Enhancement of saccharification in transgenic *Acacia mangium* overexpressing xyloglucanase

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Abstract

Transgenic mangiums (*Acacia mangium*) overexpressing *Aspergillus* xyloglucanase were generated by *Agrobacterium* method. The overexpression of xyloglucanase decreased xyloglucan content in the walls. The levels of saccharification and successive ethanol production were increased in the wood of the transgenic *Acacia mangium* overexpressing xyloglucanase compared with that of the wild type plant, and even after delignification of the wood. We propose that removal of xyloglucan intercalated into cellulose microfibrils facilitates degradation of cellulose microfibrils with a different action from lignin. Biotechnology that increases the level of saccharification could be effective for the improvement of feedstock in cellulosic ethanol production.

Introduction

Hemicelluloses are instrumental in the formation of the cellulose microfibril network in higher plants, and genetic alteration of hemicelluloses contents can both disrupt or enhance cellulose formation. One of the functional hemicelluloses is xyloglucan, which occurs as bridges between cellulose microfibrils by surface adsorption and chain intercalation (Hayashi, 1989). Overexpression of xyloglucanase caused the degradation of xyloglucan tethers, which decreased wall pressure and accelerated cell enlargements in the growing plant cells (Park *et al*., 2004). Xyloglucan intercalated into cellulose microfibrils could be one of the recalcitrant components in the saccharification of lignocelluloses (Kaida et al. 2009a). Transgenic *Paraserianthes falcataria* expressing poplar cellulase in the cell walls did not decrease cellulose content but caused a decrease in xyloglucan content (Hartati et al. 2008). The level of saccharification and successive ethanol production was increased in the wood of the transgenic *P. falcataria* (Kaida et al. 2009b). Genetic modification biotechnology that decreases the level of xyloglucan could be effective for the improvement of saccharification and ethanol production.

Lignocellulosic biomass from woody plants has the potential to become a major source of fermentable sugars for the production of bioethanol because trees are the most abundant biomass on the earth. The conversion of lignocellulosic biomass

into biofuels remains a significant challenge because it is still hard to hydrolyze lignocellulose with enzymes. Cellulose in lignocellulosic biomass is well protected from chemical, enzymatic and microbial digestion by a hemicellulose and lignin complex.

Mangium (*Acacia mangium*) is one of the tropical forest trees that grows symbiotically with nitrogen-fixing *Rhizobium* at colonizing infertile sites. The tree grows well not only in cleared forest sites but also in degraded sites, such as weedy *Imperata* grasslands and mining sites. The attribute of the tree include rapid early growth, high cellulose recovery (for pulp, timber and fuel), and tolerance of a range of soil types and pH. The specific gravity of the wood is more than 0.5 because of high cellulose density. As its good characteristics, it is selected as one of Indonesian Industrial Timber Estate (HTI) commodities to promote the production of raw material for wood-based industry. However, the levels of enzymatic hydrolysis of cellulose from *A. mangium* wood are substantially low (Kaida et al. 2009c). In this study, transgenic mangiums (*Acacia mangium*) overexpressing *Aspergillus* xyloglucanase were generated and saccharification levels of the xylem were determined.

Results and Discussion

1. Grafted seedlings

Vegetative seedlings were obtained from the branched stems of 12 year-old mangium trees (16 to 20 m) at 1-m from shoot top through the grafting method by peeling skin, removing cambium and covering with at wet kadaka fibers with plastic wrap. It took about 3 months to obtain rooting in the plastic wrap. Upper branches from the top to 2.5 m of main stem could produce higher rooting efficiency at about 80% than the lower branches at less than 50%. Nevertheless, the grafted seedlings derived from the upper branches have higher survival rate at 60 to 80% than those from the lower branches at less than 50%, when they were transfer to soil. The result showed that higher rooting efficiencies and survival vegetative plant seedlings were obtained from the branches which occurred at the top to 2.5 m of main stem.

2. Transformation

When one-year-old grafted seedlings formed flower bud, *Agrobacterium* solution was sprayed in the closed bags in the closed greenhouse. After they were incubated in the bags for three days, the bags were removed. Seeds were formed in the pods during 25 to 30 weeks after flowering.

Four independent transgenic seeds were resistant to grow in the MS medium

in the presence of kanamycine from 149 seeds after spraying *Agrobacterium* solution. Control seed showed a germination rate at 65.5% in the MS medium in the absence of kanamycine. Kanamycine-resistant seeds could germinate and develop to become normal seedlings. The transgenic number obtained represents a transformation efficiency at about 4.1%, although the transformation efficiencies of acacia plants were obtained as those at 7% (*A. crassicarpa,* Yang, 2008), 4% (*A. sinuate,* Vengadesan *et al*., 2006) and 30% (*A. mangium*, Hong, 2002) by tissue culture via organogenesis using selectable and reporter genes. Although the transformation efficiency of our procedure was not high so far, the efficiency could be increased by the timing of spraying to pads.

3. Xyloglucanase expression

We obtained three independent transgenic lines that expressed a xyloglucanase from *Asperigillus aculeatus* (*AaXEG2*) under the control of a constitutive promoter and the *PopCel1* signal sequence. To assay the expression of the transgene, we used an antibody against the *AaXEG2* gene product, which recognizes as a single 28 kDa band on a western blot. The xyloglucanse was present in their leaf and petiole, running at a position corresponding to the size of the mature xyloglucanase. No protein was detected in control plants. The level of expression was different among the three transgenic plants, in which two transgenic plants (trg2 and 3) showed stronger signals than XEG1. They also showed different levels of xyloglucanase activities, which were about ten to twenty-fold higher than that of the control.

Based on the carbohydrate analysis of whole leaves including rachis of pinna with petiole, the transgenic plants contained less wall-bound xyloglucan in non-cellulosic polysaccharides than those in the wild-type plants. A decrease in xyloglucan content corresponded to the increased level of xyloglucanase expression and activity in their cell walls. In the secondary wall with high expression, the wall had relatively a little bit decreased amount of hemicellulose and lignin with the increased amount of cellulose. Based on methylation analysis, 4,6-linked glucose due to xyloglucan backbone was found at a low proportion in the transgenic lines, although one of the major sugar in the hemicellulose was 4-linked xylose, due to xylan.

4. Growth response

The two transgenic plants (trg2 and trg3) with high expression grew faster in their heights and diameters of stems than the wild type, although one line with low expression (trg1) grew lower than the wild type. The transgenic seedlings (trg2 and trg3) showed an early leaf development, in which the stage at three bipinnate leaves

proceeded to that at enlarged petiole with those by 2-weeks-faster than that in the wild types.

5. Effect of overexpression of xyloglucanase on enzymatic hydrolysis of wood

Enzymatic hydrolysis of woody meal was significantly increased by the overexpression of xyloglucanase during experimental time course. When 100 mg of woody meal was subjected to saccharification for 48 h, free reducing sugar was yielded from the transgenic plants (trg1, 2 and 3) at 13.0 mg and from the wild type at 9.4 mg. The level of glucose residue in the sugar was 9.2 mg in the transgenic wood and 6.8 mg in the wild type. The level of saccharification in the transgenic plants was 1.4-fold higher than that in the wild type. During the saccharification, the level of cellulose hydrolysis was accelerated for the transgenic plants compared with the wild type plants. This result showed that the overexpression of xyloglucanase accelerated the saccharification of wood, in which cellulose hydrolysis was also effectively increased.

6. Ethanol production

When saccharification was accompanied by fermentation with yeast, ethanol production was accelerated for the wood overexpressing xyloglucanase compared with the wild type. The ethanol production was increased 1.4-fold by the overexpression of xyloglucanase, in which the increased level of the production was similar to that of succharification.

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 xyloglunase.

7. Saccharification of delignificated wood

Delignification of wood with sodium chlorite accelerated the enzymatic hydrolysis of meal about 2-fold higher than in the non-treated samples. When 100 mg of woody meal was subjected to delignification and 48-h-saccharification, free reducing sugar was formed from the transgenic plants at 29.3 mg and from the wild type at 26.4 mg of it. The level of released glucose was 19.4 mg in the transgenic wood and 16.9 mg in the wild type. These results show that the efficiency of transgenic reduction of xyloglucan on saccharification is kept in delignification sample.

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 placed horizontally are unable to bend upward, but cellulose microfibrils could also be highly hydrolyzed by cellulase (Kaida et al. 2009a, b). These effects would be in agreement with the finding 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.

Experimental methods

1. Plant materials

Flower buds which is generated from 1 year old grafted mangium (*A. mangium*) trees grown on soil in pots were used as plant material for transformation. The branches of several mangium trees (12-years old) were grafted by peeling branch skin and covering with root plant fiber and plastic wrap.

2. Transgenic constructs and plant transformation

Aspergillus aculeatus cDNA for xyloglucanase was amplified from the first strand cDNA as a template by polymerase chain reaction (PCR) using a forward primer containing a *Xba*I site (5P-GCTGCCAGTCTAGAGC CCGCAGCGAC-3P) and a reverse primer containing an internal *Sac*I site (5P-CTCCCGTCAGCCGCGGTCCACGCAAC- 3P) complementary to the DNA sequence of *AaXEG2* (accession number AY160774). The *Xba*I-*Sac*I fragment for *AaXEG2* was subcloned into pBluescript II (SK3). The xyloglucanase exhibits 98% identity at the DNA level and 99% identity at the amino acid level to AaXEG1 (accession number AF043595). The signal peptide (Met1 to Leu30) of *Populus alba* cellulase was cloned in pGEM-T Easy vector from PaPopCel1 cDNA (accession number D32166) by PCR using a forward primer containing a *BamH*I site (5P-CTAGTGGATCCTTTGGAG-3P) and a reverse primer containing an *Xba*I site (5P-AGCATAGTCTAGAGAAGTGAAGGC-3P). The *BamH*I-*Xba*I fragment was ligated into the pBluescript II (SK3) harboring the *Xba*I-*Sac*I fragment for *AaXEG2*. The chimeric DNA for PopCel1 signal peptide and XEG2 mature protein was excised with *BamH*I and *Sac*I, and inserted into the *BamH*I-*Sac*I site of the binary vector pBE2113-GUS, under control of the CaMV35S promotor and E12-6 enhancer sequences. The

plasmid constructs were electroporated into *Agrobacterium tumefaciens* LBA4404 which was to transform mangium by spraying bacterial solution of OD 0.6 to flower bud.

3. Transformant selection from seeds

When one-year-old grafted seedlings formed flower bud, the bud was sealed with a plastic bag. A hole was made in the bag close to the flower bud, and *Agrobacterium* solution was sprayed into the bag. Then, the bag was sealed and incubated between the bacterium and bud for three days. Seeds were formed in the pods from 25 to 30 weeks. Seeds produced by spraying the solution in the absence of *Agrobacterium* were used as a control.

Mangium seeds derived from the buds were washed with water five times, soaked in hot water at 80 ^{o}C for 10 min. The seeds were then sterilized with 2% benlate for 30 min and 4% dithane for 30 min followed with 70% ethanol for 3 minutes and 0.05% $HgCl_2$ for 3 min. The seeds were washed five times with sterilized water and germinated in the MS agar medium containing 300 μg/ml kanamycin. After germination, the meristems of kanamycine-resistant shoots were subjected to propagation in the presence of BAP and IBA in order to form multiple shoots. Each shoot were then transferred to the MS medium containing IBA in order to form roots.

4. Western blot analysis

Young shoots of about 0.3 g fresh weight was homogenized in 20 mM sodium phosphate buffer (pH 6.2) in a mortar, and the wall residue was washed three times. The wall-bound proteins were extracted from the wall residue with a buffer containing 1 M NaCl. The proteins were then subjected to electrophoresis with 10% SDS-PAGE, electrotransferred to Hybond-C Extra (Amersham), and probed with an antibody against the AaXEG sequence, followed by a second antibody using the Toyobo ABC High-HRP immunostaining kit. 29 number lines of plants were assayed.

5. Assay of xyloglucanase activity

Each enzyme preparation was obtained from the wall residue of leaves with a buffer containing 1 M NaCl, and its activity was assayed viscometrically at 28^{o}C for 4 h, using 0.1 mL of the enzyme preparation plus 0.9 mL of 10 mM sodium phosphate buffer (pH 6.2) containing 0.5 % (w/v) tamarind xyloglucan in semimicroviscometers from Cannon Instruments. One unit of activity is defined as the amount of enzyme required to cause 0.1% loss in viscosity in 2 h under such conditions. Protein was determined using the Coomassie Plus protein assay reagent (Pierce), according to the method described by Bradford.

6. Wall analysis

Polysaccharides of hemicelluloses of woody meal were successively extracted three 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. 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.

Whole leaves including rachis of pinna with petiole were ground in liquid nitrogen and successively extracted four times with 10 mM sodium phosphate buffer (pH 7.0) and three times with 24% KOH containing 0.1% NaBH4 at less than 45^{o}C for 3 h in an ultrasonic bath. Non-cellulosic polysaccharides were determined in the 24% KOH extracts by the phenol/sulfuric acid method. Xyloglucan was determined by the iodine/sodium sulfate method.

7. Growth measurement

The growth response of the transgenic plants was monitored after they were transplanted in soil and habituated for 2 weeks under nonsterile conditions. Each stem (around 15 cm) was marked at a height of 5 cm, which was used as a reference point for measuring its height and diameter every third day. The length of stem was determined from the top to the reference point. The timing of leaf development was determined from 2-cm seedlings containing one bipinnate by observing the development of leaves every day for 14 weeks.

8. Preparation of woody meal

Pieces of *A. mangium* 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 30 min. The meal samples were used for saccharification alone or in combination with fermentation.

9. 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 FPU of the cellulase preparation (2.0 mg). 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 Somogyi-Nelson method. The amount of glucose was also determined by the action of glucose oxidase using the Glucose C2 (Wako, Osaka, Japan).

10. 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 FPU 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. Butanol was used as an internal standard.

11. Treatment for delignification of woody meal

The woody meal (100 mg) that was autoclaved at 120°C for 3 min, and delignification 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. The meal was washed five times by centrifugation at 3,000 rpm for 5 min. The meal samples were confirmed as lignin-free (nondetectable) by the Klason method, and then used for enzymatic hydrolysis.

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References

Hayashi T (1989) Xyloglucans in the primary cell wall. Annu Rev Plant Physiol Plant Mol Biol 40, 139-168

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

Kaida R, Kaku T, Baba K, Oyadomari M, Watanabe T, Nishida K, Kanaya T, Shani Z, Shoseyov O, Hayashi T (2009a) Loosening xyloglucan accelerates the enzymatic degradation of cellulose in wood. Mol Plant 2, 904-909.

Kaida R, Kaku T, Baba K, Hartati S, Sudarmonowati E, Hayashi T (2009b) Enhancement of saccharification by overexpression of poplar cellulase in sengon. J Wood Sci 55, 435-440

Kaida R, Kaku T, Baba K, Hayashi T (2009c) Enzymatic saccharification and ethanol production of *Acacia mangium* and *Paraserianthes falcataria* wood, and oil palm trunk. J Wood Sci 55, 381-386

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

Vengadesan G, Amutha S, Muruganantham M, Perm Anand R, Ganapathi A (2006) Transgenic Acacia sinuate from *Agrobacterium tumefacience*-mediated transformation of hypocotyls. Plant Cell Report 25, 1174-1180

Xie DY, Hong Y (2002) *Agrobacterium*-mediated genetic transformation of Acacia mangium. Plant Cell Rep 20, 917-922

Yang M, Xie X, Zheng C, Zhang F, He X, Li Z (2008) *Agrobacterium tumefaciens*-mediated genetic transformation of Acacia crassicarpa via organogenesis. Plant Cell Tiss Organ Cult 95, 141-147