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ORIGINAL PAPER

Clonal variation in heartwood norlignans of *Cryptomeria japonica*: evidence for independent control of agatharesinol and sequirin C biosynthesis

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Abstract
• ***Introduction*** In *Cryptomeria japonica*, heartwood properties are considered to be affected by specific extractives. It remains unclear whether traits of specific heartwood compounds are under genetic control.
• ***Methods*** Two major heartwood norlignans, agatharesinol (A) and sequirin C (S), were determined quantitatively and qualitatively in 29 *C. japonica* plus-tree clones to evaluate their clonal variations and clonal repeatability.
• ***Results*** The content of two norlignans and their composition (S)mol/((A)mol + (S)mol) varied significantly depending on the clone, suggesting that the biosynthesis of norlignan is genetically regulated in *C. japonica*. In particular, the clonal repeatability of sequirin C was higher than that of both agatharesinol and total norlignan content. In addition, the clonal repeatability of the norlignan molar ratio was quite high. These results suggested that genetic involvement is greater in the accumulation of sequirin C than agatharesinol. No significant correlation was found between agatharesinol and sequirin C content, or between the total norlignan content and the norlignan molar ratio, suggesting that the formation of agatharesinol and sequirin C in norlignan biosynthesis is independently controlled in *C. japonica*.
• ***Conclusions*** It was suggested that the traits of the specific heartwood extractive norlignans were under genetic control in *C. japonica*.

Keywords Biosynthesis · Clonal variation · *Cryptomeria japonica* · Heartwood extractives · Norlignan

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1 Introduction

Chemical compounds of wood, especially heartwood extractives, may have critical roles in wood properties such as heartwood color and resistance against wood rots. *Cryptomeria japonica* D. Don (sugi or Japanese cedar) is an indigenous conifer in Japan and one of the most popular species for plantation forestry. A number of studies have identified the major *C. japonica* heartwood extractives, which are classified as norlignans and terpenoids (diterpenephenols). The norlignans in *C. japonica* heartwood are particularly associated with both normal heartwood color and discoloration of the heartwood, so-called black-heart or kuro-jin (Kai et al. 1972; Kai and Teratani 1977; Takahashi 1996, 1998; Ishiguri et al. 2003; Takahashi et al. 2003; Takahashi and Mori 2006). The heartwood norlignan sequirin C turns black on treatment with alkaline solution, indicating that it is involved in blackening (Takahashi and Mori 2006). The norlignans are also considered to contribute to resistance of the wood against butt-rot disease (Ohtani et al. 2009), termite feeding (Kano et al. 2004), and damage due to feeding by snails (Chen et al. 2001).

Although heartwood extractives could affect some wood properties in *C. japonica*, the factors regulating their qualitative and quantitative variations have not been well investigated. Tamura et al. (2004, 2005) found that the yield of alcohol-benzene extracts from *C. japonica* heartwood varied depending on the clone and that genetic factors are likely to lead to improvement in carbon sequestration capacity. Shibutani et al. (2007) reported that the *C. japonica* heartwood terpenoid content is regulated not only by genetic factors but also by environmental factors, i.e., the growth sites of the trees. In other studies, the composition of *C. japonica* heartwood terpenoids was also reported to be regulated both genetically (Nagahama et al. 2001) and environmentally (Nagahama et al. 2002).

Ogiyama et al. (1983) surveyed norlignans in the heartwood of 432 *C. japonica* trees grown in 18 districts over almost all of the Japanese islands. They found that heartwood norlignan in *C. japonica* is composed mainly of agatharesinol and sequirin C, accompanied occasionally by sugiresinol and hydroxysugiresinol (chemical structures in Fig. 1), and that the composition did not vary significantly with growth site.

It is thought that in *C. japonica* the heartwood norlignans are formed in the intermediate wood, "the inner layers of the sapwood that are transitional between sapwood and heartwood in color and general character" as defined by IAWA (1964), and this part of the wood is observable as a narrow white zone, especially in *C. japonica* (Nobuchi and Harada 1983). The content of agatharesinol increases gradually from the middle part of intermediate wood toward the heartwood, whereas the content of sequirin C increases rapidly from the innermost intermediate wood toward the heartwood, indicating that the formation of agatharesinol is initiated at the middle stages of heartwood formation, followed by the formation of sequirin C at the latest stage of heartwood formation (Imai et al. 2005). Recently, Imai et al. (2009) demonstrated in vitro hydroxylation of agatharesinol to sequirin C using a microsomal preparation from *C. japonica* intermediate wood. In particular, the hydroxylase activity was higher on the

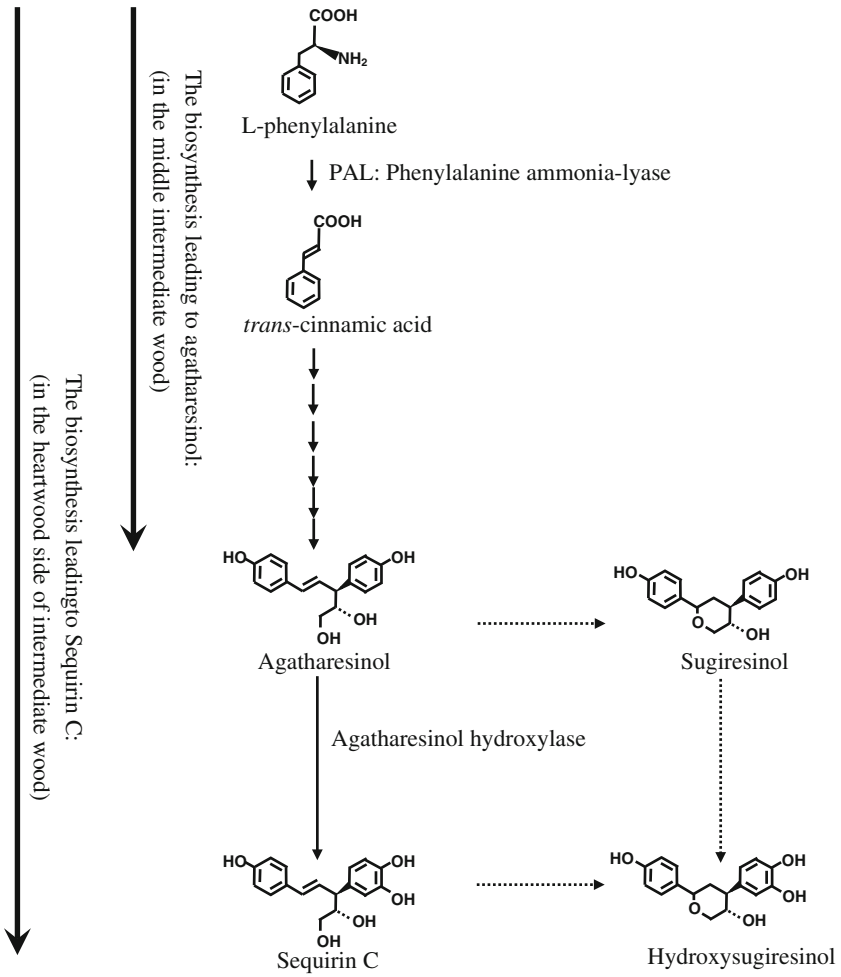

Fig. 1 Proposed biosynthetic pathway of norlignan

heartwood side of intermediate wood, indicating that the conversion of agatharesinol to sequirin C occurs at the later stage of heartwood formation. Thus, the two *C. japonica* norlignans change quantitatively and qualitatively during heartwood formation.

Because the formation and accumulation of heartwood extractives are among the most remarkable changes during heartwood formation, qualitative and quantitative variations in norlignan could reflect major factor affecting variation during heartwood formation and affect consequential variation in its properties. Environmental influences on qualitative variation in *C. japonica* heartwood norlignans have been investigated (Ogiyama et al. 1983); however, neither the influence of the environment on quantitative variation nor the influence of genetics is well understood.

Although heritability or clonal variation of the amounts of heartwood extracts in tree species such as *Pinus radiata* (Shelbourne 1997), *Pinus pinaster* (Pot et al. 2002), and *Eucalyptus globulus* (Poke et al. 2006) have also been reported, such studies have not been well extensive. In particular, inheritance of the traits of specific heartwood compound is open to further investigation.

Our objective was to estimate genetic effects on regulation of the traits of specific heartwood extractive norlignans in *C. japonica* for the first time. Investigation of qualitative and quantitative variations in two major heartwood norlignans, agatharesinol and sequirin C, in 29 *C. japonica* plus-tree clones led to the evaluation of clonal repeatability of the norlignan composition, which indicates the magnitude of their inheritance (van Buijtenen 1992). The influence of genetics on the variation is discussed. In addition, the association of norlignan traits (norlignan content and norlignan composition) with four conventional selection traits (tree height, diameter at breast height (DBH), wood density, and heartwood moisture content) is discussed to estimate the influence of selection for these conventional traits during breeding on norlignan diversity.

2 Materials and methods

2.1 Materials

Plus-tree clones of *Cryptomeria japonica* had been planted in clonal archives at the Okubo Breeding Material Management Garden of the Forest Tree Breeding Center, Forestry and Forest Product Research Institute in Hitachi, Ibaraki Prefecture, Japan. The clonal archives were established in 1994 under a row plot design, in which seven ramets (individual trees in each clone) are planted in one row without any replication. Plus-trees are selected based on their superior growth and stem form in the Japanese tree breeding program. A 40-m^2 study plot was selected from a 2.8-ha clonal archive. The study plot consisted of four subplots including 11 to 13 clones each. The spacing of planting was 1.1 m between ramets within a row (one clone per one row), 3.0 m between rows (clones), and 3.0 m between subplots. Therefore, the stocking was ca. 2,300 stems per hectare. A total of 29 clones, including 22 clones selected from Fukushima Prefecture and seven clones from Kochi Prefecture, were used. The trees selected for investigation were average sized trees within the clone that had the least damage by insects or weather hazards.

Average tree height and average diameter at breast height (with coefficient of variation in parentheses) of the sample trees were 10.5 m (14.7%) and 16.1 cm (21.0%), respectively. From 85 trees (three ramets from 27 clones and two ramets from two clones), increment cores were collected at breast height with an increment borer (inner diameter, 5 mm) in May 2008, and were divided into heartwood, sapwood, and intermediate wood based on color. Samples were stored in methanol containing 0.1% (*w*/*w*) ascorbic acid at 4°C until use. Wood disks were obtained from the same trees at breast height in November 2008 for the measurement of wood density and heartwood moisture content. Fan-shaped wood blocks (fan-shape × height: r=3–5 cm, θ=10°, h=5 cm (approx.)) were taken from heartwood where no effect of core sampling was observed, and the fresh weight was measured immediately after felling the sample trees. The oven-dried (at 105°C until the measured mass was stable) heartwood density was determined by the mercury displacement method as the value of oven-dried weight per oven-dried volume. The moisture content (MC) of the specimen was determined as follows: MC = (Wg − Wd)/Wd, where Wg is the fresh weight and Wd is the oven-dried weight.

2.2 Extraction and gas chromatography–mass spectrometry analysis

The heartwood samples from increment cores were cut into small pieces with a knife, and then extracted thoroughly with hot (55°C) methanol (MeOH) for 4 days, during which solvent was exchanged twice a day. All MeOH solutions and the prestorage MeOH solution (mentioned in Section 2.1) obtained from each sample were combined and evaporated to give a residue (MeOH extract). The MeOH extracts were extracted with ethyl acetate (EtOAc), and the EtOAc solutions were evaporated to give EtOAc extracts. A portion of the EtOAc extract was dissolved in pyridine, and an aliquot of the solution was trimethylsilylated using *N,O*-bis (trimethylsilyl)-trifluoroacetamide (Wako, Japan). The trimethylsilyl derivatives were analyzed by gas chromatography–mass spectrometry (GC-MS) on a Shimadzu GC-MS QP 2010 Plus instrument (Shimadzu, Kyoto, Japan) equipped with a DB-1 capillary column (30 m×0.32 mm, i.d.; film thickness, 1 μm) using helium gas as the mobile phase. The

oven temperature was 280°C, and the ionization energy was 70 eV. Chromatogram peaks were assigned by comparing their retention times and mass fragmentation patterns with those of authentic samples. The content of agatharesinol and sequirin C was determined using 4,4′-dihydroxychalcone as an internal standard.

2.3 Statistical analysis

One-way analysis of variance (ANOVA) was used to test for significance of the genetic (clonal) involvement in the variation in norlignan content and norlignan composition. Clonal repeatability was calculated as an indicator of the magnitude of inheritance as follows:

$$h^2 = \sigma^2{}_c / \left(\sigma^2{}_c + \sigma^2{}_e\right)$$

where h^2 is the clonal repeatability, $\sigma_c{}^2$ is the variance due to interclonal differences, and $\sigma_e{}^2$ is the error variance. Standard error for the heritability of each trait was calculated using the delta method (Lynch and Walsh 1998). The estimation of variances and standard errors was carried out using ASReml software (Gilmour et al. 2009).

Because each clone was planted in only one row with several ramets, there was no environmental replication. Therefore, it was not possible to separate the variance due to genotype from variance due to that portion of environmental effects that might have varied orthogonally along the row in the clonal archive. Estimates of clonal repeatability were thus conducted assuming that the environmental effects influencing norlignan content were randomly distributed over the site.

Pearson product–moment correlation on coefficients between norlignan traits (norlignan content and norlignan composition) and conventional selection traits (tree height, DBH, wood density, and heartwood moisture content) were calculated based on 29 *C. japonica* plus-tree clonal averages.

3 Results

GC-MS analysis demonstrated that agatharesinol and sequirin C were the major norlignans in all 85 *C. japonica* heartwood samples; neither sugiresinol nor hydroxysugiresinol was detected in any samples, consistent with a previous report that *C. japonica* wood only infrequently contains sugiresinol and hydroxysugiresinol (Ogiyama et al. 1983). In this study, therefore, the total content of heartwood norlignan was expressed as the sum of agatharesinol and sequirin C content (μmol/g of oven-dried sample weight). The composition of heartwood norlignan was expressed as the molar ratio of agatharesinol (A) to sequirin C (S): S(mol)/(A(mol) + S(mol)). The total norlignan content should represent the ability of the norlignan biosynthetic pathway to form agatharesinol and the norlignan molar ratio should represent the activity of agatharesinol hydroxylase in the conversion of agatharesinol to sequirin C (Fig. 1).

Quantitative variation in heartwood norlignan was shown by the frequency distribution of agatharesinol content, sequirin

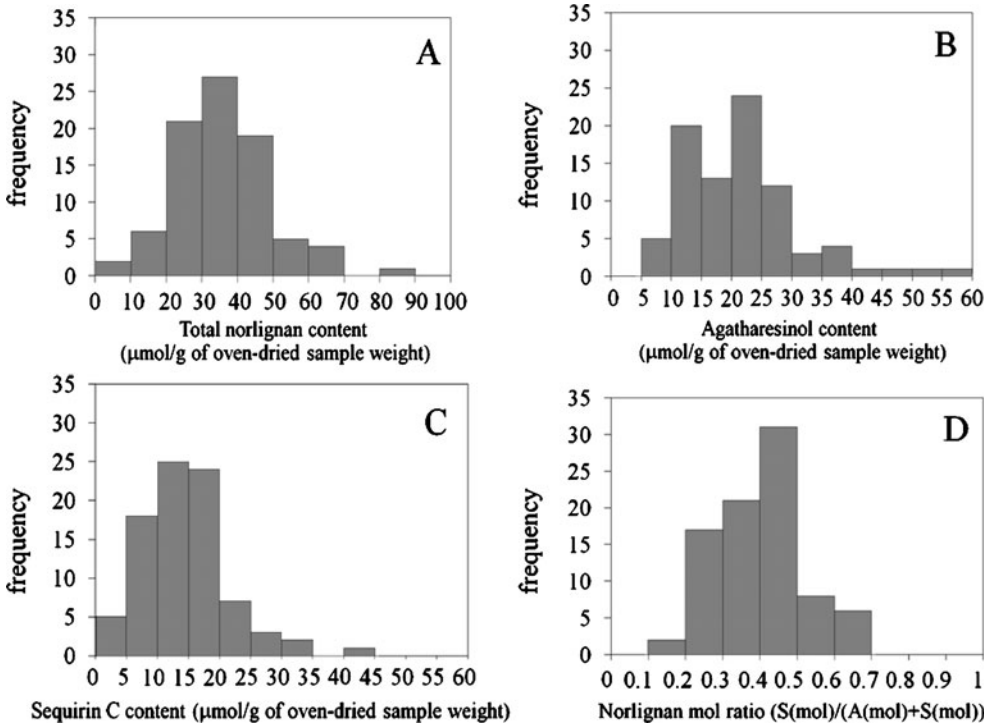

Fig. 2 **a**–**d** Frequency distribution of **a** total norlignan content, **b** agatharesinol content, **c** sequirin C content, and **d** norlignan molar ratio (S(mol)/(A(mol) + S(mol))) in 85 samples

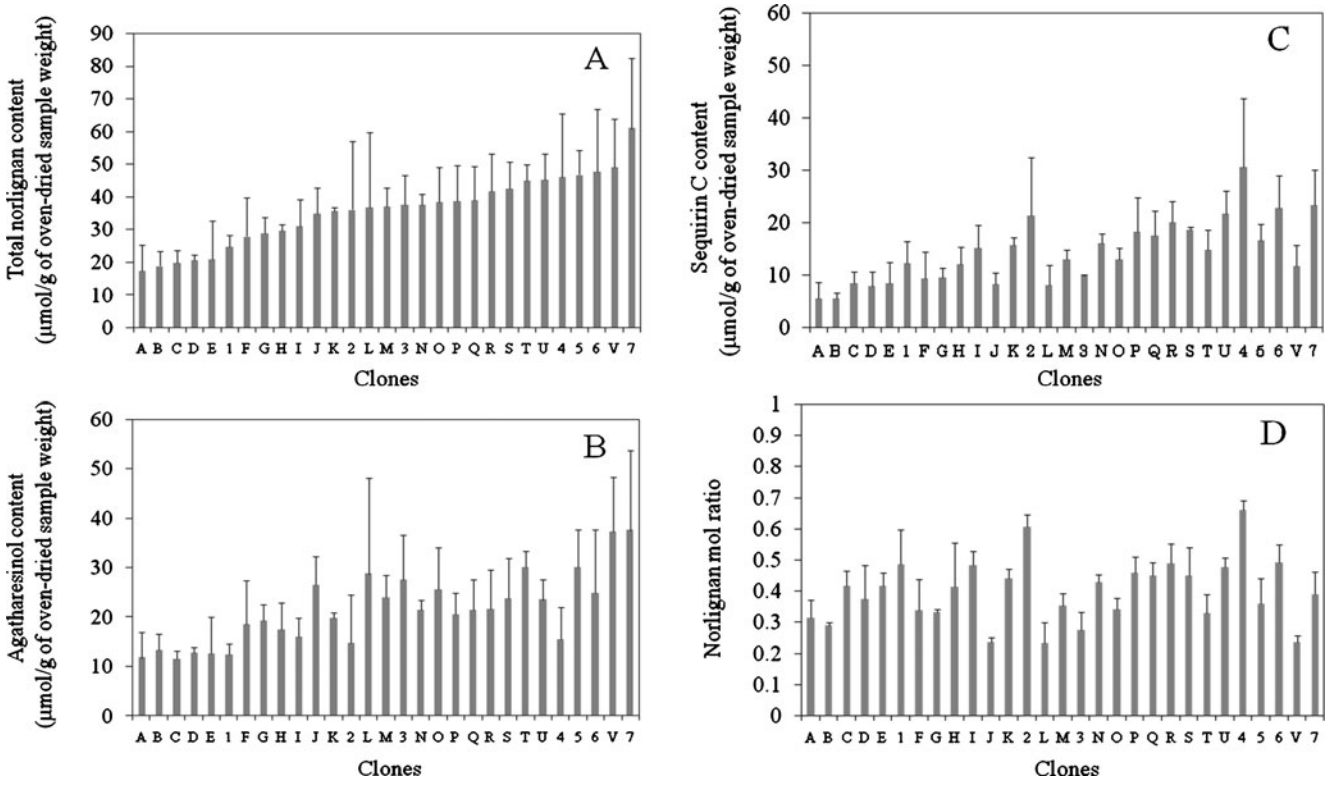

Fig. 3 Clonal variations of **a** total norlignan content, **b** agatharesinol content, **c** sequirin C content, and **d** norlignan ratio among the 29 *C. japonica* plus-tree clones. *Error bars* show standard deviation. Plus-tree clones from Fukushima Pref. are indicated as letters and those from Kochi Pref. as numbers. The order of the clone in the figures follows the total norlignan content

C content, and total norlignan content, and qualitative variation was shown by the frequency distribution of the norlignan molar ratio (S/(A + S)) among the 85 *C. japonica* heartwood samples (Fig. 2a–d). The coefficients of variation (standard deviation/mean value) of agatharesinol, sequirin C, and total norlignan content were 45%, 50%, and 39%, respectively, and that of the norlignan molar ratio was 28%.

The clonal average agatharesinol content varied from 11.5 to 37.6 μmol/g (0.33% to 1.07% (*w*/*w*)), sequirin C content from 5.5 to 30.6 μmol/g (0.17% to 0.92% (*w*/*w*)), and total norlignan content from 17.4 to 61.0 μmol/g (0.51% to 1.78% (*w*/*w*)) (Fig. 3a–c). The total average of agatharesinol, sequirin C, and total norlignan content were 21.4, 14.3, and 35.7 μmol/g, respectively (0.61%, 0.43%, and 1.05% (*w*/*w*)).

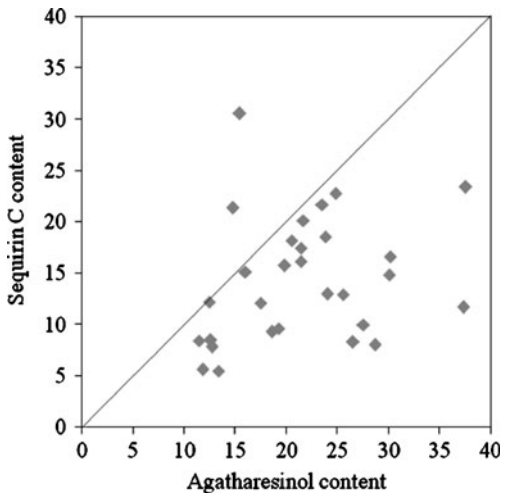

Fig. 4 Relation between agatharesinol content and sequirin C content. All 29 clonal means are plotted. r=0.237; p>0.05. The *diagonal line* shows $x = y$

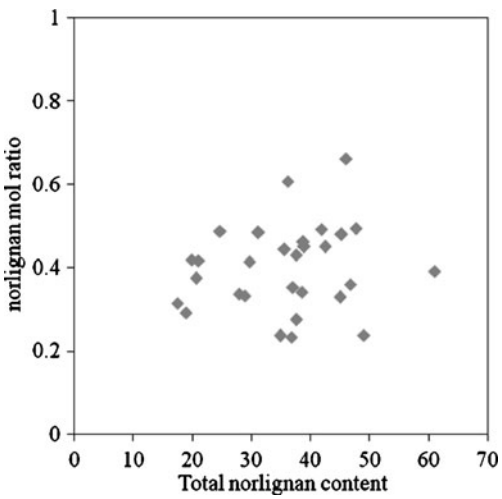

Fig. 5 Relation between the total norlignan content and the norlignan molar ratio. All 29 clonal means are plotted. r=0.155; p>0.05

Table 1 Analysis of variance for norlignan content and norlignan molar ratio of *C. japonica* plus-tree clones

Factor	SS	*df*	MS	*F* value	*P* value	Clonal repeatability (standard error)
			Agatharesinol content			
Clones	4,298	28	153.5	2.44	<0.01	0.33 (0.12)
Residuals	3,517	56	62.81			
			Sequirin C content			
Clones	3,028	28	108.1	4.54	<0.001	0.55 (0.10)
Residuals	1,333	56	23.81			
			Total norlignan content			
Clones	8,913	28	318.3	2.38	<0.01	0.32 (0.12)
Residuals	7,485	56	133.7			
			Norlignan molar ratio			
Clones	0.882	28	0.0315	8.49	<0.001	0.72 (0.08)
Residuals	0.208	56	0.0037			

SS sum of squares, *df* degrees of freedom, *MS* mean squares

The clonal average of the norlignan molar ratio varied from 0.23 to 0.66, and its total average was 0.40 (Fig. 3d).

No significant correlation was found between the clonal average agatharesinol content and the sequirin C content (Fig. 4; r=0.237; p>0.05). Data for almost all clones fell below a line showing the relationship $y = x$. The higher average content of agatharesinol than sequirin C as well as an average norlignan molar ratio smaller than 0.5 (Fig. 3d) revealed that *C. japonica* tends to accumulate more agatharesinol than sequirin C. Only two clones appeared to accumulate more sequirin C than agatharesinol.

No significant correlation was found between the total norlignan content and the norlignan molar ratio (Fig. 5; r= 0.155; p>0.05).

One-way ANOVA (Table 1) revealed significant differences between clones for all norlignan traits: agatharesinol content (p<0.01), sequirin C content (p<0.001), total norlignan content (p<0.01), and norlignan molar ratio (p<0.001).

The clonal repeatability (standard error) of agatharesinol content, sequirin C content, and total norlignan content were 0.33 (0.12), 0.55 (0.10), and 0.32 (0.12), respectively, and that of the norlignan molar ratio was 0.72 (0.08). The clonal repeatability of tree height, DBH, heartwood density, and heartwood moisture content was 0.61 (0.10), 0.31 (0.12), 0.34 (0.12), and 0.40 (0.12), respectively.

Table 2 shows correlation coefficients between the norlignan traits and the four conventional selection traits. No significant correlation was found between the norlignan and selection traits (Table 2; p>0.05), except for a low correlation between the total norlignan content and heartwood density (r=0.383; p<0.05) and between agatharesinol content and heartwood density (r=0.396; p<0.05).

4 Discussion

Qualitative variation in *C. japonica* heartwood norlignan and environmental influences on this variation have been studied (Ogiyama et al. 1983). In the present study, we investigated genetic involvement in the quantitative and qualitative variation in heartwood norlignan using *C. japonica* plus-tree clones.

One-way ANOVA (Table 1) revealed apparent clonal variations in agatharesinol content (p<0.01), sequirin C content

Table 2 Pearson product–moment correlation on coefficients between norlignan traits and conventional selection traits of 29 *C. japonica* plus-tree clones (n=29)

Conventional selection traits	Norlignan traits			
	Total norlignan content	Agatharesinol content	Sequirin C content	Norlignan molar ratio
Tree height	−0.167 ns	−0.179 ns	−0.076 ns	0.017 ns
DBH	−0.200 ns	−0.252 ns	−0.046 ns	0.101 ns
Heartwood density	0.383*	0.396*	0.190 ns	−0.030 ns
Heartwood moisture content	0.157 ns	0.042 ns	0.221 ns	0.131 ns

ns not significant at p=0.05

*0.01<p<0.05, significant

($p<0.001$), total norlignan content ($p<0.01$), and norlignan molar ratio ($p<0.001$). These results strongly suggest that the accumulation of norlignan is genetically regulated in *C. japonica*. The clonal repeatability of the norlignan composition was similar to that of heartwood extracts of *C. japonica* (Tamura et al. 2005).

As Imai et al. (2009) demonstrated recently in vitro, sequirin C is biosynthesized by a one-step enzymatic hydroxylation of agatharesinol, which is biosynthesized through multi-step enzymatic reactions in the phenylpropanoid pathway (Fig. 1, Imai et al. 2006a, b). Although the biosynthesis of sequirin C requires one additional step beyond agatharesinol, the clonal repeatability of sequirin C content ($h^2=0.55$) was higher than that of both agatharesinol content ($h^2=0.33$) and total norlignan content ($h^2=0.32$; Table 1). In addition, the clonal repeatability of the norlignan molar ratio was quite high ($h^2=0.72$; Table 1). These results suggest that genetic involvement in the accumulation of sequirin C is more significant than that of agatharesinol, which is likely attributable to high genetic regulation of agatharesinol hydroxylase activity.

Significant correlations were not found between agatharesinol content and sequirin C content (Fig. 4), or between the total norlignan content and the norlignan molar ratio (Fig. 5). These results suggest that the biosynthetic steps leading to agatharesinol formation and the following conversion of agatharesinol to sequirin C is regulated separately, resulting in independent control of biosynthesis of these two norlignans in *C. japonica*; sequirin C biosynthesis might be regulated apart from agatharesinol biosynthesis in spite of sequirin C being formed through agatharesinol as a biosynthetic intermediate (Imai et al. 2009). This hypothesis of independent control of the biosynthesis is supported by previous studies: Ohashi and Imai (1990) and Ohashi et al. (1990) found that agatharesinol accumulated in the sapwood of *C. japonica* stem logs allowed to stand after cutting the trees down, and Imai et al. (2005, 2006a, b) found that agatharesinol accumulated in sapwood sticks of *C. japonica* kept under high humidity after preparing the sticks from fresh wood. It is assumed that the biosyntheses of heartwood extractives including norlignan occurs in dying ray parenchyma cells in intermediate wood during the transition from sapwood to heartwood. Therefore, Imai et al. concluded that the accumulation of agatharesinol in the sapwood must be due to biological action, probably by dying ray parenchyma cells, which would resemble the phenomenon in intermediate wood. In these experiments, however, sequirin C was not accumulated in the sapwood. Thus, the biosynthesis of agatharesinol can be induced experimentally, but the following one-step conversion of agatharesinol to sequirin C has never occurred.

Nobuchi and Harada (1983) reported that the percentage of dead parenchyma cells increased gradually from the sapwood side toward the heartwood side in intermediate wood of *C. japonica*. This observation demonstrates that in intermediate wood cells, aging cells gradually progress toward cell death, and in this process, ray parenchyma cells alter their physiological functions. In particular, sequirin C biosynthetic ability must develop after cell aging has progressed substantially, because the content of sequirin C increases rapidly from the innermost intermediate wood toward the heartwood, indicating preferential formation of sequirin C at the later stage of heartwood formation (Imai et al. 2005, 2009). Furthermore, the ray parenchyma cells in *C. japonica* intermediate wood might have a different physiological ability for sequirin C biosynthesis, depending on the position of the ray cells, as Nakaba et al. (2006, 2008) observed in *Abies sachalinensis, Pinus densiflora*, and *Pinus rigida*. Nakaba et al. proposed that the differentiation and cell death of ray parenchyma cells starts in the upper or lower radial cell lines of a ray. Thus, the spatial and temporal dynamics of the biosynthesis of norlignan during heartwood formation should also be taken into consideration as well as regulation of agatharesinol hydroxylase activity to understand the variation in heartwood norlignan.

Almost no correlations were significant between norlignan traits (norlignan content and norlignan molar ratio) and conventional selection traits (tree height, DBH, and wood density) (Table 2), suggesting that norlignan accumulation is not genetically associated with these traits. Therefore, the diversity of norlignan accumulation in heartwood will not be lessened even when tree breeding programs improve the conventional selection traits associated with cell wall formation.

To the best of our knowledge, this is the first report suggesting genetic control of the traits of specific heartwood norlignans in *C. japonica*. Further studies are under way to reveal the inheritance of norlignan traits precisely using control-pollinated families of *C. japonica*.

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