temperature and precipitation at Miyazaki, Japan in the year of the study were 19.2 °C and 2681 mm, respectively. To meet objective (1), five trees were used to assess the effect of applying tZ alone and tZ in combination with other phytohormones on tracheid differentiation in June–July. To meet objective (2), another five trees were used to assess the effects of applying tZ alone on tracheid differentiation in August–November.

To meet objective (1), IAA, gibberellin A3 (GA3) and tZ were applied alone and in combination with each other (IAA+GA3, GA3+tZ and IAA+tZ) to the stem at breast height (Experiment I in Fig. 1). The concentrations of applied phytohormones may affect the response in xylem formation. Therefore, each phytohormone was prepared at five different concentrations [1.00, 0.50, 0.25, 0.10 and 0.05 % in lanolin (w/w)] and then applied to each tree (total five trees) in June. In this study, there was no replication for each concentration, because we try to examine the difference of the effects among phytohormones in spite of the large variations of concentrations of phytohormones. Each phytohormone was applied with a constant interval in tangential directions ("Treatment" in Experiment I of Fig. 1). In this study, we did not planed the experiments for varying the ratios between two phytohormones (IAA:GA3, GA3:tZ and IAA: tZ = 1:1). Lanolin alone was applied in the upper 10 cm of each phytohormone application as a control ("Control" in Experiment I of Fig. 1). All treatments have each control in longitudinally parallel to all treatments. Pin insertion into the cambium was done at each treatment and control position in June and July to examine the tracheids formed in June–July. Samples were cut from each treatment and control position in August for the measurement of indexes for evaluating tracheid differentiation.

To meet objective (2), tZ [0.1 % in lanolin (w/w)] was applied alone to the stem at breast height in August, September, October and November with a constant interval in tangential directions ("Treatment" in Experiment II of Fig. 1). Based on the results in Experiments I of Fig. 1, it was recognized that the effect of the difference of tZ concentrations on tracheid differentiation was small. We selected 0.1 % tZ for objective (2), because of relative smaller concentration. Lanolin alone was applied in the upper 10 cm of the application as a control ("Control" in Experiment II of Fig. 1). The effects of tZ application on tracheid differentiation were examined in five trees. Pin insertion into the cambium was done at each treatment and control position in August, September, October and November, to examine the tracheids formed in August–September, September–October, October–November and November–December. Samples were cut from each treatment and control position in December for the measurement of indexes for evaluating tracheid differentiation.

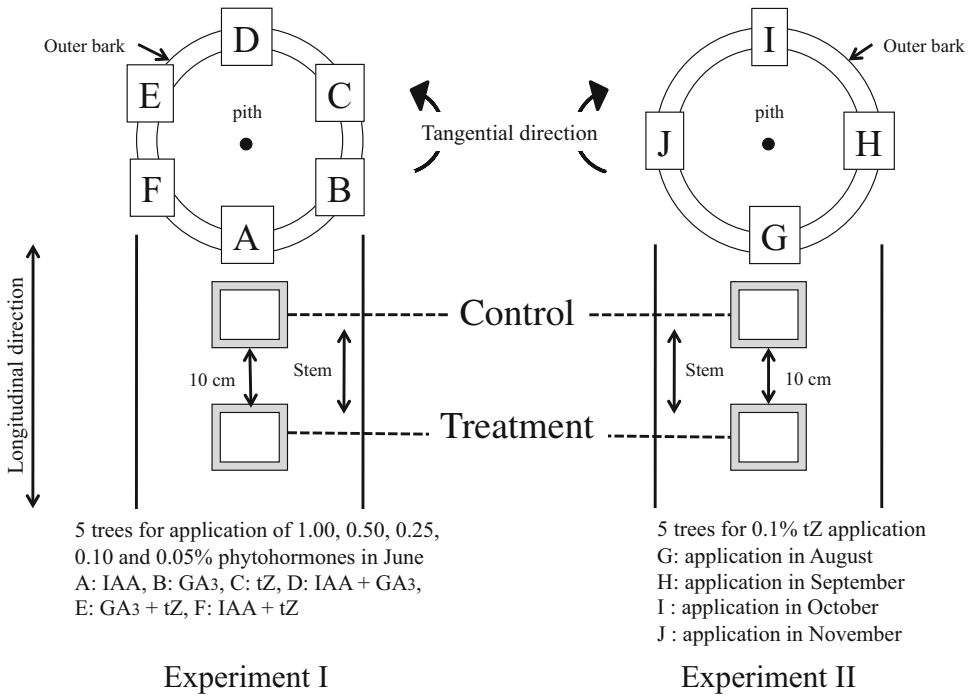

Fig. 1 Scheme of phytohormone application to the intact stem. Pin insertion to cambial tissues was done to distinguish the xylem cells formed after the treatment in the annual rings

According to the results for tZ-type cytokinins in *Arabidopsis* [19], injection of tZ to xylem sap assumed to be better than application of tZ in lanolin to the stem surface. However, we also tried to compare the effect of IAA and GA3, which were applied in lanolin to stem surface, to the effect of tZ simultaneously. In this study, we applied tZ in lanolin to the stem surface, because the difference of application method might affect the tree response to the applied phytohormones.

Measurements of indexes evaluating tracheid differentiation

To meet objectives (1) and (2), we examined the effects of applied phytohormones on tracheid differentiation during latewood formation. Therefore, the cell wall ratio (%) and the microfibril angle (MFA) of the S_2 layer in the secondary wall of tracheids formed during each experimental period were measured as indexes for evaluating tracheid differentiation. Based on the Mork's definition, earlywood has a cell wall ratio <50 % and latewood has a cell wall ratio ≥50 % [26]. Based on the variation of MFA in the annual rings of sugi cultivars [27], the MFAs of earlywood were larger than those of latewood in each annual ring. We assumed that the cell wall ratio and MFA were suitable indexes for evaluating the effects of the applied phytohormones on tracheid differentiation.

The tracheids formed during each experimental period were determined by the pinning method [28]. Small specimens including pin insertion points were cut from the obtained samples, and cross-sections were obtained by sliding microtome. Cross-sections of the wound tissues formed by pin insertion into the cambial-region tissues were obtained. The sites of the cambial initials at the time of pinning were determined by observation of the increasing tracheid row at the wound tissues. Based on the sites of the cambial initials at the pinning times (June, July, August, September, October and November), the tracheids formed during each experimental period (June–July, August–September, September–October, October–November and November–December) were determined.

The cell wall ratio of tracheids formed in June–July was measured in samples in Experiment I of Fig. 1. Samples were embedded in spurr resin (Polysciences, Inc.), and then 7-μm-thick cross-sections were obtained and stained with safranin. As previously reported [29], the cell wall ratio was obtained as the ratio of cell wall area to total cell area, using microscopic digital images of the cross-sections. In this study, for simple measurements of cell wall ratio, microscopic digital images of the cross-sections were obtained and processed to generate binary images (cell wall+cell corner: black, cell lumen: white), and the cell wall ratio was obtained as the ratio of cell wall+cell corner area to the total area (cell wall, cell lumen, cell corner) was measured using image J [30]. The accuracy of the measurement was 0.3 μm/pixel and the cell wall ratio was obtained by averaging ten measurements of different positions in the cross-section.

The MFA of the tracheids formed after the treatments administered in August–November was examined in samples in Experiment II of Fig. 1. The MFA was measured by the iodine-staining method [31]. I_2 crystallized in the gaps between microfibrils in tangential sections of the xylem formed after treatments, and the sections were observed with a light microscope. Under light microscopy, MFA was measured using image analysis software (Image J [30]). The MFA of each position in the xylem formed after treatment was obtained by averaging the measurements of 30 tracheids.

Statistical analysis

For statistical analysis of the obtained data, statistical analysis software (SPSS ver. 16 with Regression and Advanced Models) was used. Using one-way ANOVA and multiple comparisons tests (Tukey's HSD test and Bonferroni test), the significant differences in the cell wall ratio of tracheids (Table 1) between treatments were examined. The *t* test was used to examine the significant differences in MFA between samples with tZ application and controls.

Table 1 Effects of applied phytohormones on tracheid differentiation in June–July

Treatment	*n*	Cell wall ratio (%)
IAA	5	77.3 (13.8)[a]
$Control_{IAA}$	5	80.7 (5.7)[a]
GA3	5	76.1 (4.7)[a]
$Control_{GA3}$	5	77.5 (8.0)[a]
tZ	5	41.6 (9.2)[b]
$Control_{tZ}$	5	74.5 (3.9)[a]
IAA+GA3	4[c]	78.3 (9.4)[a]
$Control_{IAA+GA3}$	5	77.8 (5.4)[a]
GA3+tZ	5	45.6 (9.3)[b]
$Control_{GA3+tZ}$	5	72.6 (12.6)[a]
IAA+tZ	5	69.7 (12.2)[a]
$Control_{IAA+tZ}$	5	75.7 (9.6)[a]

The values represent the averages in 4–5 trees, and the values in parentheses represent the standard deviations. In the cell wall ratio, different characters show significant differences ($p < 0.01$)

n number of samples

[c] One tree with missing of wounding tissue by pin insertion was excluded

Results

Effects of applied phytohormones alone or in combination with each other on tracheid differentiation in June–July

Latewood formation of sample trees has just initiated at the beginning of Experiment I. As shown in Table 1, there were significant differences in the cell wall ratios of tracheids formed after treatment among the types of treatment (ANOVA, $p < 0.01$). It was recognized that the tracheids formed after the application of tZ or GA3+tZ had significantly smaller cell wall ratios than those formed after other treatments including controls (Table 1, multiple comparisons tests, $p < 0.01$). As previously described, earlywood had a cell wall ratio <50 % and latewood had a cell wall ratio ≥50 % [26]. Therefore, the application of tZ or GA3+tZ induced earlywood-type tracheid formation during latewood formation in June–July. However, IAA+tZ did not have a significant effect on the cell wall ratio. As shown in Fig. 1, the concentrations of phytohormones applied in Experiment I (application in June) varied from 0.05 to 1.00 % (w/w in lanolin). However, the standard deviations of the cell wall ratio were not large values (Table 1).

Effects of applied tZ on tracheid differentiation in August–November

To examine the effect of tZ on tracheid differentiation in August–November, in which the activity of xylem formation decreased, we planned Experiment II shown in Fig. 1. Tracheids formed after tZ application in August and September had larger radial diameters and thinner cell walls in comparison with the controls (Fig. 2). The results shown in Fig. 2 were recognized in all tested trees. These results were consistent with the observed effects of tZ on tracheid differentiation in June–July. However, tracheids that formed after tZ application in October had the same radial diameter and cell wall thickness in comparison with those of the controls. In November, xylem formation ceased, and tZ application could not induce the reactivation of the cambium.

The MFA of earlywood is usually larger than that of latewood in sugi trees [27]. The MFA variations after the tZ application in August are shown in Fig. 3. The MFAs of tracheids formed after tZ applications were significantly larger than those of the controls. Although the effects of the application of tZ on MFA seen in August were also recognized in September, the effects were restricted in tracheids formed just after the application (data not shown).

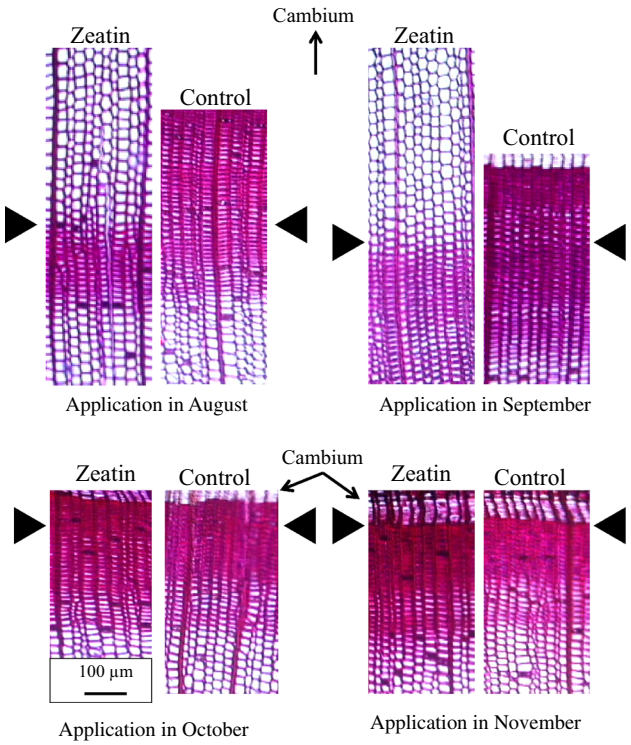

Fig. 2 Effect of applied *trans*-zeatin on cross-sectional dimensions of tracheids. *Arrowheads* show the positions of the application dates in annual rings. The *upper* part of *pictures* showed the tracheids near the cambium

Discussion

We examined the effects of applied phytohormones on tracheid differentiation in intact sugi trees forming latewood in this study. The obtained results showed that the application of tZ induced earlywood-type tracheid formation (Table 1; Figs. 2, 3). The results of application of phytohormones in combination with each other were very interesting (Table 1). There were significant effects of the application of GA3+tZ on the cell wall ratio in June–July. GA3 alone did not significantly affect the cell wall ratio. Therefore, the significant effects of GA3+tZ show that GA3 did not inhibit the effects of applied tZ. However, IAA+tZ did not significantly affect the cell wall ratio. We assumed that the application of IAA inhibited the effects of applied tZ. Based on the studies on transgenic plants, it was reported that auxin-overproducing plants contained many more vessel elements of smaller size [32], and plants with lowered IAA levels contained fewer vessel elements of larger size [33] than did control plants. In ring-porous trees, cytokinins enhances cambium sensitivity to low level IAA streams originating in swelling buds and creates the special conditions that enable the differentiation of very wide earlywood vessels during limited period of time in spring

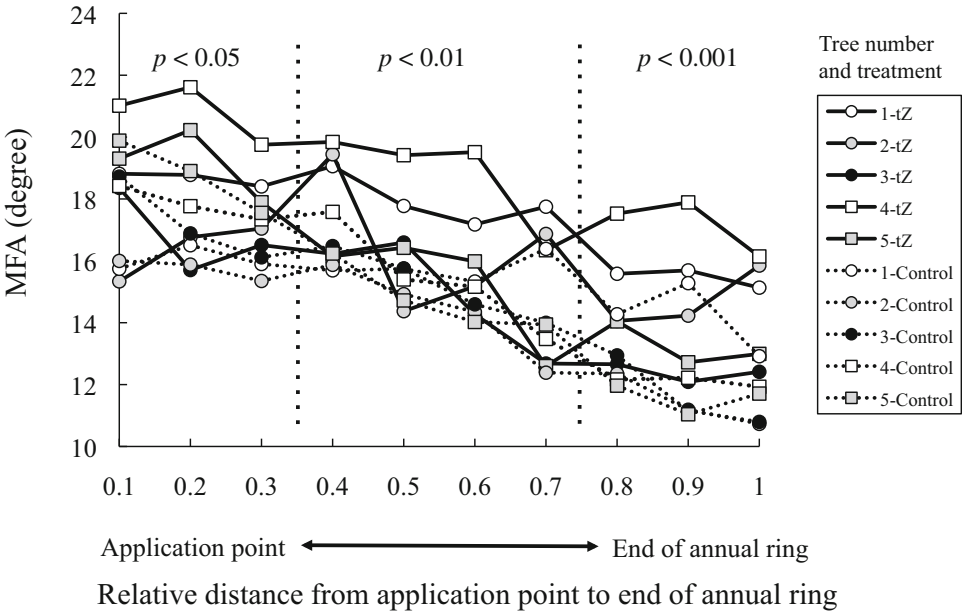

Fig. 3 Effect of applied *trans*-zeatin in August on radial variation of microfibril angle (MFA). Variation of MFA were measured at the every 0.1 relative distance from the application point to end of annual ring. The MFAs in three parts (0.1–0.3, 0.4–0.7 and 0.8–1 relative distance) were compared between the treatment and control groups

[34]. These results for IAA and cytokinins on vessels assumed to be relevant to our results on tracheids in sugi trees.

However, we did not measure endogenous phytohormones in the cambial region tissues of the samples used in this study. It was previously pointed out that conclusions drawn from studies on applied phytohormones that did not measure endogenous phytohormones must be viewed with caution [35]. The probability that the response (smaller cell wall ratios resulting from the application of tZ) is due to very high concentrations of applied tZ compared to endogenous tZ could not be excluded, although the effects of the concentrations of applied tZ were small in the range of concentrations tested (standard deviations in Table 1). It was reported that the amounts of endogenous IAA varied seasonally [11, 36], although endogenous cytokinin levels did not vary greatly between dormant tissues and actively dividing and differentiating tissues in *P. sylvestris* [37] and *Larix kaempferi* [38]. A recent study of endogenous cytokinins reported that seasonal variation of tZR was recognized in the crown of *Abies nordmanniana*, but not in the root [39]. The reason of the significant effects of the application of tZ on the cell wall ratio in our study may be the decreased amounts of endogenous cytokinins in the season of latewood formation. As previously described, water deficit increased cytokinin oxidase and implied cytokinin degradation in *Zea mays* [24]. Sugi trees may not have enough amounts of endogenous cytokinins for earlywood formation in the season of latewood formation, because of the water deficits induced by the weather condition. From the obtained results in our study, we hypothesized that the seasonal variation of the ratio of endogenous tZ to IAA may affect the tracheid differentiation (earlywood or latewood). In future studies, we will try to examine the relationship between the seasonal variation of the ratio (endogenous cytokinins amounts/IAA amounts) and the tracheid differentiation (earlywood or latewood).

In October and November, the decline of xylem formation and the dormancy of cambium were observed, respectively (Fig. 2). The application of tZ could not inhibit the decline of xylem formation and the dormancy of the cambium. It was reported that localized heating induced reactivation of cambium in coniferous species from late winter to early spring [40]. However, in December, localized heating could not induce accelerated reactivation of cambium in sugi, because of the low sensitivity of cambium [41]. Therefore, the observed effects of applied tZ in this study may be recognized only when the environmental factor (temperature) is optimal for xylem formation and the sensitivity of cambium is relatively high.

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