Transgenic Note

Increasing fiber length and growth in transgenic tobacco plants overexpressing a gene encoding the *Eucalyptus camaldulensis* HD-Zip class II transcription factor

Tetsuya Sonoda¹, Hisako Koita¹, Shiho Nakamoto-Ohta¹, Keiko Kondo¹,
Tazuko Suezaki¹, Tomohiko Kato¹, Yoko Ishizaki¹, Kana Nagai¹, Nanae Iida¹,
Shigeru Sato¹, Toshiaki Umezawa², Takashi Hibino^{1,*}

¹ Forestry Research Institute, Oji Paper Co. Ltd., Nobono 24-9, Kameyama, Mie 519-0212, Japan; ² Research Institute for Sustainable Humanosphere, Kyoto University, Uji, Kyoto 611-0011, Japan

* E-mail: takashi-hibino@ojipaper.co.jp Tel: +81-595-85-0122 Fax: +81-595-85-1528

Received October 20, 2008; accepted November 27, 2008 (Edited by T. Ohno)

Abstract Since the woody plants are important bio-materials for paper, chemical and energy production, strategies for improvement of their properties are of considerable interest. We examined the expression of *Eucalyptus camaldulensis* transcription factor (TF) genes in xylem tissues using an oligo-microarray gene analysis technique. As a result, we isolated 21 TF genes related to xylem development. Following a functional investigation of 7 TFs *in vitro*, we selected the xylem TF gene *EcHBI*, which encodes a HD-Zip class II, for investigation as a transgene. *EcHBI* expression was driven by the CaMV35S promoter in transgenic tobacco plants, which showed greater fiber length ($\leq 20\%$) and plant height ($\leq 50\%$) than wild-type plants. In addition, leaf, root and stem growth were significantly enhanced in the transgenic lines, which also had a lower acid-soluble lignin and hemicellulose content than wild-type. Our results indicate that metabolic flexibility might be involved in these improvements to xylem cell wall biosynthesis and that in addition to providing a growth advantage, such modifications may confer long-term structural integrity to the woody perennials.

Key words: Cell wall, *Eucalyptus*, fiber growth, transcription factor.

Plant use atmospheric carbon dioxide to synthesize carbohydrates via photosynthesis, and some of these carbohydrates such as cellulose, hemicellulose and lignin, are components of cell walls. In trees, which are perennial and have completed dilation growth, cell wall components represent at least 90% of the fixed carbon dioxide. Conversion of this plant biomass into fossil energy sources such as oil has a fundamental impact on world development. Thus, research into the mechanisms of cell wall formation, which represents the majority of the convertible biomass, is important for the 21st century (Li et al. 2008; Pauly and Keegstra 2008). With respect to cell wall biosynthesis, the mechanisms underlying lignin biosynthesis have been investigated biochemically (Higuchi 1990) and genetically (Boerjan et al. 2003), with significant advances occurring during the latter half of the 1980's as gene analysis technology progressed. Elucidation of the mechanisms underlying cellulose and hemicellulose biosynthesis, which account for more

than 50% of cell wall components, has been slower. *Arabidopsis thaliana* was used as a model plant, in which processes and gene functions were determined. Currently, many cell wall biosynthesis genes have been characterized and with recent progress in DNA recombination techniques, these genes have been used in various improvement trials (Somerville 2006). Following publication of the complete *A. thaliana* genome sequence (The Arabidopsis Genome Initiative 2000), several additional plant genome sequencing projects were undertaken. In woody plants, the poplar genome has been completed (Tuskan et al. 2006) and *Eucalyptus* genome sequencing projects are underway in Japan and the USA. Artificial genetic modification technologies have led to the creation of new crops exhibiting herbicide resistance and tolerance to insects (Dale et al. 2002). In response to a demand from a paper pulp production industry, trees have also been the focus of genetic modification efforts, specifically with respect to

Abbreviations: *4CL*, 4-coumarate: CoA ligase; *C4H*, cinnamate 4-hydroxylase; *CAD*, cinnamyl alcohol dehydrogenase; CaMV, cauliflower mosaic virus; *CCoAOMT*, caffeoyl CoA 3-O-methyltransferase; *CCR*, cinnamoyl CoA reductase; *CesA*, cellulose synthase; *EGase*, endo- β -1,4-glucanase; fL, femto liter; HD, homeodomain motif; RT-PCR, reverse transcription polymerase chain reaction; S/G ratio, ratio of the abundances of the syringyl β -aryl ether subunits to those of the guaiacyl equivalents; *SuSy*, sucrose synthase; TF, transcription factor; *XTH*, xyloglucan endotransglucosylase/hydrolase; Zip, leucine-zipper motif

This article can be found at <http://www.jspcmb.jp/>

increasing and decreasing the cellulose and lignin contents, respectively (Boerjan 2005). During the 1990's, anti-sense-mediated reduction in the expression of lignin biosynthesis enzymes was reported by several research groups (Hibino et al. 1995; Baucher et al. 1996; Kajita et al. 1996; Ralph et al. 1998; Lapierre et al. 1999). In 1999, Hu et al. demonstrated that control of the 4-coumarate: coenzyme A ligase (*4CL*) gene could provide improvements in the growth potential of poplar, with a relative increase in cellulose content and a corresponding decrease in lignin content. Although there are many targets for improvement of the growth potential in woody plants, such as fiber length, cell wall thickness and lignin S/G ratio, in this report we have focused on the TFs that control the expression of multiple genes encoding enzymes for different cell wall biosynthesis pathways. Specifically, we considered that a practicable mechanism for eliciting continued biomass production in trees might be achieved by artificially increasing expression of certain TF genes. Although the model plant *A. thaliana* has represented the primary source of genetic information on plant cell wall biosynthesis, we chose *Eucalyptus* as a more appropriate model for analysis of

these processes in woody plants.

Eucalyptus camaldulensis trees were grown in the experimental field at the Forestry Research Institute (Oji Paper Co. Ltd.). RNA and DNA were prepared from 2- to 5-year-old plants. RNA was extracted from about 1 g of each tissues used e.g. stem, leaf and root using the Plant RNA Isolation Reagent (Invitrogen, USA), according to the supplier's protocol. The SMART kit (Clontech, USA) was then used to generate five tissue-specific cDNA libraries. Sequencing, data assembly and annotation were performed by Takara-Bio (Japan). All original results were reported to the *Eucalyptus* EST database (Sato et al. 2001) and our microarray analysis methodology was established using this sequence information.

Sixty nucleotide sequences were used as probes for searching the 10,000 *Eucalyptus* cDNA sequences within two xylem specific cDNA libraries. As a result, 7,538 independent array probe sequences were identified, designed and used to construct a glass slide oligo-array (Agilent Technology, USA). Hybridization experiments were performed using the Agilent Technology labeling and hybridization kits, according to the supplier's

Figure 1. Expression trends of genes involved in cellulose and lignin biosynthesis. All expression ratios are shown in $\log_2$ scale. We examined three different gene expression profiles from *Eucalyptus* trees, i.e., the lower part of a 2-year-old tree (Ec2y), and the lower and upper parts of a 5-year-old tree (Ec5y-L and Ec5y-U, respectively), using our *Eucalyptus* oligo-microarray analysis system. Group 1 (Red lines) showed the highest expression in the lower part of 2-year-old tree. Group 2 (Blue lines) showed the highest expression in the upper part of the 5-year-old tree. (A) Expression trends of the CesaA family were shown. (B) Expression trends of EGase, Chitinase, Dynamine and Susy. (C) Expression trends of the CAD family. (D) Expression trends of the Laccase family. (E) Expression trends of OMT. (F) Expression trends of 4CL, CCR and C4H.

protocol. Fluorescence intensity was measured with the Model G2505A scanner (Agilent Technology) and multivariate data analyses were performed using the Luminator software (Rosetta Biotechnology, USA). We collected the gene expression profiles from two different *Eucalyptus* trees (the lower part of a 2-year-old tree, and the lower and upper parts of a 5-year-old tree) using our *Eucalyptus* oligo-microarray. To identify the trends in expression of genes involved in cellulose and lignin biosynthesis, we performed trend data analysis between the three samples. These trends could be divided into two groups (Figure 1): Group 1 showed the highest expression in the lower part of 2-year-old tree and Group 2 showed the highest expression in the upper part of the 5-year-old tree.

Next, we identified gene clusters for each of the groups and found that each cluster contained several TF genes. From these results, we selected 21 TF genes for *in vitro* analysis using the Fluorescence Correlation Spectroscopy method (Palo K et al. 2002). This single molecule fluorescent detection system (model MF20; Olympus, Japan) enabled the analysis of interactions between 7 TF proteins and the promoters of 15 genes encoding different lignin and cellulose biosynthesis enzymes. This method can be used to obtain the translational diffusion time, i.e., the average time

required for the molecules to pass through the very small volume (1 fl) in the sample. The size of fluorescent molecules can be determined by the translational diffusion time and DNA-protein interactions detected as a change in that diffusion time. This method allowed us to analyze DNA-protein interactions easily and more quickly than conventional systems such as gel shift

Figure 2. *EcHB1* expression profile. *EcHB1* was expressed in secondary xylem developing tissues of *E. camaldulensis*. Total RNA was extracted from mature leaf (ML), young leaf (YL), flower (F), seedling (Se), hypocotyl (H), 1-year-old mature root (MR), 6-month-old young root (YR), 6-month-old young stem (YS) and xylem of a 5-year-old mature stem (MX). Expression levels of samples and experimental control actin were analyzed by semi-quantitative RT-PCR using the Access RT-PCR System (Promega, USA) by supplier's protocol. PCR amplification was performed using the primers HB1RTF (5'-CGAAGCTCAGTGGTAACTCC), and HB1RTR (5'-GGCTCTCCTATTCTGGAACC) for *EcHB1* and *EcACT2F* (5'-CTTCGTGTAGCCCCAG), and *EcACT2R* (5'-CTCGTATATGGGCACAGT) for actin.

Figure 3. Phenotypes of the transgenic and wild-type tobacco plants. (A) Growth after 6 weeks. (B) Flower shapes of the transgenic and wild-type tobacco plants. Bar=1 cm

assays. Clear diffusion time changes were detected in reactions containing a TF annotated as the HD-Zip class II (Rueda et al. 2005).

We isolated a full-length cDNA (*EcHB1*; accession No. AB458829) encoding *E. camaldulensis* HD-Zip class II transcription factor. The protein encoded by *EcHB1* belongs to the HD-Zip class II subfamily and is predicted to contain both a homeodomain and a leucine-zipper motif. RT-PCR analysis indicated that *EcHB1* is expressed in the mature stem and root tissues (Figure 2).

In order to characterize the function of *EcHB1*, the gene was over-expressed *in vivo* in tobacco and *Eucalyptus*. The Gateway binary vector pGWB2 (Invitrogen) was used to construct *35S::EcHB1*, which was introduced into *Agrobacterium tumefaciens* EHA101 (Hood et al. 1986) by Bevan's method (1984). The construct was then transferred from the bacterium into tobacco (*Nicotiana tabacum* L. Xanthi-nc) and eucalypt (*Eucalyptus grandis* × *urophylla* hybrid) by the leaf disk method (Horsh et al. 1985). Inoculated tobacco leaves were cultured on a regeneration medium containing carbenicillin (500 mg ml⁻¹) and hygromycin (100 mg ml⁻¹) at 25°C. Regenerated plants were grown on the MS medium containing hygromycin (100 mg ml⁻¹) for three weeks and then transferred to soil. Although

inoculated *Eucalyptus* tissues were cultured in the same manner as tobacco, PCR screening was performed on the transgenic calluses. The introduced *EcHB1* gene was detected using the primers HB1F (5'-CACCACGCGTGATATCATGTGCCCTATCGAT), and HB1R (5'-GGGTCGACTTAACAAGCGGCTGAT).

To obtain multiple transgenic tobacco plants with the same genetic background, the stems of plants were cut into several pieces, transplanted into soil to generate new plants and then, grown for six weeks. The height and diameter of transgenic tobacco stems were measured every week and the fiber lengths were determined using a weighing device (model: FS-200; Kajaani, Finland). After acid hydrolysis of cellulose and hemicellulose, the content of these polysaccharides in the tobacco stem was determined from total sugars (Chiang and Sarkanen 1983). Lignin content was determined by the Klason method (Chiang and Funaoka 1990).

In all transgenic tobacco plants, overexpression of recombinant *EcHB1* was driven by the CaMV35S promoter and clearly conferred improvement in plant growth potential (Figure 3). The mean height of six transgenic tobacco plants was *ca.* 1.5-fold greater than two wild-type tobacco plants and fiber lengths were *ca.* 1.2-fold greater in the former than the latter with

Figure 4. Fiber lengths in the transgenic and wild-type tobacco plants. About 30 mg of xylem tissues were dissociated by immersed with an acid solution (10% (v/v) nitric acid and 10% (w/v) chromium (IV) oxide) for 24 hours. The treated xylem tissues were washed with distilled water in twice, and immersed with distilled water. (A) Fiber cells were observed with a microscope and then photographed. Bar=200 μ m. (B) Fiber length measurements were determined using a weighing device (model: FS-200; Kajaani, Finland).

statistically significantly (Figure 4). The cell wall of transgenic tobacco plants exhibited reduced acid-soluble lignin and hemi-cellulose contents, although no change was observed in the cellulose content or in the amount of Klason-lignin (data not shown).

Gene expression was investigated in approximately 50 mg of fresh the transgenic *Eucalyptus* callus tissue using the oligo-microarray analysis system. Preliminary findings indicate the expression of major genes related to lignin biosynthesis (*CCoAOMT*, *CAD*, *CCR* and *C4H*) was reduced. However, we also observed increased the expression of several *XTH* genes, which are involved in cell elongation. Statistically significantly, expression of *XTH1*, *XTH2* and *XTH3* were *ca.* 3.0-fold, *ca.* 2.3-fold and *ca.* 1.5-fold greater than a wild-type *Eucalyptus*, respectively (data not shown).

To clarify *EcHb1* function in related to cell wall biosynthesis genes, mature plants have been regenerated from the transgenic *Eucalyptus* callus. Our results from the analysis of transgenic tobacco plants indicate that metabolic flexibility might be involved in our observed improvements to xylem cell wall biosynthesis and that these modifications confer a growth advantage, as well as the long-term structural integrity to the woody perennials.

Acknowledgements

We gratefully acknowledge Dr. Takashi Shiina (Kyoto Pref. University) for the gift of the tobacco plant (*Nicotiana tabacum* L. Xanthi nc). This work was supported by the New Energy and Industrial Technology Development (NEDO) program, which is part of the "Development of Fundamental Technologies for Controlling the Material Production Process of Plants" project.

References

- Baucher M, Chabbert B, Pilate G, Van Doorselaere J, Tollier MT, Petit-Conil M, Cornu D, Monties B, Van Montagu M, Inze D, Jouanin L, Boerjan W (1996) Red xylem and higher lignin extractability by down-regulating a cinnamyl alcohol dehydrogenase in Poplar. *Plant Physiol* 112: 1479–1490
- Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. *Nucl Acids Res* 12: 8711–8721
- Boerjan W (2005) Biotechnology and the domestication of forest trees. *Curr Opin Biotechnol* 16: 159–166
- Boerjan W, John Ralph J, Baucher M (2003) Lignin biosynthesis. *Annu Rev Plant Biol* 54: 519–546
- Chabannes M, Barakate A, Lapierre C, Marita JM, Ralph J, Pean M, Danoun S, Halpin C, Grima-Pettenati J, Boudet AM (2001) Strong decrease in lignin content without significant alteration of plant development is induced by simultaneous down-regulation of cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) in tobacco plants. *Plant J* 28: 257–270
- Chabannes M, Ruel K, Yoshinaga A, Chabbert B, Jauneau A, Joseleau JP, Boudet AM (2001) In situ analysis of lignins in transgenic tobacco reveals a differential impact of individual transformations on the spatial patterns of lignin deposition at the cellular and subcellular levels. *Plant J* 28: 271–282
- Chiang VL and Funaoka M (1990) The dissolution and condensation reaction of guaiacyl and syringyl units in residual lignin during kraft delignification of sweetgum. *Holzforschung* 44: 147–188
- Chiang VL and Sarkanen KV (1983) Ammonium sulfate organosolv pulping. *Wood Sci Technol* 17: 217–226
- Dale PJ, Clarke B, Fontes EM (2002) Potential for the environmental impact of transgenic crops. *Nat Biotechnol* 20: 843
- Hibino T, Takabe K, Kawazu T, Shibata D, Higuchi T (1995) Increase of cinnamaldehyde groups in lignin of transgenic tobacco plants carrying and antisense gene for cinnamyl alcohol dehydrogenase. *Biosci Biotechnol Biochem* 59: 929–931
- Higuchi T (1990) Lignin biochemistry: biosynthesis and biodegradation. *Wood Sci Technol* 24: 23–63
- Hood EE, Helmer GL, Fraley RT, Chilton MD (1986) The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. *J Bacteriol* 168: 1291–1301
- Horsh RB, Fry JE, Hoffmann NL, Eihholtz D, Roger SG, Fraley RT (1985) A simple and general method for transferring genes into plants. *Science* 227: 1229–1231
- Hu W-J, Harding SA, Jung J, Popko JL, Ralph J, Stokke DD, Tai C-J, Chiang VL (1999) Repression of lignin biosynthesis promotes cellulose accumulation and growth in transgenic trees. *Nat Biotechnol* 17: 808–812
- Kajita S, Katayama Y, Omori S (1996) Alterations in the biosynthesis of lignin in transgenic plants with chimeric genes for 4-coumarate: coenzyme A ligase. *Plant Cell Physiol* 37: 957–965
- Lapierre C, Pollet B, Petit-Conil M, Toval G, Romero J, Pilate G, Leple JC, Boerjan W, Ferret VV, De Nadai V, Jouanin L. (1999) Structural alterations of lignins in transgenic poplars with depressed cinnamyl alcohol dehydrogenase or caffeic acid O-methyltransferase activity have an opposite impact on the efficiency of industrial kraft pulping. *Plant Physiol* 119: 153–164
- Li X, Weng JK, Chapple C (2008) Improvement of biomass through lignin modification. *Plant J* 54: 569–581
- O'Connell A, Holt K, Piquemal J, Grima-Pettenati J, Boudet A, Pollet B, Lapierre C, Petit-Conil M, Schuch W, Halpin C (2002) Improved paper pulp from plants with suppressed cinnamoyl-CoA reductase or cinnamyl alcohol dehydrogenase. *Transgenic Res* 11: 495–503
- Palo K, Brand L, Eggeling C, Jager S, Kask P, Gall K (2002) Fluorescence intensity and lifetime distribution analysis: toward higher accuracy in fluorescence fluctuation spectroscopy. *Biophys J* 83: 605–618
- Pauly M, Keegstra K (2008) Cell wall carbohydrates and their modification as a resource for biofuels. *Plant J* 54: 559–568
- Pilate G, Guiney E, Holt K, Petit-Conil M, Lapierre C, Leple JC, Pollet B, Mila I, Webster EA, Marstorp HG, Hopkins DW, Jouanin L, Boerjan W, Schuch W, Cornu D, Halpin C (2002) Field and pulping performances of transgenic trees with altered lignification. *Nat Biotechnol* 20: 607–612
- Ralph J, Hatfield RD, Piquemal J, Yahiaoui N, Pean M, Lapierre C, Boudet AM (1998) NMR characterization of altered lignins extracted from tobacco plants down-regulated for lignification enzymes cinnamylalcohol dehydrogenase and cinnamoyl-CoA reductase. *Proc Natl Acad Sci USA* 95: 12803–12808
- Rueda EC, Dezar CA, Gonzalez DH, Chan RL (2005) Habb-10, a

- sunflower homeobox-leucine zipper gene, is regulated by light quality and quantity, and promotes early flowering when expressed in Arabidopsis. *Plant Cell Physiol* 46:1954–1963
- Sato S, Horikiri K, Miyashita K, Ishige K, Asada T Hibino T (2001) Analysis of wood development with a genomic approach: *Eucalyptus* ESTs and TAC genomic library. In: Morohoshi N, Komamine A (eds) *Molecular Breeding of Woody Plants*, Elsevier, Tokyo, pp 223–228
- Somerville C (2006) Cellulose Synthesis in Higher Plants. *Annu Rev Cell Dev Biol* 22: 53–78
- The Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* 408: 796–815
- Tuskan GA, Difazio S, Jansson S, Bohlmann J, Grigoriev I, et al. (2006) The Genome of Black Cottonwood, *Populus trichocarpa* (Torr. & Gray). *Science* 15: 1596–1604