J Wood Sci (2009) 55:435–440
DOI 10.1007/s10086-009-1048-y
© The Japan Wood Research Society 2009

ORIGINAL ARTICLE

Rumi Kaida · Tomomi Kaku · Kei'ichi Baba · Sri Hartati
Enny Sudarmonowati · Takahisa Hayashi

Enhancement of saccharification by overexpression of poplar cellulase in sengon

Received: May 27, 2009 / Accepted: June 24, 2009 / Published online: September 7, 2009

Abstract Lignocellulosic material from trees has great potential to form the basis of the second generation for bioethanol production because trees produce most of the biomass on the earth. We modified the wall structure of sengon (*Paraserianthes falcataria*) through overexpression of poplar cellulase in the cell walls. The overexpression did not decrease cellulose content but caused a decrease in xyloglucan bound to the walls. The level of saccharification and successive ethanol production was increased in the wood of the transgenic sengon overexpressing poplar cellulase compared with that of the wild type plant, and even after delignification of the wood. We propose that a xyloglucan intercalated into cellulose microfibrils could be one of the recalcitrant components in the saccharification of lignocelluloses.

Key words Cellulase · Sengon · Xyloglucan · Saccharification · Overexpression

Introduction

With more than half of the world's original oil reserves consumed, many countries now add ethanol to gasoline to levels of up to 25% to 30%.[1] Using bioethanol-blended gasoline for automobiles not only saves petroleum but also reduces exhaust greenhouse gas emissions. The ethanol used is made mainly from sucrose extracted from sugarcane and sweet sorghum and starch extracted from corn, wheat, and cassava. However, the use of these feedstocks competes with food manufacture for humans and livestock and tends to limit the supply for ethanol production. Therefore, it is considered that lignocellulosic biomass (e.g., wood, straw, and grasses) is the most promising material in second-generation bioethanol production. Trees can grow on marginal land unsuited to food production and produce more than 90% of the biomass on earth.[2–4] We believe that trees, in comparison to grasses, could represent a key feedstock for bioethanol production.

Trees grow faster in tropical areas with lots of rain and high humidity than in other regions on earth. Thus, woody plant biomass in tropical areas is becoming more attractive as an alternative fuel resource from the viewpoint of global warming mitigation and fossil resource conservation. Among the trees under consideration, we have proposed cultivating sengon (*Paraserianthes falcataria*) as a feedstock in tropical areas because its wood can be converted to ethanol at the same or a notably higher level than that of poplar.[5] Poplar has been recommended as a feedstock for production of cellulosic ethanol in temperate regions,[6] so we propose using sengon as the standard feedstock in tropical regions. Sengon is one of the fastest growing tree species on earth. The plant belongs to the subfamily Mimosoideae of Leguminosae, and is native to Haiti, Indonesia, Papua New Guinea, and the Solomon Islands. Like *Acacia mangium*, sengon thrives even in marginal land, where the plant grows symbiotically with nitrogen-fixing *Rhizobium* and phosphorus-promoting mycorrhizal fungi.[7]

Lignocellulose has been subjected to either alkaline, acid, or fungal treatment to facilitate its enzymatic degradation because the cellulose is difficult to hydrolyze by enzymatic means. Rather than studying such treatment, we focus on the effect of wall modification by the genetic recombination on lignocelluloses.

The poplar cellulase (*endo*-1,4-β-glucanase) was purified to homogeneity from suspension-cultured poplar cells.[8] The purified enzyme specifically cleaved the 1,4-β-glucosyl linkages of carboxymethylcellulose, phosphor-swollen cellulose, lichenan, xyloglucan, and xylan, but the latter two substrates were hydrolyzed very slowly. The enzyme hydrolyzed xyloglucan with a low K_m at a low rate. A cDNA clone

R. Kaida (✉) · T. Kaku · K. Baba · T. Hayashi
Research Institute for Sustainable Humanosphere, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan
Tel. +81-77-438-3622; Fax +81-77-438-3618
e-mail: kaida@rish.kyoto-u.ac.jp

S. Hartati · E. Sudarmonowati
Research Centre for Biotechnology, LIPI, Cibinong 16911, Indonesia

Part of this article was presented at the 59th Annual Meeting of the Japan Wood Research Society, Matsumoto, March, 2009

encoding the *endo*-1,4-β-glucanase was isolated,[9] and transgenic sengons overexpressing the cellulase constitutively were generated using the cDNA.[10]

In this study, we examined the level of saccharification of wood from transgenic sengon overexpressing poplar cellulase. Xylem matured for about 1 year was used to test for saccharification. Our aim was to assess whether the modification of wall structure by overexpression of plant cellulase enhances saccharification.

Materials and methods

Sengon

Sengon seeds were obtained from West Java, Indonesia, and immersed in water at 90°C for 30 s to break the dormancy of the seeds before planting them on sterile agar medium. This technique is used to remove wax from the seeds, so that the seeds can easily absorb the water they need to germinate. Hypocotyls of seedlings (about 3 weeks old) were finely cut into small pieces (1 cm) and used for transformation.

Transgenic plants

We used transgenic sengons that were produced previously.[10] The poplar cellulase (*PaPopCel1*, accession number D32166) cDNA fragment[9] was substituted for the GUS-coding sequence in the binary vector pBE2113.[11] The plasmid was transformed into *Agrobacterium tumefaciens* LBA4404. Pieces of sengon stems excised from their hypocotyls were dipped in the *Agrobacterium* solution for 10 min and cocultivated for 1 day on half strength hormone-free Murashige-Skoog (MS) medium. Then the pieces of stem were placed on MS agar medium containing 600 mg/l kanamycin for 2 weeks after they were washed with a water solution containing 400 mg/l Claforan. The stems were cultured several times by transplantation on MS agar media containing 50 mg/l kanamycine and 4 µM benzylaminopurine for 2–4 months under 14-h day (4000 lx)/10-h night cycles after they were placed on a medium containing 300 mg/l kanamycine for 2 weeks. Shoots 5 mm in length were excised from the medium and cultured again on a medium containing 50 mg/l kanamycine in the absence of plant hormone. Roots were then formed in the medium for 2–4 weeks and the plantlets were further cultured for growth in the medium for 2 months. Plantlets approximately 10–15 cm long were planted in soil. This study employed three kinds of transgenic plants: Trg 1, Trg 2, and Trg 6, which correspond to the transgenic sengons reported by Hartati et al.[10]

Preparation of sengon woody meal

Pieces of sengon stem wood were excised from 1-year-old trees grown in biosafety containment. Their bark was peeled off, and samples of wood were dried in an oven at 70°C for 16 h, and then milled into a powder using a ball mill (MM400, Retsch, Haan, Germany) at a speed of 15 rps for 10 min. The meal samples were used for saccharification alone or in combination with fermentation.

Wall analysis

Polysaccharides of hemicelluloses were successively extracted four times with 24% KOH containing 0.1% $NaBH_4$. The insoluble wall residue (cellulose fraction) was washed twice with water and solubilized with ice-cold 72% sulfuric acid. The amount of cellulose was also determined by measuring the acid-insoluble residue; the samples were extracted with acetic/nitric reagent (80% acetic acid/concentrated nitric acid, 10:1) in a boiling water bath for 30 min.[12] The resulting insoluble material was washed in water and solubilized with ice-cold 72% sulfuric acid. Total sugar in each fraction was determined by the phenol–sulfuric acid method. The alkali-soluble fraction was neutralized, dialyzed, and freeze-dried for use in methylation analysis.[13] Partially methylated alditol acetates were analyzed using an Agilent gas chromatography-mass spectrometer apparatus (Santa Clara, CA, USA) with a glass capillary column (DB-225, (0.25 mm i.d. ×15 m, Agilent). Each alditol acetate was identified by its retention time and mass spectrum. Lignin content was determined by the Klason method.[14]

Enzymatic hydrolysis

One hundred milligrams of meal was autoclaved at 120°C for 3 min to impregnate it with water, and washed once with water by centrifugation. A commercial cellulase preparation (Meicelase, Meiji Seika, Tokyo, Japan) derived from *Trichoderma viride* was used to digest the meal samples. The enzyme preparation contained endocellulases, exocellulases (CBHI and CBHII), xyloglucanase, xylanase, galactanase, and polygalacturonase. Enzymatic hydrolysis of the meal samples was performed in 2 ml of 50 mM sodium acetate buffer, pH 4.8, containing 0.02% Tween 20 and 0.4 filter paper units of the cellulase preparation (2.0 mg). One filter paper unit is defined as 1 µg of glucose released per minute from filter paper. The mixture was incubated at 45°C in a rotary shaker set at 135 rpm. About 100 µl of the supernatant was collected at 6, 24, and 48 h after the start of hydrolysis and used for sugar analysis. The quantity of sugar released was estimated as reducing sugar by the Nelson-Somogyi method. The amount of glucose was also determined by the action of glucose oxidase using the Glucose C2 reagent (Wako, Osaka, Japan). Furthermore, free sugars released were directly analyzed according to their alditol acetates using gas chromatography.

Ethanol production

For simultaneous enzymatic saccharification and fermentation, a seed culture of *Saccharomyces cerevisiae* (SH1089)

and yeast nutrients [4 mg $(NH_4)_2HPO_4$, 0.2 mg $MgSO_4 \cdot 7H_2O$, and 8 mg yeast extract] were added to each meal sample for enzymatic hydrolysis (2 ml of 50 mM sodium acetate buffer, pH 4.8, containing 0.02% Tween 20, and 0.4 filter paper units of cellulase preparation). Each mixture was incubated at 40°C in a rotary shaker set at 135 rpm. About 100 µl of the supernatant was collected at 6, 24, and 48 h after the start of incubation. The ethanol formed was measured by gas chromatography on a Supelcowax-10 column (0.53 mm i.d. ×15 m, Supelco, Bellefonte, PA, USA) at 50°C using an Agilent gas chromatograph. Isopropanol was used as an internal standard.

Treatment for delignification

Delignification of the woody meal (100 mg) that was autoclaved at 120°C for 3 min was performed in 5 ml of 8% sodium chlorite solution containing 1.5% acetic acid by shaking at 50 rpm at 35°C for 40 h. During the delignification, the solution was exchanged for a new one prepared just before use every 8 h. This delignification procedure was repeated five times in total. The meal was washed five times by centrifugation at 3000 rpm for 5 min. The meal samples were confirmed as lignin-free (nondetectable) by the Klason method,[14] and then used for enzymatic hydrolysis.

Results

Chemical analysis of wood

Based on chemical analyses (Table 1), the level of cellulose in sengon wood was not affected by the overexpression of poplar cellulase; nor was lignin content changed by the overexpression. Based on methylation analysis of the hemicellulose fraction, the transgenic sengon overexpressing poplar cellulase contained only half as much 4,6-linked glucose as well as 4-linked glucose as the wild type, indicating that the transgenic plant had less wall-bound xyloglucan than the wild type. The predominant component was 4-linked xylose in the walls of both the wild type and transgenic plant, showing that xylan is the major hemicellulose component in the woods of sengons. It should be noted that the overexpression did not affect xylan and glucomannan content in the walls, because levels of 4-linked xylose and 4-linked mannose were not changed in the transgenic plant.

Effect of overexpression of poplar cellulase on enzymatic hydrolysis of wood

Enzymatic hydrolysis of woody meal was significantly increased by the overexpression of poplar cellulase during the experimental time course (Fig. 1). The transgenic plants (Trg 1, 2, and 6) gave 41 mg (average of three lines) of reducing sugar when 100 mg of woody meal was subjected to saccharification for 48 h. The level of saccharification in the transgenic plants was 1.4-fold higher than that in the wild type. The level of glucose liberated was about 76% of total sugar released from the transgenic wood overexpressing poplar cellulase and about 69% of total sugar from the

Table 1. Composition of walls in sengon wood

Components	Wild type	Transgenic
Cellulose	52.6	54.4
Lignin	26.5	25.3
Hemicellulose	21.0	20.3
Fucosyl terminal	n.d.	n.d.
Arabinosyl terminal	0.5	0.6
Xylosyl terminal	0.3	0.4
2- or 4-Linked	14.2	15.4
Glucosyl 4-linked	3.1	1.4
4,6-Linked	0.5	0.2
Galactosyl terminal	0.1	0.1
4-Linked	1.4	1.3
3,6-Linked	0.2	0.2
Mannosyl 4-linked	0.7	0.7

Data given in units of mg/ 100 mg wood. Hemicelluloses extracted with 24% KOH were used for methylation analysis. Individual values represent the mean (three wild type lines and transgenic 1, 2, and 6 lines) varying from the mean by <0.8%

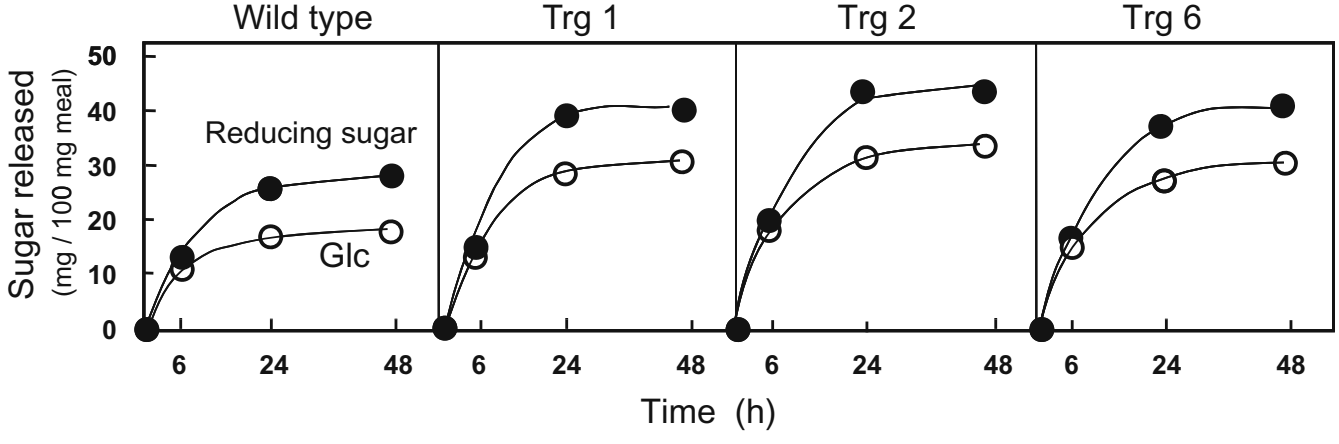

Fig. 1. The time course of enzymatic hydrolysis of sengon woody meal. *Closed symbols*, reducing sugars; *open symbols*, glucose (*Glc*) determined by the glucose oxidase method. Individual values represent the mean of three different experiments varying from the mean by <0.5%

Table 2. Monosaccharides released by enzymatic hydrolysis at 48 h

Monosaccharide	Wt	Trg 1	Trg 2	Trg 6
Glc	20.2	30.9	32.1	29.5
Gal	0.7	0.9	1.0	0.9
Man	0.4	0.3	0.3	0.3
Xyl	7.4	8.4	8.6	8.5
Ara	0.2	0.3	0.3	0.2
Fuc	0.1	0.1	0.1	0.1
Rha	0.3	0.3	0.3	0.3
Total	29.3	39.5	42.7	39.8

Data given in units of mg/100 mg wood meal. Individual values represent the mean of three different experiments varying from the mean by <0.5%
Wt, Wild type; Trg, transgenic; Glc, glucose; Gal, galactose; Man, mannose; Xyl, xylose; Ara, arabinose; Fuc, fucose; Rha, rhamnose

Table 3. Monosaccharides released by enzymatic hydrolysis from delignified wood meals

Monosaccharide	Wt	Trg 1	Trg 2	Trg 6
Glc	37.9	48.6	50.6	48.4
Gal	2.2	2.2	2.2	2.1
Man	1.7	1.5	1.4	1.6
Xyl	11.9	15.9	16.3	15.8
Ara	0.5	0.5	0.4	0.4
Fuc	0.1	0.1	0.1	0.1
Rha	0.3	0.3	0.3	0.3
Total	54.6	69.1	71.3	68.7

Data given in units of mg/100 mg wood meal. Individual values represent the mean of three different experiments varying from the mean by <0.5%

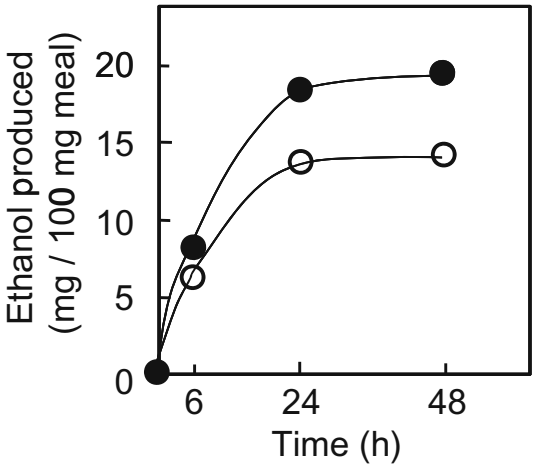

Fig. 2. Ethanol production from woody meals. Time course of ethanol production was due to simultaneous enzymatic saccharification and fermentation. *Closed circles*, transgenic sengons; *open circles*, wild type sengons. Individual values represent the mean (three wild type lines and transgenic 1, 2, and 6 lines) varying from the mean by <0.8%

wild type (Fig. 1 and Table 2). During the saccharification of the walls, because the level of cellulose hydrolysis was higher for the transgenic plants than wild type plants, cellulose hydrolysis was increased 1.4-fold in the transgenic plant than in the wild type; that is, cellulose hydrolysis increased to 57% (31 mg/54 mg) in the transgenic and 38% (20 mg/52 mg) in the wild type, respectively. This result showed that the overexpression accelerated the saccharification of wood, in which cellulose hydrolysis was effectively increased.

Ethanol production

Ethanol production was higher in the enzymatic hydrolysate of the wood overexpressing poplar cellulase than in the wild type when saccharification was accompanied by fermentation with yeast (Fig. 2). The relative pattern of ethanol production was similar to that of the level of saccharification among the transgenic and the wild type plants. The ethanol production was increased 1.4-fold by the overexpression.

There may have been some product inhibition during saccharification rather than simultaneous enzymic saccharification and fermentation, because the levels of ethanol production were theoretically 10% to 15% higher than those of fermentable sugars (glucose, mannose, and galactose) produced during saccharification. The higher the level of saccharification rose, the more inhibition occurred. Therefore, the inhibition was notably higher for the wood overexpressing poplar cellulase. It should be noted that although ethanol fermentation was reported to be inhibited by 5-hydroxymethyl furfural, furfural, and vanillin formed in acid hydrolysis or thermal pretreatment,[15] no corresponding inhibitory compounds were found in the enzymic hydrolysate.

Saccharification after removal of lignin

Delignification of wood with sodium chlorite accelerated the enzymatic hydrolysis of meal in all the samples from the transgenic and the wild type plants (Fig. 3 and Table 3). Nevertheless, the levels of saccharification in the transgenic plants were kept at 1.4-fold higher than that in the wild type. In addition, the amount of glucose released during enzyme degradation was about 69% in all the samples, showing that the delignification caused the acceleration of saccharification equally in all the wall components.

Discussion

In the transgenic sengons overexpressing poplar cellulase, the amount of wall-bound xyloglucan in the secondary wall of the stem (Table 1) as well as in the primary wall of the petiole pulvinus and the main vein[10] was decreased. Nevertheless, increased cellulase activity in the walls did not affect the content of cellulose and glucomannan. The result is in agreement with the previous finding[10,16,17] that the overexpression of plant cellulase did not cause a decrease in cellulose content, but loosened xyloglucan intercalation between cellulose microfibrils, the paracrystalline sites of which could be efficiently removed by poplar cellulase. Here, we demonstrated that the modification of wall struc-

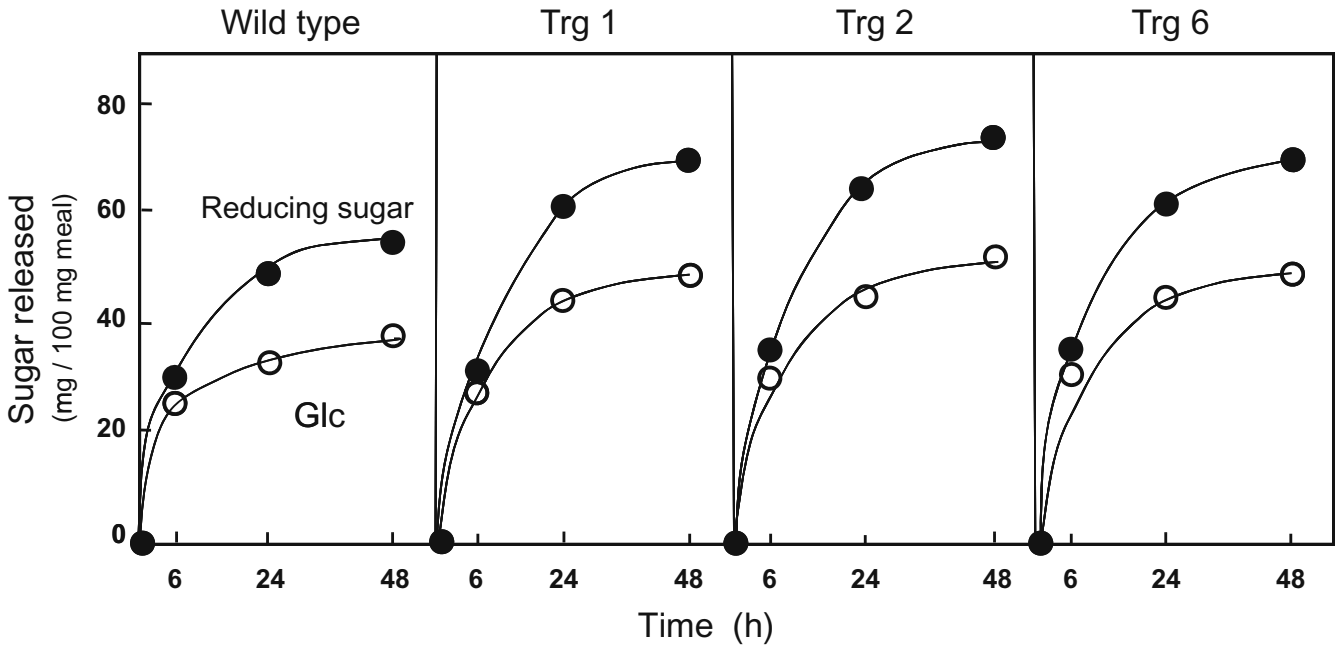

Fig. 3. The time course of enzymatic hydrolysis of sengon woody meal in which lignin was removed. Closed symbols, reducing sugar; open symbols, glucose (*Glc*) determined by the glucose oxidase method. Individual values represent the mean of three different experiments varying from the mean by <0.8%

ture by the overexpression of poplar cellulase enhanced the levels of saccharification and fermentation in sengon. It should be noted that the overexpression accelerated the saccharification of wood, in which cellulose hydrolysis was more effectively increased. Therefore, biotechnology that increases the level of saccharification could be effective for the improvement of feedstock in cellulosic ethanol production.

It is well known that the removal of lignin results in an increase in the level of saccharification of plant cell walls, because lignin occurs in close association with cellulose microfibrils. The removal of lignin certainly increased the enzymatic hydrolysis levels in both of the transgenic and the wild type plants. Nevertheless, the level of enzymatic hydrolysis was still higher in the transgenic plant (Fig. 1) than that in the wild type after delignification (Fig. 2). This result shows that xyloglucan could be recalcitrant for the enzymatic hydrolysis.

Our results identify xyloglucan as a key hemicellulose that tightens as a tether of cellulose microfibrils in the secondary walls. If the tether could be loosened rather than tightened during growth, not only could the trees grow faster[10,16–18] but cellulose microfibrils could also be highly hydrolyzed by cellulase. These effects would be in agreement with the finding[19–21] that xyloglucan tightens gelatinous layers to the S2 layer in the secondary walls and provides tension to the wall structure. The genetic reduction of xyloglucan in xylem has been confirmed to accelerate the hydrolysis of paracrystal 1,4-β-glucans in cellulose microfibrils. Such technology could be applied as *in fibril* modification rather than *in wall* modification, such as reduction of lignin, or *in planta* modification, such as autohydrolysis during postharvest. All of these modifications are required, step by step, to facilitate bioprocess consolidation for bioethanol production.

Acknowledgments Funding for this work was provided by the NEDO program: New Energy Technology Development of Technology for High-Efficiency Conversion of Biomass and other Energy Converting Fast-Growing Tropical Trees into Feedstock for Cellulosic Ethanol and JSPS Kakenhi (grants 19208016 and 19405030). This work is also part of the outcome of the JSPS Global COE Program (E-04): In Search of Sustainable Humanosphere in Asia and Africa.

References

1. Najafi G, Ghobadian B, Tavakoli T, Buttsworth DR, Yusaf TF, Faizollahnejad M (2009) Performance and exhaust emissions of a gasoline engine with ethanol blended gasoline fuels using artificial neural network. Appl Energ 86:630–639
2. Gray KA, Zhao L, Emptage M (2006) Bioethanol. Curr Opin Chem Biol 10:141–146
3. Lin Y, Tanaka S (2006) Ethanol fermentation from biomass resources: current state and prospects. Appl Microbiol Biotechnol 69:627–642
4. Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J, Eckert CA, Frederick WJ Jr, Hallett JP, Leak DJ, Liotta CL, Mielenz JR, Murphy R, Templer R, Tschaplinski T (2006) The path forward for biofuels and biomaterials. Science 311:484–489
5. Kaida R, Kaku T, Baba K, Hartati S, Sudarmonowati E, Hayashi T (2009) Enzymatic saccharification and ethanol production of *Acacia mangium* and *Paraserianthes falcataria* wood, and *Elaeis guineensis* trunk. J Wood Sci. DOI 10.100T/s10086-009-1038-0
6. Huang H-J, Ramaswamy S, Al-Dajani W, Tschimer U, Cairncross RA (2009) Effect of biomass species and plant size on cellulosic ethanol: a comparative process and economic analysis. Biomass Bioenerg 33:234–246
7. Binkley D, Senock R, Bird S, Cole TG (2003) Twenty years of stand development in pure and mixed stands of *Eucalyptus saligna*

and nitrogen fixing *Falcataria moluccana*. Forest Ecol Manag 182:93–102
8. Nakamura S, Hayashi T (1993) Purification and properties of an extracellular *endo*-1,4-β-glucanase from suspension-cultured poplar cells. Plant Cell Physiol 34:1009–1013
9. Ohmiya Y, Samejima M, Shiroishi M, Amano Y, Kanda T, Sakai F, Hayashi T (2000) Evidence that *endo*-1,4-β-glucanase act on cellulose in suspension-cultured poplar cells. Plant J 24:147–158
10. Hartati S, Sudarmonowati E, Park YP, Kaku T, Kaida R, Baba K, Hayashi T (2008) Overexpression of poplar cellulase accelerates growth and disturbs the closing movements of leaves in sengon. Plant Physiol 147:552–561
11. Mitsuhara I, Ugaki M, Hirochika H, Ohshima M, Murakami T, Gotoh Y, Katayose Y, Nakamura S, Honkura R, Nishimiya S, Ueno K, Mochizuki A, Tanimoto H, Tsugawa H, Otsuki Y, Ohashi Y (1996) Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants. Plant Cell Physiol 37:49–59
12. Updegraff DM (1969) Semimicro determination of cellulose in biological materials. Anal Chem 32:420–424
13. Hayashi T (1989) Measuring β-glucan deposition in plant cell walls. In: Linskens HF, Jackson JF (eds) Modern methods of plant analysis: plant fibers. Springer, Berlin Heidelberg New York, pp 138–160
14. Chiang VL, Funaoka M (1990) The difference between guaiacyl and guaiacyl–syringyl lignins in their responses to kraft delignification. Holzforschung 44:309–313
15. Miyafuji H, Nakata T, Ehara K, Saka S (2005) Fermentability of water-soluble portion to ethanol obtained by supercritical water treatment of lignocellulosics. Appl Biochem Biotechnol 121–124: 963–971
16. Park YW, Tominaga R, Sugiyama J, Furuta Y, Tanimoto E, Samejima M, Sakai F, Hayashi T (2003) Enhancement of growth by expression of poplar cellulase in *Arabidopsis thaliana.* Plant J 33:1099–1106
17. Shani Z, Dekel M, Tsabary G, Goren R, Shoseyov O (2004) Growth enhancement of transgenic poplar plants by overexpression of *Arabidopsis thaliana endo*-1,4-β-glucanase (*cel1*). Mol Breed 14:321–330
18. Park YW, Baba K, Furuta Y, Iida I, Sameshima K, Arai M, Hayashi T (2004) Enhancement of growth and cellulose accumulation by overexpression of xyloglucanase in poplar. FEBS Lett 564:183–187
19. Nishikubo N, Awano T, Banasiak A, Bourquin V, Ibatullin F, Funada R, Brummer H, Teeri TT, Hayashi T, Sundberg B, Mellerowicz EJ (2007) Xyloglucan *endo*-transglycosylase (XET) functions in gelatinous layers of tension wood fibers in poplar – a glimpse into the mechanism of the balancing act of trees. Plant Cell Physiol 48:843–855
20. Baba K, Park YW, Kaku T, Kaida R, Takeuchi M, Yoshida M, Hosoo Y, OjioY, Okuyama T, Taniguchi T, Ohmiya Y, Kondo T, Shani Z, Shoseyov O, Awano T, Serada S, Norioka N, Norioka S, Hayashi T (2009) Xyloglucan for generating tensile stress to bend tree stem. Mol Plant. doi:10.1093/mp/ssp054
21. Taniguchi T, Omiya Y, Kurita M, Tsubomura M, Kondo T, Park YW, Baba K, Hayashi T (2008) Biosafty assessment of transgenic poplars overexpressing xyloglucanase (AaXEG2) prior to field trials. J Wood Sci 54:408–413