Reduction of Lignin Content by Suppression of Expression of the LIM Domain Transcription Factor in *Eucalyptus camaldulensis*

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(Received: July 26, 2006/Accepted: October 14, 2006)

Abstract

We report a reduction of lignin content in the woody plant *Eucalyptus camaldulensis* by the suppression of gene expression of the LIM domain transcription factor. Previously, we identified a cDNA encoding the tobacco (*Nicotiana tabacum*) LIM domain transcription factor, Nt1iml, involved in lignin biosynthesis and that specifically binds to an important *cis*-acting element, the PAL-box sequence. The orthologous Eucalyptus gene of *Ntliml*, namely *Ecliml*, was isolated from the *E. camaldulensis* cDNA library (84% amino acid identity). The antisense *Ntliml* construct with a kanamycin-resistant gene was introduced into *E. camaldulensis*. The transgenic Eucalyptus plants grown in the greenhouse showed decreased expression levels of severallignin biosynthesis genes, phenylalanine ammonialyase (*PAL*), cinnamate-4-hydroxylase (*C4H*) and 4-hydroxycinnamate CoA ligase (*4CL*). The abnormal phenotypic changes and a 29% reduction of lignin content were observed in the line LG12, in which the transcript level of *Ecliml* was most1y suppressed. *Ec1iml* is one of the key transcription factors involved in lignin biosynthesis.

Key words: Eucalyptus camaldulensis; *Ecliml* gene, transgenic Eucalyptus, lignin, holocellulose.

Introduction

Plant cell walls are composed of mostly cellulose, hemicellulose and lignin. Cellulose provides strength and flexibility to plant tissue and is also of great importance to chemical, textile, pulp and paper industries. In woody plants, high levels of cellulose are synthesized and account for about 50% of the dry weight of wood materials. However, the precise biosynthetic pathway of cellulose is still unclear, although a study, following a genome-level approach, is progressing on *Arabidopsis thaliana* as a model plant. Lignin represents 20–30% of wood components and is the second-most abundant natural biopolymer after cellulose. The ability to synthesize lignin has been essential in the adaptation of plants from an aquatic environment to land. Lignin has crucial roles in higher plants, providing structural integrity to the cell wall, enabling transport of water and solutes through the vascular system, and protecting plants against insects and pathogens. For over the past decade, a number of molecular analyses of the genes encoding enzymes involved in lignin biosynthesis have been studied. Many approaches to modifying lignin content and/or deposition have been reported (BOERJAN et al., 2003). Most structural genes involved in lignin biosynthesis have already been identified in many plant species including woody plants. However, there are still many unresolved aspects of monolignol biosynthesis (BOERJAN et al., 2003).

Many transcription factors of lignin biosynthesis have been described (ROGERS and CAMPBELL, 2004; GOICOECHAE et al., 2005). One of those, expressed from the tobacco *Ntlim1* gene, was isolated as a transcription factor that can specifically bind to a PAL-box element. This element is thought to be an important *cis*-acting element of promoter regions that regulates the expression of genes associated with lignification (KAWAOKA et al., 2000; KAOTHIEN et al., 2002). Moreover, *Ntlim1* transiently activated the transcription of a β-glucuronidase reporter gene driven by the PAL-box sequence in tobacco protoplasts. The transgenic tobacco plants with antisense *Ntlim1* showed a low concentration of transcripts from some key phenylpropanoid pathway genes, such as phenylalanine ammonia-lyase (*PAL*), 4-hydroxycinnamate CoA ligase (*4CL*) and cinnamyl alcohol dehydrogenase (*CAD*). Furthermore, a 27% reduction of lignin content was observed in the transgenic tobacco with antisense *Ntlim1* (KAWAOKA et al., 2000). The Arabidopsis has six LIM genes, one of these *AtWLIM2* is preferentially expressed in a developing silique (RIDER JR. et al., 2003). Lignification of valve margin cells adjacent to the dehiscence zone and of an internal valve cell layer in a silique is proposed to contribute to pod shatter (LILJEGREN et al., 2000). On the other hand, tobacco WLIM1 has a function as a F-actin binding protein involved in actin cytoskeleton remodeling (THOMAS et al., 2006). Thus, plant LIM proteins have been reported with dual functions.

Eucalyptus species constitute the most widely planted hardwood tree in the temperate and subtropical regions of the world and their wood is used as a raw material for production of cellulose. Developing a transformation strategy for this species would be an important step towards the improvement of its features for the species' utilization by the pulp and paper industries. Several reports regarding transformation methods in Eucalyptus species are available (HO et al., 1998; MULLINS et al., 1997). For the first time, genetic engineering in *E. camaldulensis* has been reported, using antisense CAD grown in a greenhouse (VALERIO et al., 2003).

In this study, we isolated a gene coding *Eclim1* that is 82% homologous with the *Ntlim1* gene at the nucleotide level. Antisense *Ntlim1* was introduced into *E. camaldulensis*, one of the important tree species for afforesta-

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DOI:10.1515/sg-2006-0035
edited by Thünen Institute of Forest Genetics

tion. We investigated the effect of LIM gene on transcript levels of several genes associated with lignin biosynthesis in transgenic Eucalyptus plants. Interestingly, suppression of the LIM gene caused a simultaneous reduction in the levels of transcripts of some lignin pathway genes and low lignin content in the transgenic plants.

Materials and Methods

Plant material

The seeds of *Eucalyptus camaldulensis* L. DENN harvested in Australia were obtained from Koei Syubyo Boeki Co. Ltd. (Tokyo, Japan). The *E. camaldulensis* plants were grown in a greenhouse at 25°C under a 16 hours light and 8 hours dark photo-period.

Isolation of the Eclim1 gene

A cDNA library was constructed using the mRNA purified from the stem region of four-month-old *E. camaldulensis* grown in the greenhouse. A cDNA clone was screened by the plaque hybridization method. An inserted DNA fragment of a positive clone was amplified by PCR using forward and reverse primers for the λZAPII vector (Stratagene, La Jolla, CA, USA). The genomic DNA fragment containing *Eclim1* was then amplified by PCR.

Chimeric gene construction

A pAlim-121 was constructed by a DNA fragment containing 1.0 kb of *Ntlim1* ligated between CaMV 35S promoter and *Nos* terminator of pBI121 (Clontech, Polo Alto, CA) at the *Bam*HI restriction enzyme site. A direction of *Ntlim1* cDNA was checked by restriction enzyme pattern or sequencing. A pAlim-121-GUS-Hm was constructed by insertion of the chimeric gene of CaMV 35S promoter::antisense *Ntlim1*::Nos terminater amplified before by PCR using primers 5'-AAGCTTGCATGCCTGCAGGTCCCCAGATTA and 5'-CCCCGAATTCGCCTTTTTCAATTTCAGAAAG, at the restriction enzyme site *Sse*8387I site (Takara Bio, Otsu, Japan) into the pBI121-Hm (gift from Dr. K. NAKAMURA, Nagoya University). The pBI121-Hm is containing the chimeric genes, CaMV 35S promoter::hygromycin phosphotransfrase:: Nos terminater and CaMV 35S promoter::GUS (β-glucuronidase)::Nos terminator.

Transformation of Eucalyptus

Seeds were sterilized by immersion in 2% (v/v) sodium hypochlorite following 70% (v/v) ethanol treatment and finally rinsed three times with sterile water. The sterilized seeds were sown on Murashige and Skoog (MS) medium (half-strength macronutrients) and the hypocotyl explants of the germinated seedlings were used as material for transformation. Eucalypts was transformed with *Agrobacterium tumefaciens* LBA4404 strain. The *Agrobacterium* culture broth was diluted one fifth with sterile water and soaked the hypocotyls explants for 10 minutes. After removing the culture broth, the explants were placed on a shoot regeneration medium at 25°C under a 16 hours light and 8 hours dark photo-period. After three days, to remove *Agrobacterim* the explants were placed on a shoot regeneration medium containing 100 mg/L kanamycin and 500 mg/L carbenicilline and replaced on a fresh shoot regeneration medium every 4 weeks. After 3 months, adventitious shoots were generated and transferred to the rooting medium. As a basal medium, half-strength macronutrients of MS medium containing 10 mM $NH4^+$, 30 mM NO_3^-, 2% sucrose, 0.8% (w/v) agar was used. For shoot regeneration, basal medium supplemented with 2 mg/L trans-zeatin and 0.3 mg/L naphthaleneacetic acid (NAA) was used. As rooting medium the basal medium added with indole butyric acid (IBA) (50 µg/L) was applied. Regenerated Eucalyptus plants were grown in a greenhouse and propagated by cutting method.

Southern and Northern analyses

Genomic DNA was isolated from leaves and a Southern blot analysis was performed as described by Maniatis et al. (1982). Total RNA was extracted from the plant tissue (CHOMCZYNSKI and SACCHI, 1987), fractionated on a formaldehyde-agarose gel and blotted onto a nylon membrane, Hybond N (Amersham Pharmacia Biotech, Bucks, UK). The membrane was hybridized with a digoxigenin (DIG)-dUTP labeled probe using a DIG labeling kit (Roche Diagnostics, GmbH, Mannheim, Germany). Hybridization was performed according to the manufacturer's protocol. A Eucalyptus ubiquitine gene was used as an internal control.

Isolation of Eucalyptus PAL, 4CL, C4H, CCoAOMT, COMT, CCR and CAD

For Eucalyptus *PAL* cDNA (GenBank accession number AF167487), 5'-AAGCAGGACAGGTACGCTCTCAGGACTTCT and 5'-CTCCCTCACCAGCTTGTACAATGGATAGGA, and for Eucalyptus 4CL (E64536) 5'-CGTTGTTCCACATATACTCCCTCAAC and 5'-ATTAGCGGTCGCTTCGGGGTCGTTCAGA were synthesized. For the C4H, (CB967509) 5'- GCCAATTCAGCTTGCACATATTGAAG and 5'-TCAAAAGATAAGTTTCAGTTCTCAAAG, for the CCoAOMT (AF168780), 5'-TTAAGAAGCATCAATGGCAGCCAACGCAGA and 5'-TCGAGATCTCCTTTCAGTTGCGATCAGCTG, and for the COMT (AF046122) 5'-GAGTTTGAATCAATGGCCACCGCCGGAGAG and 5'-TAGATGCTCAGCTGATCCGACGGCAGAGAG were used. For the CCR (CB967622), 5'- GTGGCAAGAACGGGGGTGATGCCAAAAATG and 5'-GTTGCTGCTGTTGCTGCTGCTGCTGCTGCT, and for the CAD (AF038561) 5-AGCTTGGATCTTTGAGCAAAAATGGGCAGT and 5'-TATGGGGAAAGGACAAAACTAATCAAGCTT were used. The PCR amplification was carried out using a cDNA (2 ng) synthesized from Eucalyptus stem as a template. The identity of these genes was checked by sequencing.

Enzyme assays

Enzyme assays were carried out using the crude protein extract from leaves. A 4CL activity was determined using ferulic acid as a substrate (HARDING et al., 2002). Protein was quantified with a protein assay kit (Bio-Rad, Hercules, CA, USA).

DOI:10.1515/sg-2006-0035
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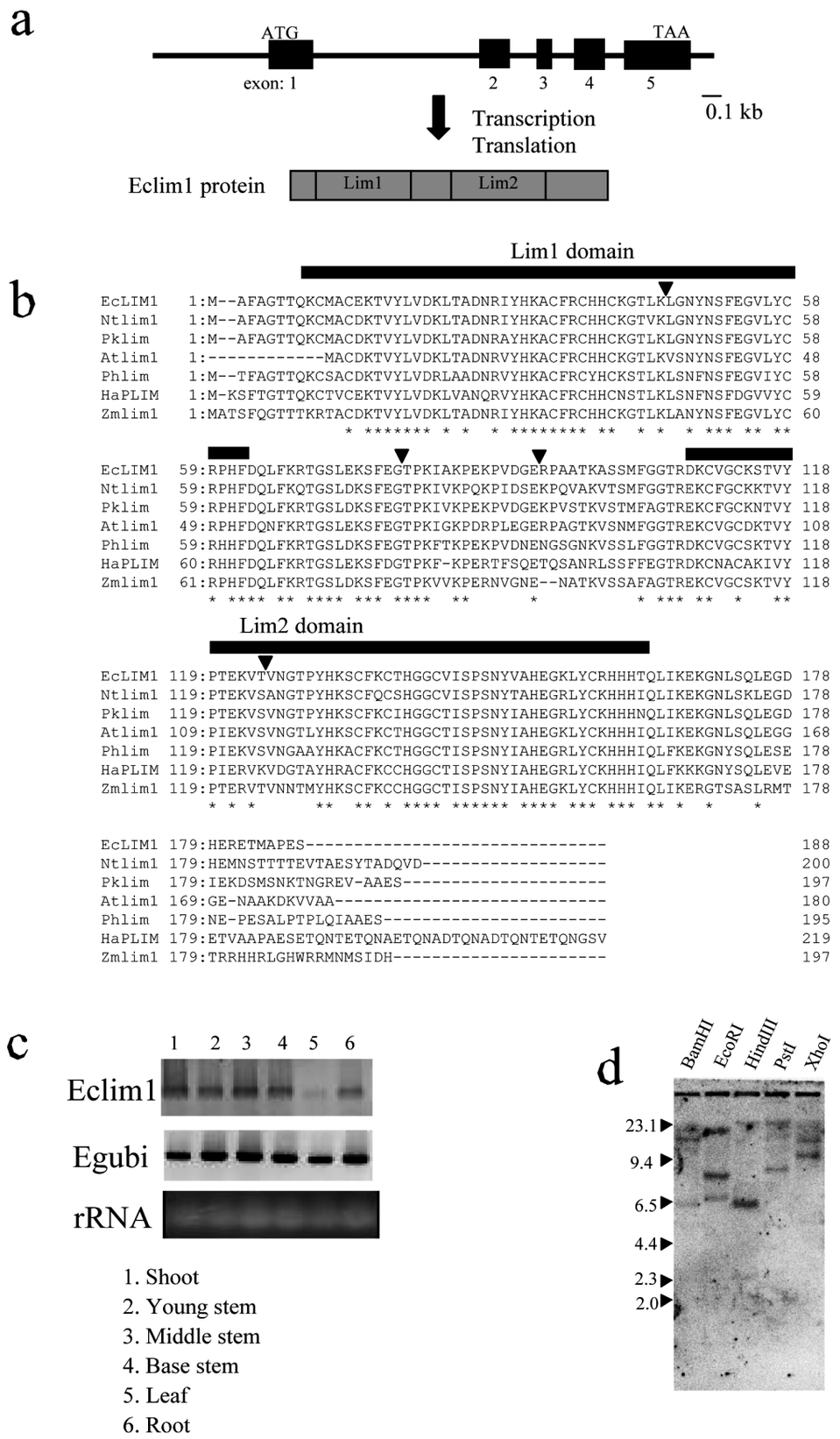

Figure 1. – (A) Organization of the Eclim1. (B) Alignment of the amino acid sequences of the plant LIM domain proteins. The conserved regions are indicated with the asterisks below them. The accession numbers of each protein are provided in the results. Arrows indicate intron splicing sites. (C) RT-PCR analysis of the Eclim1 gene expression in *E. camaldulensis*. (D) Southern hybridization of Eclim1.

DOI:10.1515/sg-2006-0035
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Phloroglucinol staining

Stem sections were stained in 1% phloroglucinol: 70% ethanol (5 min). Excess phloroglucinol was removed and replaced with 18% HCl.

Cell wall composition analysis

Lignin determination was performed on the dried insoluble cell wall residues (CWR) of samples that had been soxhlet extracted with toluene/ethanol, ethanol and then water. Klason lignin was measured by the method of EFFLAND (1977). Holocellulose was determined by sulfide acid extraction. Glucan and other polysaccharides (hemicelluloses) were quantified by high-pH anion exchange chromatography after hydrolysate composition assay (DAVIS, 1988).

Results

Ntlim1 homologous gene in Eucalyptus camaldulensis

To isolate a cDNA that encodes the gene homologous to *Ntlim1*, a cDNA library constructed from *E. camaldulensis* young stems was screened by using a cDNA fragment containing the coding region of *Ntlim1* as a probe. Two positively hybridized clones were obtained from the screening of one million recombinant phages. The 1.0- and 0.7-kb cDNA inserts of the clones were amplified by PCR and subcloned in the TA-cloning vector pCR4 (Invitrogen, Carlsbad, CA, USA). These two cDNA inserts had identical sequences and contained an open reading frame that encodes a protein of 188 amino acid residues and the nucleotide sequence (accession no. AB208711) was shown to be highly homologous with that of *Ntlim1* (*Figure 1b*). We designated the protein encoded by this cDNA '*Eclim1*'. The genomic DNA containing *Eclim1* was isolated by PCR using optimal primers. The 1.8-kbp DNA fragment was amplified and was found to consist of five exons (*Figure 1a*, accession no. AB208712). The genomic sequence of the coding region completely matched that of the *Eclim1* cDNA. From a GenBank database search, the deduced amino acid sequence of *Eclim1* proved to be highly homologous to those of other plant LIM proteins, the PLIM-1 of late pollen genes in the sunflower (BALTZ et al., 1992) and Arabidopsis L2 (GenBank accession no. X91398). Results from the motif search showed that the deduced amino acid sequence of the *Eclim1* protein contains two repeats of conserved amino acid sequences of the LIM domain (LIM1 and LIM2; *Figure 1b*). The alignment of amino acid sequences of *Ntlim1* and other LIM proteins, e.g. sunflower PLIM-1 (BALTZ et al., 1992), poplar Pklim (accession no. AB079510), petunia PhLIM (accession no. AF049917) and maize ZmLIM (accession no. AF135591), are shown in *Figure 1b*. The amino acid sequences of the LIM domain were highly conserved in plants. To determine the number of genes in the Eucalyptus genome that are related to the *Eclim1* gene, Southern hybridization was carried out. Three hybridizing bands were obtained with genomic DNA digested with *Bam*HI, *Eco*RI, or *Hind*III, indicating that there are at least three copies of *Eclim1* in the Eucalyptus genome (*Figure 1d*).

Total RNA was extracted from Eucalyptus shooting buds, roots, leaves, and young or middle or basal part of stems. The transcript level in each organ was assayed by RNA gel blot. The *Eclim1* mRNA was abundant in shoots, each part of the stems and roots, but not in the leaves (*Figure 1c*).

Production of transgenic Eucalyptus plants

Since the LIM domains of *Eclim1* and *Ntlim1* were highly homologous (82%) at the nucleotide level, we constructed two types of the binary vectors based on pBI121 with antisense *Ntlim1* (*Figure 2*). pAlim contains the neomycin phosphotransferase (NPTII) gene and the DNA fragment of coding region of *Ntlim1* ligated in an antisense direction driven by the cauliflower mosaic virus (CaMV) 35S promoter. pAlim-121-GUS-Hm has the 35S promoter::GUS and a hygromycine phosphotransferase (HPT) gene also driven by the 35S promoter, in addition to the components of the pAlim. These constructs were introduced into hypocotyles by *Agrobacterium* mediated transformation. Over ten thousand hypocotyles of seedlings germinated from seeds were prepared as transformation materials. Forty-five independent primary transgenic shoots were produced and all lines contained their respective T-DNA by PCR analysis (data not shown). The kanamycin-resistant shoots were selected and placed on a medium for root

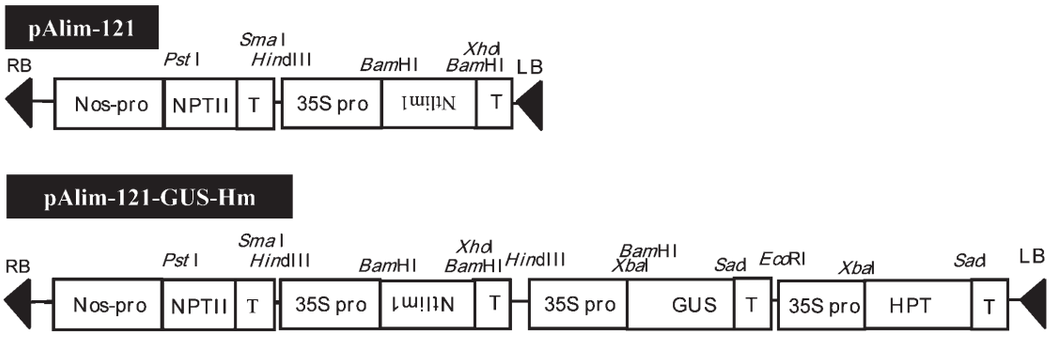

Figure 2. – Schematic representation of the constructs. The full length tobacco Ntlim1 cDNA is driven by CaMV35S promoter. The restriction site *Xho*I is unique on the T-DNA. RB, right border; Nos-pro, nopaline synthase gene promoter; NPTII, neomycin phosphor transferase-coding sequence; T, nopaline synthase gene terminater; 35S pro, CaMV 35S promoter; Ntlim1, tobacco Ntlim1 coding-sequence; GUS, β-glucronidase coding-sequence; HPT, hygromycin phosphotranspherase coding sequence; LB, left border.

DOI:10.1515/sg-2006-0035
edited by Thünen Institute of Forest Genetics

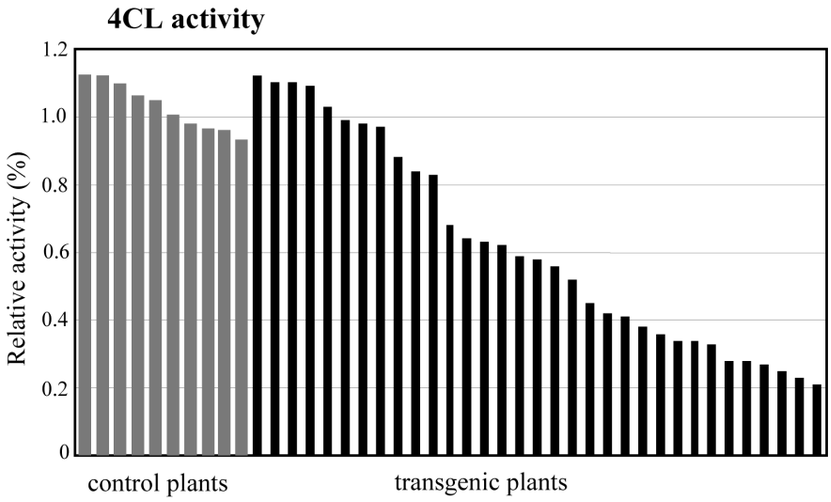

Figure 3. – Relative 4CL activity in control and transgenic plants with the antisense Ntlim1. Soluble protein fraction was prepared from four-month-old plants grown in the greenhouse. The enzyme activities were relative to the specific activity in the wild-type plants (= 1.0, 140.8 nmol min^{-1} mg-$protein^{-1}$). Each data point represents the average of three replicates.

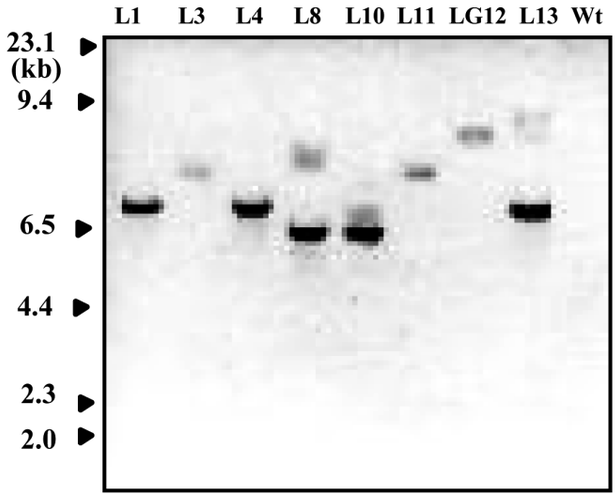

Figure 4. – Southern blot analysis using genomic DNA extracted from leaves in the transgenic plants. The genomic DNAs were digested with a restriction enzyme *Xho*I. A DNA fragment of the coding sequence of Ntlim1 was used as a probe.

regeneration. Forty PCR-positive or GUS-positive staining independent lines were finally transferred to soil and grown in a greenhouse.

4CL activities in transgenic plants

As a first screening, 4CL activity in the four month-old transgenic plants (about 80 cm tall) was measured. The soluble protein fraction was extracted from the upper part leaves of and the 4CL activity was assayed using a ferulic acid as a substrate. *Figure 3* shows relative activity where average specific activity of the control plants (wild-type and transgenic plants with pBI121-Hm) is 1.0. The 4CL activity in thirty-three transgenic plants showed wide variation and several transgenic plants exhibited less than 40% of 4CL activity as compared with control plants.

Southern analysis

To determine the number of copies of the transgene that had been integrated, Southern blots were performed. Genomic DNA was extracted from leaves in the

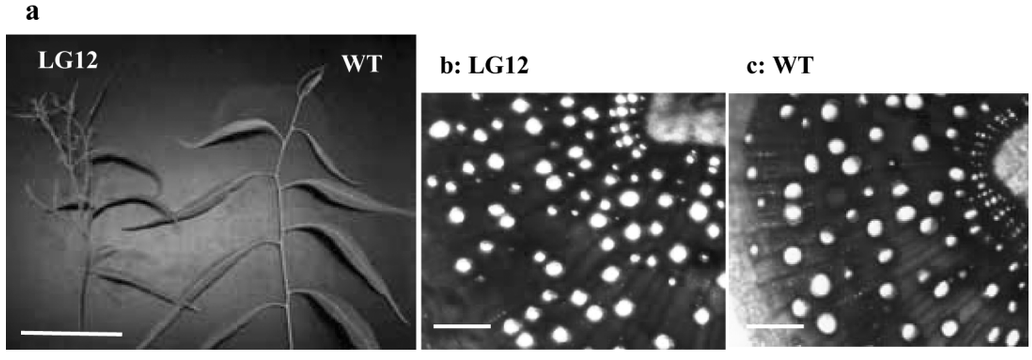

Figure 5. – Morphology and cross section of stem in the control and the LG12 plant. The control and LG12 plants appeared to grow normally under the greenhouse conditions. Stem sections were stained with phlorogrucinol-HCl to show anatomy. Vessel elements in both control and LG12 were not collapsed. Bars = 10 cm in (A) and 500 µm (B-C).

DOI:10.1515/sg-2006-0035
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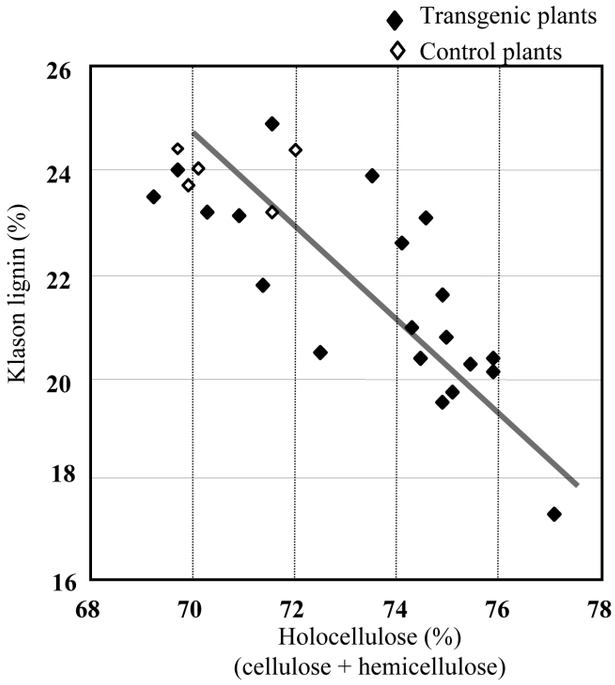

Figure 6. – Holocellulose and lignin contents in CWR of the control and transgenic plants. Lignin content is expressed as a percentage (w/w) of cell wall residues (CWR) by Klason lignin. Holocellulose content was determined by sulfide acid extraction. All values represent the results from the analysis of three measurements.

transgenic plants with lower levels of 4CL activity and digested with the restricted enzyme *Xho*I (one restriction site in the T-DNA). Hybridization with a digoxigenin (DIG)-labeled *Ntlim1* probe revealed that all analysed plants exhibited at least one copy of the *Ntlim1* gene (*Figure 4*).

Phenotype of transgenic plants

No obvious phenotypic differences in development or growth were found between control and transgenic plants grown in the greenhouse for six months, except in one line, LG12. The transgenic LG12 plants grew abnormally, exhibiting changing leaf shape and readily dropping their upper leaves (*Figure 5*). Stem cross sections were prepared and vessel elements were investigated. However, there were no collapsed xylem vessels in the cross section of the LG12 stem. Obviously, the phenotype of this plant may lack water transport ability.

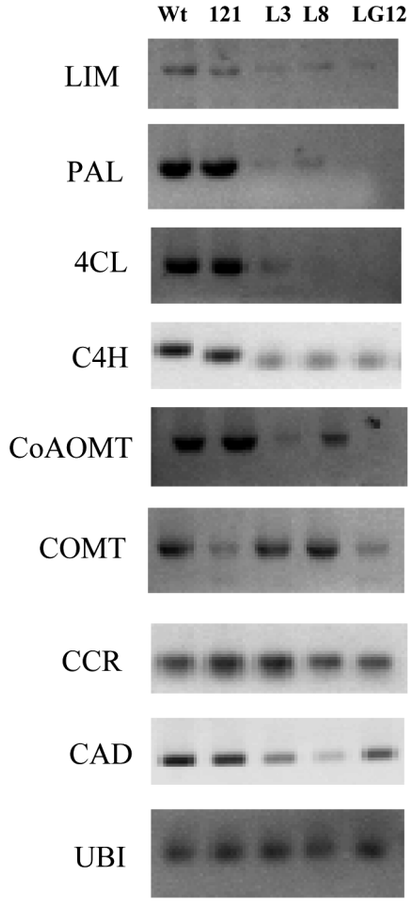

Figure 7. – RNA gel blot analysis showing expression of genes in the phenylpropanoid biosynthesis pathway. Total RNA was extracted from stems in wild-type (Wt), a transgenic plant (121) with control plasmid pBI121-Hm and plants with antisense Ntlim1 (L3, L8 and LG12). The transgenic plants were grown in the greenhouse for six months. Accumulations of mRNA of Eclim1, PAL, 4CL, C4H, CoAOMT, COMT, CCR and CAD were investigated. Eucalyptus ubiquitine gene was used as an internal control.

Wood composition in transgenic plants

Next, we measured Klason lignin and holocellulose content of six-month-old twenty transgenic and five control (wild-type and pBI121-Hm) Eucalyptus plants grown in the greenhouse. *Figure 6* showed lignin and holocellulose content of cell wall residues (CWR). Holocellulose value exhibited a sum of cellulose and hemicel-

Table 1. – Percentage weight composition of wood, including various neutral sugars from acid hydrolysis (% of cell wall residue).

Ec lines	lignin	holocellulose	glucose	mannose	arabinose	galactose	xylose
Wt	24.3 ± 0.4	69.5 ± 1.2	50.4 ± 0.7	1.1 ± 0.1	0.7 ± 0.1	2.4 ± 0.1	15.0 ± 0.4
121HPT	24.0 ± 0.1	70.1 ± 1.0	50.5 ± 0.2	1.1 ± 0.1	0.7 ± 0.1	2.2 ± 0.1	14.8 ± 0.1
L3	19.8 ± 0.3	75.4 ± 0.2	54.6 ± 0.6	1.3 ± 0.2	0.7 ± 0.2	1.6 ± 0.1	15.8 ± 0.2
L8	19.6 ± 0.4	75.2 ± 0.2	54.8 ± 0.5	1.3 ± 0.2	0.7 ± 0.1	1.6 ± 0.1	15.6 ± 0.2
LG12	17.3 ± 0.3	77.2 ± 0.2	55.8 ± 0.5	1.2 ± 0.2	0.7 ± 0.2	1.8 ± 0.1	15.5 ± 0.2

Six-month-old *E. camaldulensis* plants were used for analysis. Lignin content is expressed as a percentage (w/w) of CWR by Klason lignin. All values represent the results from the analysis of three measurements. As control plants, wild-type (Wt) and pBI121-Hm were measured.

DOI:10.1515/sg-2006-0035
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lulose content. Percentage weight composition of wood, including various neutral sugars from acid hydrolysis of CWR, were measured. The control plants contained 23–25% Klason lignin and approximately 70–72% holocellulose content (*Figure 6*). Three transgenic plants with the antisense *Ntlim1* had a lignin content of less than 20%. The most suppressed plant, LG12, had percentage weight compositions of less than 18% for lignin and 77% for holocellulose.

Percentage weight composition of wood, including various neutral sugars from acid hydrolysis of CWR, were measured. Control and transgenic plants differed the most in glucose content (*Table 1*). The transgenic plants L3, L8 and LG12 demonstrated glucose contents about 5% higher than those of the controls, indicating a possibility that cellulose content was increased.

Transcript levels of lignin biosynthesis

The transcript levels of the lignin biosynthesis genes *PAL*, *4CL*, *C4H*, caffeoyl-COA 3-O-methyltransferase (*CCoAOMT*), caffeic acid O-methyltransferase (*COMT*), cinnamyl:CoA reductase (*CCR*) and *CAD* were investigated among these transgenic plants with low level 4CL activity. Total RNAs were extracted from the stem of the six-month old transgenic and control (wild-type and pBI121-Hm) plants grown in the greenhouse. Controls showed a high level of expression of these genes (*Figure 7*). However, in the transgenic plants with the antisense *Ntlim1*, low transcript levels of the LIM gene were observed. Simultaneous down regulation was observed in the gene expression of the *PAL*, *C4H* and *4CL*. Levels of the other gene transcripts (*CCoAOMT*, *COMT*, *CCR* and *CAD*) were not affected in the plants, including those with low levels of *Eclim1* gene transcripts.

Discussion

We have succeeded in isolating the homologous gene of the tobacco transcription factor, *Ntlim1*, from *E. camaldulensis*. The *Eclim1* gene is present in *E. camaldulensis*, as indicated by a RNA gel blot analysis, and exhibits a gene expression mode like that of *Ntlim1*. (Kawaoka et al., 2000). A high level expression was observed in the shoots and entire stem region, indicating that *Eclim1* may be involved in lignification (*Figure 1c*). The amino acid sequence of *Eclim1* was revealed to be highly homologous to other known LIM proteins (*Figure 1b*). Notably, the LIM domain showed more than 80% identity with it. This result indicates the LIM domain may be conserved contractually. However, the C-terminal region of *Eclim1*, which is an activation domain, is 12 amino acids shorter than that of *Ntlim1* (Kawaoka et al., 2000).

There have been several reports regarding the transformation method and *E. camaldulensis* using a *GUS* reporter gene (Mullins et al, 1997; Ho et al., 1998). A high transformation efficiency has been documented for a selected clone of *E. camaldulensis* (Valerio et al., 2003). We introduced the antisense *Ntlim1* gene into hypocotyls prepared from germinated young seedlings. The resulting transformation efficiency was not extremely high (0.43%) as compared with Valerio's protocol. However, we produced kanamycin-resistant Eucalyptus plants. As a first screening of transgenic tree plants, we measured 4CL activity because 4CL activity is a reliable indicator of lignin content in higher plants (Kajita et al., 1998; Hu et al., 1998). An 80% decrease of 4CL activity was observed in the transgenic plants with the antisense *Ntlim1* (*Figure 3*).

The expression levels of genes involved in lignin biosynthesis showed intriguing results. The transcript levels of *PAL*, *C4H* and *4CL* in the LIM down-regulated plants, L3, L8 and LG12, were simultaneously reduced. In contrast, the *CAD* mRNA level was same as that of the control plants. This result is different from that found for tobacco, indicating a different gene expression mechanism by *Ntlim1* regulation. The AC-rich element, PAL-box, is present in the 5'-upstream region of the *CAD* gene of another Eucalyptus species, *E. gunni* (Piquemal et al., 1998). The detailed function of the sunflower LIM protein WLIM1 has been analyzed using a specific antibody. The WLIM1 protein was located in both the cytosol and the nucleus. Being found there, the WLIM1 protein acts as one of the proteins of the transcriptional complex (Briere et al., 2003). Recently, tobacco WLIM1 has reported as a novel F-actin binding protein involved in actin cytoskeleton remodeling (Thomas et al., 2006). Further analysis will be needed to determine the precise function of the LIM protein in lignification.

Eucalypts are the most commonly planted trees by the pulp and paper industries for providing hardwood material for pulp production. Wood is composed of cellulose, hemicellulose and lignin. The study of lignin biosynthesis has progressed well and there are numerous reports on transgenic woody plants with modifications of their lignin content or composition. Several genes, such as *4CL*, *COMT*, *CCR* and *CAD*, have been introduced into poplar trees and the trees' lignin composition analyzed (Hu et al., 1998; Li et al., 2003). Double transgenic aspen plants, with down-regulation of 4CL and up-regulation of coniferaldehyde 5-hydroxylase (CAld5H), showed low lignin content and a high S/G ratio (Li et al., 2003). Furthermore, down-regulation of CAD by the antisense method has been investigated in poplars, revealing that the plants with strongly reduced CAD activity had large amounts of free phenolic compounds. However, no obvious quantitative changes in lignin content have been found to date (Baucher et al., 1996; Lapierre et al., 1999; Pilate et al., 2002). Industrial interest is a strong driver of the nature of research undertaken on woody plants. Here, we described that a regulatory gene of lignin biosynthesis, a LIM-type transcription factor, affected lignin content in *E. camaldulensis*. The most dramatic reduction of lignin content in the transgenic plants with the antisense *Ntlim1* resulted in a content of 17.3% of CWR. The control plants showed approximately 24% lignin content, indicating a 28% reduction. Holocellulose content was increased by 5% in inverse proportion to lignin content (*Table 1*). Furthermore, glucose content (assessed by neutral sugar analysis) in the transgenic plant increased (*Table 1*). This result indicates the possibility of an increase in the final pulp yield. Chemical pulp is made by the removal

DOI:10.1515/sg-2006-0035
edited by Thünen Institute of Forest Genetics

of lignin with harsh chemicals. Therefore, woody plants with low lignin and high cellulose and hemicellulose contents would be desirable for efficient pulp production. Our results indicate that the down-regulation of the LIM gene is one of the effective methods of reducing lignin content in Eucalyptus trees. Naturally, the lignin content of woody plants grown in greenhouses is dependent on growing conditions. Since woody plants have longer growth periods than herbaceous plants, field studies of the growth rate of Eucalyptus with antisense *Ntlim1* are required.

Acknowledgements

We thank to ETSUKO MATSUNAGA, KEIKO YAMADA-WATANABE, KOICH SUGITA, SAORI ENDO-KASAHARA for transformation of Eucalyptus and MIDORI TAKAHASHI for sugar analysis. We also thank to TOSHIYUKI TAKANO and TOSHIAKI UMEZAWA for helpful discussion.

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DOI:10.1515/sg-2006-0035
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DOI:10.1515/sg-2006-0035
edited by Thünen Institute of Forest Genetics