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NOTE

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Changes in levels of endogenous plant hormones in cambial regions of stems of *Larix kaempferi* at the onset of cambial activity in springtime

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Abstract The total amounts of endogenous indole-3-acetic acid (IAA), cytokinins, and abscisic acid (ABA) were quantified by gas chromatography–selected ion monitoring–mass spectrometry (GC-SIM-MS) in cambial regions of the main stems of *Larix kaempferi* during the spring season. During the sampling period, cambium in the dormant state entered the active meristematic state. The total amount of IAA did not change at the onset of cambial reactivation but increased when the active division of cambial cells became apparent. Four cytokinins – *trans*- and *cis*-ribosylzeatin (RZ), N^6-isopentenyladenine (iP), N^6-isopentenyladenosine (iPA) – were quantified, but no zeatin (Z) was detected. The total amount of the four cytokinins together and the total amount of isopentenyl-type cytokinins (iP and iPA) varied during the sampling period but did not appear to be specifically associated with cambial activity. The total amounts of *trans*- and *cis*-RZ remained relatively constant during the sampling period, as did the total amount of ABA. The results suggest that there is little correlation between total amounts of endogenous plant hormones in the cambial region and reactivation of the cambium during the spring.

Key words Indole-3-acetic acid · Cytokinins · Abscisic acid · Cambial activity · *Larix kaempferi*

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Introduction

Increases in the diameters of tree stems are due to the activity of cambium.[1] Such activity in temperate-zone conifers exhibits annual periodicity.[2,3] In these trees cambial activity resumes in the spring (cambial reactivation), with a change from the quiescent dormant state to the active state. Cambial reactivation is inhibited in debudded cuttings.[4] However, a supply of indole-3-acetic acid (IAA), an auxin, to the apical surface of debudded cuttings of conifers activates the cambium at the quiescent stage of dormancy under favorable growth conditions.[4–10] Such observations demonstrate the importance of IAA (which is transported basipetally in the cambium and its most recent derivatives from developing shoots[11,12]) during cambial reactivation of stems. The level of endogenous IAA in the cambial region varies seasonally.[9,12–21] However, measurements of temporal and spatial variations in levels of endogenous IAA in the cambial region have yielded conflicting results, so no consistent relation between endogenous IAA levels and the seasonal process of cambial activity has been established.[11,22] More information is needed to characterize the relation between endogenous IAA and cambial reactivation.

Plant hormones other than IAA also promote or inhibit cambial activity in woody plants,[11,23] but it remains unclear whether such plant hormones are directly involved in regulating the annual cycle of cambial activity. This issue is unresolved, for the most part, because of the paucity of information available about the temporal and spatial distribution of these plant hormones in the cambial region.[11,24] It has been well established that cytokinins are key factors in cambial development.[23,25,26] The application of exogenous cytokinins to the stems of trees results in stimulation of cambial activity in many but not all cases.[23] Some cytokinins have been identified in the cambial regions of conifers,[11] but there have been only a few reliable reports of endogenous cytokinin levels in the cambial regions of conifers.[27–30]

The present study was designed to investigate in further detail the relation between endogenous plant hormones and the seasonal changes in cambial activity, focusing on the

onset of cambial activity during the spring. We measured the total amounts of endogenous IAA, cytokinins, and abscisic acid (ABA), which is known to be an inhibitor of cambial growth, in the cambial regions of main stems of *Larix kaempferi*, a deciduous conifer.

Materials and methods

Plant materials

Seven healthy, approximately 20-year-old specimens of *Larix kaempferi* growing in the Nakatsugawa Plantation Forest, Chichibu, Japan were used in this study. The trees were 11.5–14.5 m in height. Bud break in the lower parts of the crown was visible at the beginning of April. A single tree was felled for quantitation of plant hormones on each sampling date, at approximately 10-day intervals between March 18 and May 17. A disk approximately 20 cm in thickness was cut from the main stem at breast height and was immediately transported to the laboratory on Dry Ice.

Two vertically adjoining blocks of an area $30 cm^2$ (5×6 cm, for quantitation of IAA and ABA) and $60 cm^2$ (5×12 cm, for quantitation of cytokinins) were excised with knives and chisels from the disk. The blocks used for quantitation of cytokinins were prepared from March 28 onward. After removing the outer bark, the tissues, which included small amounts of mature phloem, differentiating phloem (when present), cambial zone cells, differentiating xylem (when present), and possibly small amounts of the previous year's xylem, were rapidly dissected out with a sharp knife and scalpel on clean filter paper. Each sample and the associated piece of filter paper were extracted three times with 80% methanol for 24 h at −20°C in darkness.

Quantitation of IAA and ABA

After methanol extraction, [$^2H_2$]-IAA (2.12 μg) and [$^2H_6$]-ABA (1.2 μg), synthesized as described by Hoskins and Pollitt[31] and Rivier et al.,[32] respectively, were immediately added as internal standards to each extract, and then each extract was concentrated in vacuo. Each residue was adjusted to pH 2.8 with 0.5 N HCl and then extracted three times with diethyl ether. The diethyl ether fraction was concentrated in vacuo and extracted with 5% $NaHCO_3$ at < pH 8. The aqueous fraction was adjusted again to pH 2.8 with 0.5 N HCl and extracted three times with diethyl ether. The diethyl ether fraction was dehydrated over Na_2SO_4 and dissolved in 50% acetonitrile.

The solution in acetonitrile was subjected to high-performance liquid chromatography (HPLC). A Shimadzu LC-6A system equipped with an ultraviolet (UV) detector (280 nm) was used for HPLC. The reverse-phase column used was a μ Bondasphere column (Waters Associates), and it was eluted with 25% acetonitrile that contained 1% acetic acid. All samples were passed through a 0.45-μm membrane filter prior to injection onto the column. The fractions that corresponded to the retention times of authentic IAA and ABA were collected separately. The ABA fraction was further subjected to HPLC on the same column in 55% methanol that contained 1% acetic acid.

The IAA and ABA fractions were dried over P_2O_5 in vacuo. The dry residues from the IAA and ABA fractions were dissolved in absolute acetonitrile (40 μl) and *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (20 μl) (Tokyo Kasei). The mixtures were heated at 80°C for 30 min for the IAA fraction and at 70°C for 15 min for the ABA fraction.[21,33–35]

The derivatives of the IAA and ABA fractions were analyzed by gas chromatography–selected ion monitoring–mass spectrometry (GC-SIM-MS). A Shimadzu LKB 9000 gas chromatograph–mass spectrometer, equipped with a multiple ion detector, was used under the following conditions: ionizing energy 20 eV; separating port temperature 280°C; glass column (1 m $\times$ 3 mm i.d.) packed with 1.5% OV-1; carrier gas He; and flow rate 30 ml min^{-1}. The column temperature was 210°C for IAA and 195°C for ABA. Ratios of m/z 405:403 and m/z 327:321 were used to calculate the amounts of endogenous IAA and ABA, respectively, according to appropriate standard curves.[21,33–35]

Quantitation of cytokinins

After methanol extraction, as described above, [$^2H_5$]-zeatin (Z; 3.08 μg), [$^2H_5$]-ribosylzeatin (RZ; 3.74 μg), [$^2H_6$]-isopentenyladenine (iP; 3.21 μg), and [$^2H_6$]-isopentenyladenosine (iPA; 2.93 μg), synthesized as described previously,[36,37] were immediately added as internal standards to each extract. Deuterium-labeled samples of Z and RZ consisted of the *trans*-isomer (92%) and the *cis*-isomer (8%).[38]

Each extract was evaporated to dryness in vacuo. The residue was dissolved in a small amount of water and loaded onto a Sep-pak C_{18} cartridge (Waters Associates). The cartridge was washed three times with 15 ml of 40% methanol for recovery of the cytokinin fraction.[39] The solution in 40% methanol was subjected to HPLC with a UV detector (270 nm) on a μ Bondasphere reverse-phase column eluted with 40% methanol. All samples were passed through a 0.45-μm membrane filter prior to injection. Fractions that corresponded to the retention times of authentic Z and RZ and of authentic iP and iPA were collected separately. Each fraction was further purified by HPLC on the same column, with 15% acetonitrile as the mobile phase for the Z plus RZ fraction and 30% acetonitrile for the iP plus iPA fraction. Each of the two resultant fractions was subjected to HPLC on a gel-permeation column (Asahipak GS-320), with a mixture of absolute methanol and 2.5% ammonium hydroxide (3:2 v/v) as the mobile phase. In this way, we obtained four fractions that contained Z, RZ, iP, and iPA, respectively. The two fractions containing Z and RZ, respectively, were further subjected to HPLC on the μ Bondasphere column with 30% acetonitrile as the mobile phase.

The four cytokinin fractions were dried over P_2O_5 in vacuo. The Z and iP fractions were each dissolved in a mixture of absolute acetonitrile (40 μl), *N,O*-

bis(trimethylsilyl)acetamide (20μl), and trimethylchlorosilane (2μl). They were then heated at 70°C for 10 min.[29,38] The RZ and iPA fractions were dissolved in a mixture of absolute pyridine (35μl), hexamethyldisilazane (10μl) (Tokyo Kasei), and trimethylchlorosilane (5μl) and were then heated at 120°C for 1 h.

The derivatives of the four cytokinin fractions were analyzed by GC-SIM-MS as described above. The column temperature was 200°C for Z, 255°C for RZ, 165°C for iP, and 245°C for iPA. *Trans-* and *cis*-RZ were separated under the previously cited conditions for GC.[36,38] Ratios of *m/z* 440:435, *m/z* 629:624, *m/z* 353:347, and *m/z* 557:551 were used to calculate the amounts of endogenous Z, RZ, iP, and iPA, respectively, according to appropriate standard curves.[38,39]

Anatomical analysis

Small blocks, including the cambial region, were cut from the main stem at breast height near the portions that had been sampled for quantitation of plant hormones on each harvest date. The blocks were fixed in a mixture of formaldehyde, acetic acid, and ethanol (FAA), dehydrated in an ethanol series, and embedded in celloidin. Thin transverse sections (approximately 15μm thick) were cut, stained in a solution of safranin-fast green, and examined under a light microscope.

Results

The level of each plant hormone in the cambial region is expressed as the amount per square centimeter, and this level is referred to as the total amount. In general, such levels are expressed either on an area basis, which gives the total amount per unit area, or on a weight basis, which gives the concentration, (i.e., the total amount per unit weight of fresh or dry tissue). In our investigation, several cell types, including some cells of the mature phloem and possibly a small number of cells from the previous year's xylem, were included in the samples of cambial tissue. Thus, the weight of the tissue varied depending on the way in which the samples had been obtained. Such variation would reflect equivocal results in terms of concentration. In addition, it has been demonstrated that endogenous IAA is distributed with a steep gradient in its level across the cambial region, with a maximum in the cambium and its most recent derivatives.[12,40–42] Thus, estimates of the concentration of IAA (on a weight basis) are of limited use for evaluating the variations in IAA levels in the cambial region.[22,41] By contrast, the total amount of IAA (on an area basis) gives consistent results. For these reasons, only amounts on an area basis are given here.

We found IAA in the cambial region throughout the sampling period (Fig. 1). The total amount of endogenous IAA was relatively constant, ranging from 30 to 40 ng cm^{-2} by the beginning of May and then increasing. In this study, a single tree was felled on each sampling date. Although we cannot exclude the possibility of variations among trees in terms of levels of plant hormones, Sundberg et al.[17] observed that such variations in the IAA levels, calculated as the relative standard deviation, were only about 15% in specimens of *Pinus sylvestris* of the same age and the same crown shape that had been grown under the same environmental conditions. The total amount of IAA probably reached a maximum at a later date, as it has been reported that the total amount of endogenous IAA in the cambial regions of stems of other conifers is generally highest during summer.[9,12,17–19,21]

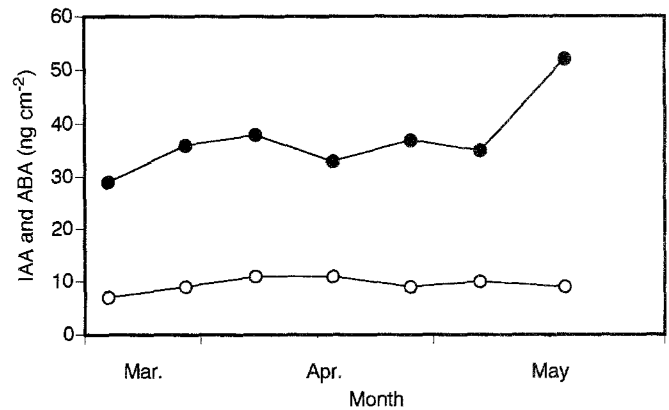

Fig. 1. Total amounts of endogenous indole-3-acetic acid (IAA) (*filled circles*) and abscisic acid (ABA) (*open circles*) in cambial regions of *Larix kaempferi* main stems. See text for details

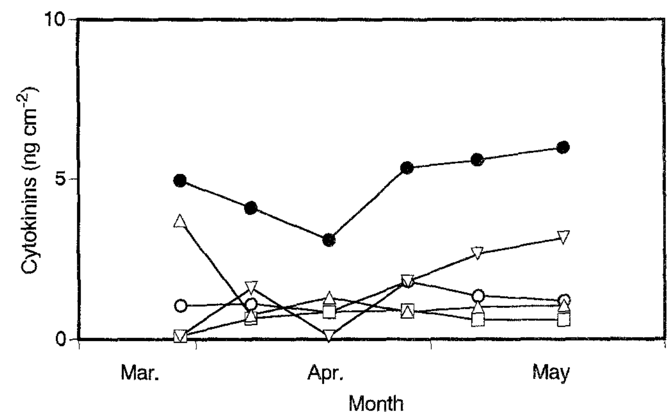

Fig. 2. Total amounts of endogenous cytokinins in cambial regions of *Larix kaempferi* main stems. *Filled circles*, total cytokinins (the sum of four cytokinins); *open circles*, *trans*-ribosylzeatin; *squares*, *cis*-ribosylzeatin; *inverted triangles*, N^6-isopentenyladenine; *triangles*, N^6-isopentenyladenosine. See text for details

We quantified *trans-* and *cis*-RZ, iP, and iPA in cambial regions of stems of *Larix kaempferi* by GC-SIM-MS (Fig. 2). We failed to detect Z, which has been regarded as an active cytokinin. Nonetheless, we cannot exclude the possible presence of undetectable low levels of Z in the cambial region of stems of *Larix kaempferi* because Z has been identified in the cambial regions of stems of other conifers,[27,29] and small amounts of Z were found in the cambial regions of the stems of *Pinus sylvestris*.[30]

The total amounts of endogenous cytokinins in the cambial region were low compared with those of IAA and ABA. The sum of the total amounts of the four cytokinins varied slightly throughout the sampling period, but the total amounts of *trans*- and *cis*-RZ were relatively constant throughout the sampling period. The total amount of iP increased after the middle of April, whereas that of iPA had decreased by the beginning of April and then varied only slightly.

The ABA was detected in the cambial region throughout the sampling period (Fig. 1). The total amount of endogenous ABA was lower than that of IAA and showed minimal variation.

Light microscopy revealed that cambial reactivation (visualized as the first periclinal divisions) was apparent by mid-April (data not shown). Centripetal cambial derivatives with radially enlarging primary walls and active cambial division were observed by the middle of May.

Discussion

We detected IAA in inactive cambial regions of stems of *Larix kaempferi* before obvious bud break; in fact, readily detectable IAA has been found in the dormant cambium of other deciduous conifers.[20,43] Moreover, exogenous [^{14}C]-IAA was transported basipetally in stems or shoots of conifers in winter.[43–45] Such observations suggest the possibility that IAA is synthesized in dormant buds and transported to the cambium. A continuous supply of IAA during winter might be required for maintenance of the morphological state of fusiform cambial cells, as fusiform cambial cells dedifferentiate into axial parenchyma cells in the absence of a continuous supply of IAA.[46]

In *Pinus sylvestris*, the total amount of IAA in cambial regions of stems increased steadily as reactivation of the cambium from the dormant state occurred.[18] By contrast, the total amount of endogenous IAA in the cambial region of *Larix kaempferi* remained constant during the resumption of cambial cell division. In this study, therefore, there was no increase in the total amount of IAA during reactivation of the cambium. This observation indicates that cambial reactivation might be independent of changes in the total amount of endogenous IAA. However, the total amount of IAA began to increase several weeks after obvious bud break. The increase in the total amount of IAA was associated with an increase in the meristematic activity of cambial cells. This result supports the observation that application of exogenous IAA, which raises internal levels of IAA temporarily,[4] promotes the frequency of cambial cell division.[11,23] Studies have demonstrated the occurrence of a steep radial gradient in the level of endogenous IAA across the cambial region.[12,40–42] In addition, a close relation has been reported between the radial width of the cambial zone and the radial width of the gradient in the IAA level.[41] The radial width of the cambial zone is closely related to the number of dividing cells there. Moreover, the radial width of the IAA gradient was closely correlated with the total amount of IAA in the cambial region.[41] Therefore, the increase in the total amount of IAA transported in the polar direction from developing buds appears to have increased the number of dividing cells in the cambial zone.

In the present study, four cytokinins, including a *cis*-isomer, were quantified by GC-SIM-MS. The sum of the total amounts of cytokinins varied slightly throughout the sampling period, but variations were not specifically associated with changes in cambial activity. Moreover, the total amount of *trans*-RZ was almost constant, and Z was not detected. Hence there appears to be little correlation between the total amount of endogenous cytokinins, which are assumed to be biologically active, and the change in cambial activity in the spring. By contrast, the total amount of endogenous cytokinins in cambial regions of stems of *Pinus sylvestris* was higher in summer, the season of greatest cambial activity, than in mid-winter, when the cambium is dormant.[30] Large amounts of endogenous cytokinins might be important for maintenance of active cambial growth.

The total amount of isopentenyl-type cytokinins (iP and iPA) changed in the opposite direction; the amount of iPA had decreased by the beginning of April, at which point the amount of iP increased (Fig. 2). The increase in iP might be attributable to conversion of iPA (the 9-riboside of iP) to iP during early spring. The change in the total amount of isopentenyl-type cytokinins occurred before bud break and before the increase in the total amount of IAA (Fig. 1). Thus, it seems that an increased rate of biosynthesis or metabolism (or both) of isopentenyl-type cytokinins might precede active biosynthesis of endogenous IAA. Further studies are needed to clarify the physiological roles of isopentenyl-type cytokinins in the control of cambial activity.

Abscisic acid is known to be an inhibitor of cambial activity, but its exact role in the regulation of cambial dormancy remains unclear.[24,47] In the present study, the total amount of ABA remained constant throughout the sampling period. Thus, there was no clear correlation between the total amount of ABA and resumption of the division of cambial cells and the differentiation of new xylem cells. Our observations support the hypothesis that the control of cambial dormancy is independent of endogenous ABA.[13,15,21,34]

Conclusions

We detected several plant hormones in cambial regions of main stems of *Larix kaempferi* throughout the sampling period. The first divisions of cambial cells (cambial reactivation) were not associated with changes in the total amounts of endogenous plant hormones. This result suggests that extrinsic factors other than plant hormones might regulate transition from the quiescent dormant state to the active state. It is probable that an increase in temperature is a limiting factor for the onset of cambial reactivation in the spring because the localized heating of stems in winter induces localized cambial reactivation in evergreen conifers, such as *Pinus contorta*,[6] *Cryptomeria japonica*,[48] and *Abies*

sachalinensis.[49] However, Oribe and Kubo[48] noted that such heat treatment for 2 weeks was insufficient for localized reactivation of the cambium in stems of *Larix kaempferi*, so additional factors might be needed for reactivation of the cambium in the stem of this deciduous conifer. An increase in the total amount of endogenous IAA appeared to be related to initiation of the active division of cambial cells, suggesting involvement of IAA in the control of cambial growth.

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