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Kazumasa Yoshida · Mitsuru Nishiguchi
Shojiro Hishiyama · Atsushi Kato · Koetsu Takahashi

Generation and alteration of norlignans in a transition zone during the drying of a *Cryptomeria japonica* log

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Abstract Sugi (*Cryptomeria japonica* D. Don) produces secondary metabolite norlignans in xylem. Several norlignans are involved in the coloration of heartwood and defense of sapwood against microbial invasion. Their biosynthetic process should be well understood so that their properties can be exploited to improve the quality and utility of *C. japonica* wood. Unfortunately, information on the norlignan biosynthesis is limited because norlignans are mainly synthesized in a particular season in the transition zone (TZ) along with the heartwood formation, and is difficult to study. Although the generation of two norlignans of *C. japonica*, agatharesinol and (*E*)-hinokiresinol, has been reported, systems for producing other norlignans are not yet known. To establish a novel norlignan generating system, we examined the changes occurring in norlignans in a TZ during the process of drying a *C. japonica* log. On the day of felling, the TZ contained agatharesinol and (*E*)-hinokiresinol, which increased until they reached a maximum on day 40 after felling. Sequirin-C appeared on day 40 and increased to day 70. The generation of sequirin-C in the TZ can be used to investigate the biosynthetic process of heartwood norlignans. This study describes for the first time the changes that occur in the composition of norlignan during the drying of the TZ.

Key words Norlignan · Transition zone · Drying · *Cryptomeria japonica* · Sugi

K. Yoshida (✉) · M. Nishiguchi · S. Hishiyama · A. Kato
Forestry and Forest Products Research Institute, Tsukuba 305-8687, Japan
Tel. +81-29-873-3211; Fax +81-29-873-0507
e-mail: ykazu@ffpri.affrc.go.jp

K. Takahashi
Faculty of Agriculture, Department of Bioenvironment, Yamagata University, Tsuruoka 997-8555, Japan

Introduction

Sugi (*Cryptomeria japonica* D. Don) contains secondary metabolite norlignans, which are a group of compounds with a diphenylpentane skeleton (Fig. 1).[1] Sequirin-C and cryptoresinol are related to the heartwood color,[2] which affects the price of sugi lumber. Antifungal (*E*)-hinokiresinol is produced when the sapwood of *C. japonica* is infected with the dieback fungus *Guignardia cryptomeriae*.[3]

Even though norlignans are involved in the properties and physiology of *C. japonica* xylem, they have not been exploited to improve the quality or utility of wood. Information on their biosynthetic process is needed so that the composition of norlignans can be altered through regulation of their biosynthesis. However, little is known about this process. Early proposals for a norlignan biosynthetic pathway based on structures lacked sufficient experimental evidence, including isolation and characterization of immediate precursors, enzymes, and genes involved in synthesis.[4–7]

Recently, (*Z*)-hinokiresinol was reported to be synthesized from 4-coumaryl alcohol and a 4-coumaroyl compound in cultured cells of *Asparagus officinalis* after fungal elicitor treatment.[8] It was also demonstrated that formation of (*Z*)-hinokiresinol and (*E*)-hinokiresinol were catalyzed from 4-coumaryl alcohol and 4-coumaroyl CoA, and from 4-coumaryl 4-coumarate by the enzyme preparations, respectively, from fungal-elicited asparagus cultured cells[9] or *C. japonica* cultured cells.[10] In spite of advances in research on norlignan biosynthesis, however, a large part of the biosynthetic process remains unknown. This is because studies of the biosynthetic process of norlignans in woody plants are difficult because norlignans are mainly synthesized in a particular season in the transition zone (TZ) along with heartwood formation.[11,12]

If the generation of norlignans can be induced in cells of woody plants, it would be useful for examining the norlignan biosynthetic process. At present, two systems in *C. japonica* are available for such a purpose. One is *C.*

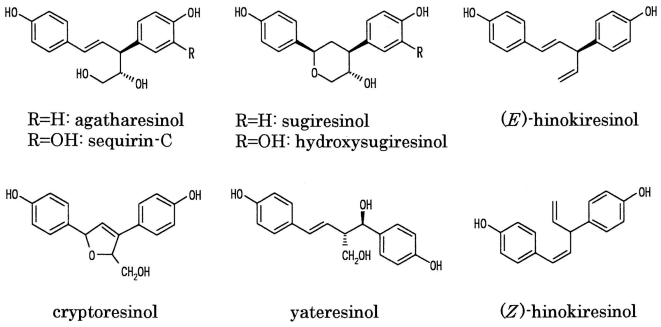

Fig. 1. Structures of norlignans

japonica cultured cells in which (*E*)-hinokiresinol is produced.[10] The other is the generation of agatharesinol in sapwood during the process of drying *C. japonica* logs that are stored indoors.[13–15] In these systems, only particular sorts of norlignans are produced, so there is only limited application of the systems to the examination of the biosynthetic process of norlignans.

We focused on the TZ, which is the site of heartwood formation and has not yet been examined in terms of norlignan generation during the drying process of logs. To determine whether norlignans are generated in the TZ during the drying of *C. japonica* logs, the changes occurring in norlignans were investigated. In addition, changes in norlignans in sapwood were analyzed, and the relationship between the production of norlignans and moisture content is discussed.

Materials and methods

Preparation of samples

A 19-year-old *Cryptomeria japonica* tree that grew in the nursery of the Forestry and Forest Products Research Institute (Tsukuba) was felled on 28 November 2003. A 0.8-m-long trunk section from a height of 1 m above the ground was excised and stored in a room at 15°–25°C with 25%–30% relative humidity. A 1-cm-thick disk was cut off from the bottom end of the log and discarded preceding the collection of a 6-cm-thick sample disk on each of postharvest days 0, 10, 20, 40, and 70. The disks were divided into sapwood and the TZ. These samples were cut into small pieces and those for extraction were soaked in methanol (MeOH).

Extraction and analysis of norlignans

The wood pieces were extracted with MeOH using a Soxhlet apparatus. Extracts were evaporated to dryness under reduced pressure and redissolved in MeOH to make the volume 1 ml/g dry weight (dw) of wood, which was determined after extraction. As an internal standard, 3,4-dimethoxybenzoic acid was added at 1 mg/g dw. Samples were trimethylsilylated according to common methods and analyzed by gas chromatography (GC) with a Hitachi G3000. The GC conditions were as follows: column NB-5 (30 m × 0.25 mm i.d.), carrier gas He, flow rate 1 ml/min, temperature rising from 180°C by 4°C/min to 280°C and held at 280°C for 25 min, injection and detector temperature 280°C. Norlignans were identified by comparing the retention times with those of authentic compounds. Agatharesinol, (*E*)-hinokiresinol, sequirin-C, and cryptoresinol were identified by gas chromatography-mass spectrometry (GC-MS) using a Jeol JMS-SX102A. The GC-MS conditions were as follows: column NB-5 (30 m × 0.32 mm i.d.), carrier gas He, flow rate 1 ml/min, temperature rising from 150°C by 4°C/min up to 280°C, and electron-impact ionization (70 eV).

Measurement of moisture content

Some of the wood pieces that were cut from the sample log were weighed before and after drying at 105°C for 24 h. Percentage moisture content was calculated as (fresh weight – dw)/dw ×100.

Results

Changes in norlignans in the transition zone

The TZ contained 512 μg/g dw of agatharesinol on day 0 (Fig. 2a). The content apparently decreased on day 10, then increased after that, reaching a maximum of 1139 μg/g dw on day 40. The content at maximum amounted to 41% of the 2759 μg/g dw found in the heartwood (Table 1).

(*E*)-Hinokiresinol was present at a concentration of 11 μg/g dw on day 0 (Fig. 2b). It increased to day 40 by a factor of approximately 4.6 and was still at the same level on day 70. Sequirin-C was not detected on days 0, 10, and 20, but it appeared on day 40 and increased to day 70 (Fig. 2c).

Changes in norlignans in sapwood

No prominent norlignans were detected in fresh sapwood. Agatharesinol content remained at low levels from day 0 to day 20 (Fig. 2a). On day 40, its content had increased to a maximum of 297 μg/g dw, which corresponded to 11% of that in the heartwood. The accumulation pattern of agatharesinol was consistent with patterns described previously.[15]

(*E*)-Hinokiresinol was not detected on days 0 and 10 (Fig. 2b). It appeared on day 20 at a concentration of 12 μg/g dw, and remained at a constant level thereafter. Sequirin-C was not found on any day (Fig 2c). Other norlignans, such as sugiresinol, hydroxysugiresinol, cryptoresinol, and yateresinol, were not observed, either in the TZ or in the sapwood (data not shown).

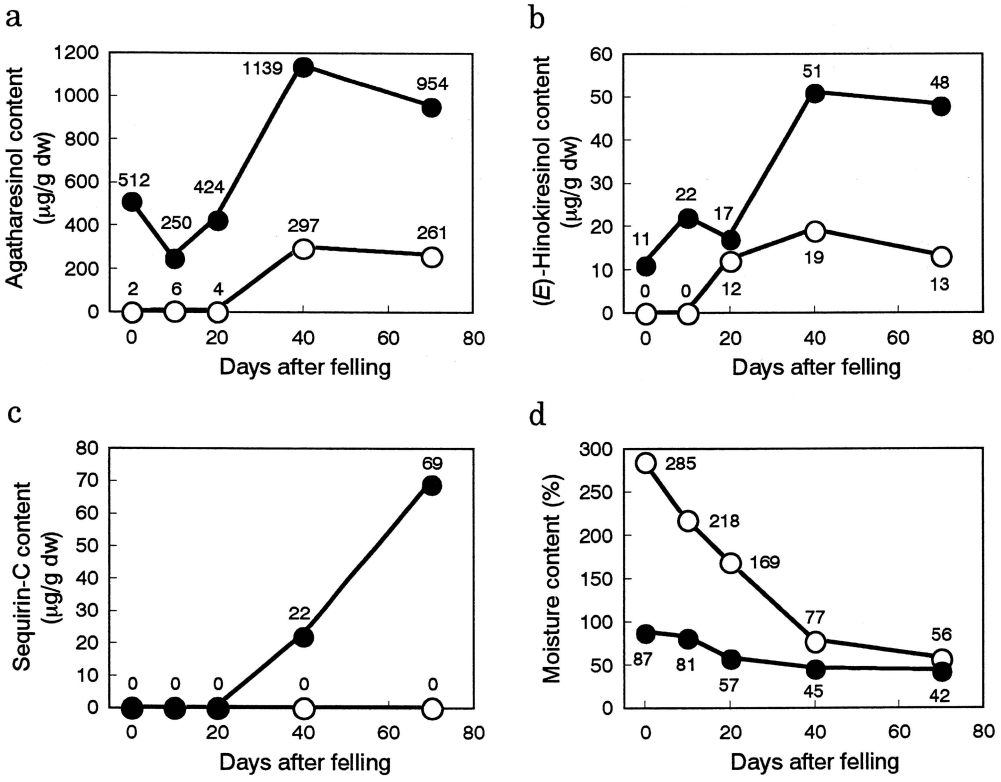

Fig. 2a–d. Changes in norlignan (**a–c**) and moisture content (**d**) in the transition zone and the sapwood during the process of drying a *Cryptomeria japonica* log. *Filled circles*, transition zone; *open circles*, sapwood. *Numerals* indicate values of content. Each data point of the moisture content represents the mean of two independent measurements

Table 1. Norlignan and moisture contents in fresh heartwood

Norlignan content (μg/g dw)				Moisture content (%)
Agatharesinol	(*E*)-Hinokiresinol	Sequirin-C	Cryptoresinol	
2759	61	53	69	94

dw, Dry wood

Changes in moisture content during the withering process

The initial moisture content was 87% in the TZ and 285% in the sapwood (Fig. 2d). The moisture content of both tissues decreased during the drying process, with the sapwood losing more of its water content than the TZ. The moisture content of the sapwood on day 40 had dropped to 77%, which was about the same level as the fresh TZ. On day 70, the moisture contents of the TZ and the sapwood were 42% and 56%, respectively.

Discussion

The TZ is thought to be the site where extractives are synthesized during heartwood formation.[16] The specimen used in this study was felled in November; therefore, we assumed that heartwood formation had progressed,[11,12] and that the TZ had started to synthesize and accumulate some norlignans. In fact, a significant amount of agatharesinol, a major heartwood norlignan in the specimen, was detected in the TZ on day 0 (Fig. 2a). This result is in accordance with immunohistochemical detection of agatharesinol in the TZ of *Cryptomeria japonica*.[17]

An increase of MeOH-soluble compounds in the TZ during drying has been suggested based on a comparison of the amount of MeOH extractives between the fresh and dried TZ.[18] However, the composition and the time course of accumulation of the generated MeOH extractives have not been investigated yet. In the present study, (*E*)-hinokiresinol and sequirin-C, in addition to agatharesinol, were accumulated in the TZ during the drying process (Fig. 2a–c). This result suggests that the TZ possesses the ability to synthesize some norlignans, and that their biosynthetic processes are stimulated by drying.

The accumulation patterns of agatharesinol and (*E*)-hinokiresinol in the TZ were similar to those of the sapwood, but the amounts were greater in the TZ than in the sapwood. Given the level of agatharesinol and (*E*)-hinokiresinol content on day 0, it is reasonable to conclude that the capacity to produce norlignans is higher in the TZ than in the sapwood.

The generation of sequirin-C in the TZ is noteworthy. Sequirin-C was not detected in the sapwood throughout the drying process. In the TZ, it was not detected on days 0, 10,

and 20, but it had appeared by day 40 and increased to day 70 (Fig. 2c). Its accumulation pattern was different from those of agatharesinol and (*E*)-hinokiresinol.

In addition to these norlignans, the heartwood contained a small amount of cryptoresinol (Table 1). This compound had not been detected in either the TZ or the sapwood at any point in the drying process. Considering the composition of norlignans in each tissue and the order of their appearance during the drying process, it appears that agatharesinol and (*E*)-hinokiresinol were synthesized in the early stage of norlignan biosynthesis in the specimen, followed by sequirin-C, and finally cryptoresinol. This speculation is in agreement with early proposals for norlignan biosynthetic pathways, in which agatharesinol is synthesized early in the norlignan pathway, then sequirin-C, sugiresinol, hydroxysugiresinol, and cryptoresinol are formed by subsequent hydroxylation and cyclization.[7,19,20]

Moisture content of the TZ (~100%) is known to be lower than that of sapwood in *C. japonica*.[21] In the sapwood, the generation of agatharesinol was prominent from day 20 to 40 when the moisture content dropped to a level similar to that of the fresh TZ (Fig. 2d). This process may mimic the transition of sapwood to the TZ that naturally occurs in standing trees.

It has been suggested that the entry of air into vessels or tracheids is one of the first processes toward heartwood formation,[22] but definite values of moisture content have not been proposed to date. Given a similar previous result,[15] it appears that the lowering of moisture content below a certain level, approximately 100%, is related to the initiation of norlignan biosynthesis in *C. japonica* xylem.

In this study, we observed the generation of three norlignans in the TZ during the process of drying a *C. japonica* log. There have been experimental systems in which agatharesinol[13–15] and (*E*)-hinokiresinol[10] have been produced, but no systems inducing other heartwood norlignans are known. Therefore, the generation of sequirin-C in the drying TZ provides a useful model system for studying their biosynthetic pathways.

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