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NOTE

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

Improvement of fermentable sugar yields of mangium through transgenic overexpression of xyloglucanase

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Abstract Recalcitrance to saccharification is a major limiting factor of the conversion of lignocellulosic biomass to ethanol. Levels of wood saccharification and subsequent ethanol production were higher in transgenic mangium (*Acacia mangium*) trees overexpressing xyloglucanase than in wild-type plants, even after delignification of the wood. We propose that a decrease in the quantity of xyloglucan that is intercalated into cellulose microfibrils could facilitate the process of saccharification.

Key words *Acacia mangium* · Saccharification · Xyloglucan · Xyloglucanase

Introduction

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 form of biomass on the Earth. However, the conversion of lignocellulosic biomass into biofuel remains a significant challenge because it is difficult to hydrolyze lignocellulose with enzymes. The cellulose in lignocellulosic biomass is bound up in a hemicellulose–lignin complex that makes it well defended against chemical, enzymatic, and microbial digestion. In seeking a way around these defenses, we focused on the genetic modification of cell walls of woody plants that are easily hydrolyzable.

Xyloglucan intercalated into cellulose microfibrils could be one of the components conferring recalcitrance to the saccharification of lignocelluloses.[1] Transgenic *Paraserianthes falcataria* expressing poplar cellulase in the cell walls did not exhibit decreased cellulose content but did exhibit decreased xyloglucan content.[2] This wood also permitted increased levels of both saccharification and subsequent ethanol production,[3] suggesting that genetic modifications which decrease xyloglucan levels may increase the efficiency of saccharification and ethanol production.

Mangium (*Acacia mangium*) is one of the fastest growing tree species in the world and is therefore a potentially useful tropical tree species for biomass production in industrial forests. One obstacle to its utility is the low level of enzymatic hydrolysis of cellulose from *A. mangium* wood.[4] In our previous work,[5] we generated transgenic mangium lines overexpressing *Aspergillus* xyloglucanase, which significantly decreased the amount of xyloglucan in the cell walls. The present study describes the levels of saccharification and ethanol production achieved using wood from these transgenic mangium trees.

Materials and methods

Plant materials

Transgenic mangium (*Acacia mangium*) plants overexpressing *Aspergillus aculeatus* xyloglucanase (AaXEG2, accession number AY160774) were produced as part of a previous study.[5] This study employed three lines of transgenic plants (trg1, trg2, and trg3).

Preparation of woody meal

We harvested 1-year-old mangium stem woods (1.2–1.8 m in length, 3–4 cm in diameter) grown in biosafety containment. Pieces of stem wood were stripped of their bark and dried in an oven at 70°C for 16 h, then milled into a powder using a ball mill (MM400; Retsch, Haan, Germany) at 15 rps for 30 min. The meal samples were used for saccharification alone or for saccharification in combination with fermentation in the presence of yeast.

T. Kaku · K. Baba
Research Institute for Sustainable Humanosphere, Kyoto University, Kyoto 611-0011, Japan

R. Kaida · T. Hayashi (✉)
Department of Bioscience, Tokyo University of Agriculture, Setagaya-ku, Tokyo 156-8502, Japan
Tel. +81-3-5477-2760; Fax +81-3-5477-2760
e-mail: t4hayash@nodai.ac.jp

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

Enzymatic hydrolysis

Meal (100 mg) was autoclaved at 120°C for 3 min to impregnate it with water and then washed once with water through 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 50 mM sodium acetate buffer, pH 4.8, containing 0.02% Tween 20 and 0.4 filter paper units (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 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.[6] The amount of glucose was also determined based on the action of glucose oxidase using the Glucose C2 (Wako, Osaka, Japan).

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 50 mM sodium acetate buffer, pH 4.8, containing 0.02% Tween 20 and 0.4 FPU cellulase preparation). Each mixture was incubated at 35°C in a rotary shaker set at 135 rpm. About 100 µl supernatant was collected at 6, 24, and 48 h after the start of incubation. The ethanol formed was measured through gas chromatography on a Supelcowax-10 column (0.53 mm i.d. × 15 m; Supelco, Bellefonte, PA, USA) at 50°C using an Agilent (Santa Clara, CA, USA) gas chromatograph. Butanol was used as an internal standard.

Treatment for delignification of woody meal

Woody meal (100 mg) that had been autoclaved at 120°C for 3 min (see above) was subjected to delignification in 5 ml 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 means of centrifugation at 3,000 rpm for 5 min. The meal samples were confirmed as lignin free (nondetectable) by the Klason method, then used for enzymatic hydrolysis.

Results

Effect of overexpression of xyloglucanase on enzymatic hydrolysis of wood

Chemical analyses of wood revealed that the overexpression of xyloglucanase in mangium slightly increased cellulose levels and slightly decreased lignin content.[5] Methylation analysis of hemicellulose revealed that the transgenic plants contained less 4,6-linked glucose than the wild type did: specifically, the amounts of 4,6-linked glucose in trg1, trg2, and trg3 were 4%, 21%, and 79%, respectively, of the amount in the wild type. The transgenic plants had less wall-bound xyloglucan in the xylem than did the wild type.

Significantly more enzymatic hydrolysis of woody meal occurred during the experimental time course in plants overexpressing xyloglucanase (Fig. 1). When 100 mg woody meal was subjected to saccharification for 48 h, the average of the three transgenic plants (trg1, trg2, and trg3) yielded 13.0 mg free reducing sugar whereas the wild type yielded only 9.4 mg. Glucose residue in the sugar was 9.2 mg in the transgenic wood and 6.8 mg in the wild type (Fig. 1). The amount of saccharification occurring in the transgenic plants was thus 1.4 times that occurring in the wild type, partly because, during saccharification, cellulose hydrolysis occurred faster in the transgenic plants than in the wild-type plants. The increase in cellulose hydrolysis in each of the three transgenic plant corresponded to a decrease at 4% to 79% levels in xyloglucan content in each transgenic plant.[5] Thus, the overexpression of xyloglucanase accelerates the saccharification of wood and effectively increases the rate of concurrent cellulose hydrolysis.

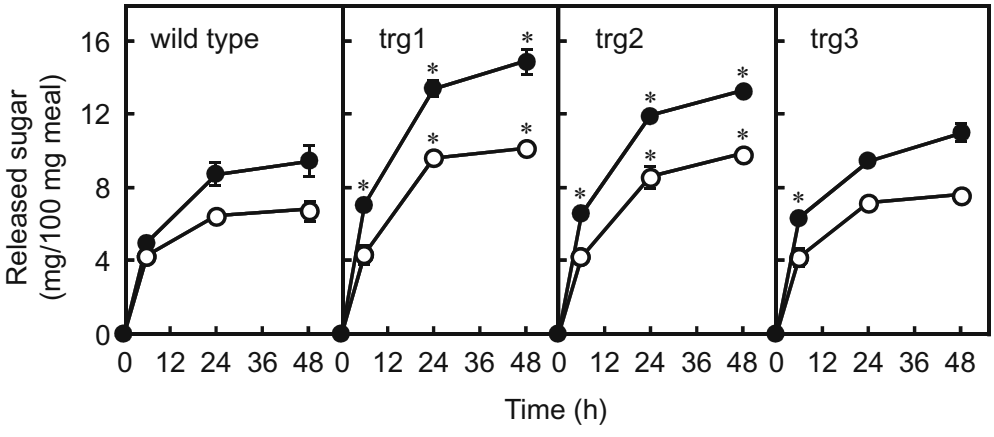

Fig. 1. Sugar released by enzymatic hydrolysis of meals of mangium wood. *Closed circles*, reducing sugars; *open circles*, glucose. Individual values represent means ± SE of three independent experiments. *Asterisks* represent a statistically significant difference (Student's *t* test, $P < 0.05$) in comparison with the wild type

Ethanol production

When saccharification was accompanied by fermentation with yeast, ethanol production was accelerated in the wood overexpressing xyloglucanase (Fig. 2). The level of ethanol production was 1.4 times greater in plants overexpressing xyloglucanase; this increase was similar in scale to the increase in saccharification associated with the overexpression of xyloglucanase.

Some product inhibition may have occurred during saccharification, although not during simultaneous enzymic saccharification and fermentation, because the theoretical levels of ethanol production were 10% to 15% higher than the levels of fermentable sugars (glucose, mannose, and galactose) produced during saccharification. The higher the level of saccharification, the more inhibition occurred. Accordingly, this inhibition was more noticeable in the wood overexpressing xyloglucanase.

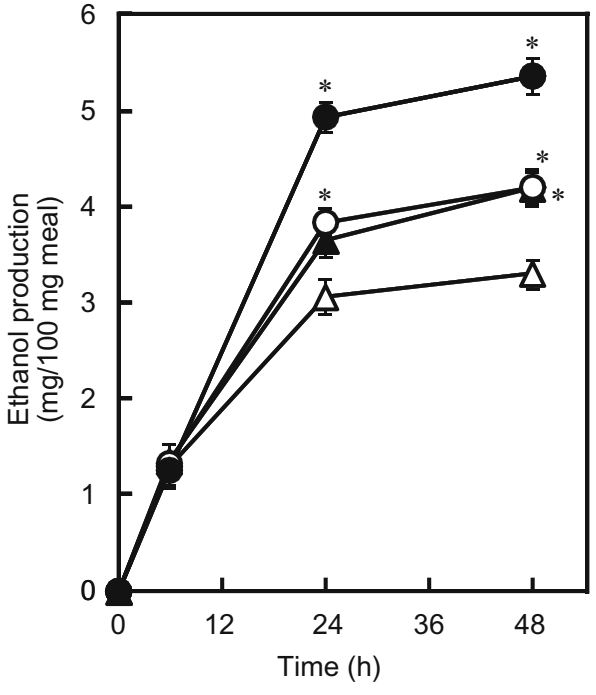

Fig. 2. Ethanol production resulting from simultaneous enzymatic saccharification and fermentation. *Closed circles*, trg1; *open circles*, trg 2; *closed triangles*, trg 3; *open triangles*, wild type. Individual values represent means ± SE of three independent experiments. *Asterisks* represent a statistically significant difference (Student's *t* test, $P < 0.05$) in comparison with the wild type

Saccharification of delignificated wood

Delignification of wood with sodium chlorite accelerated the enzymatic hydrolysis of meal to about twice the rate achieved in nontreated samples. When 100 mg woody meal was subjected to delignification and 48 h saccharification, 29.3 mg free reducing sugar was extracted from the transgenic plants and 26.4 mg from the wild type (Fig. 3). Transgenic woods released 19.4 mg glucose while the wild type released 16.9 mg. These results show that the increase in the efficiency of saccharification that results from the genetic reduction of xyloglucan persists when samples are delignificated.

Discussion

We have demonstrated that the wall structure modification resulting from the overexpression of xyloglucanase, which causes a 4% to 79% decrease in the amount of wall-bound xyloglucan in the transgenic wood,[5] enhances the levels of saccharification and fermentation in mangium. We confirmed that saccharification occurring in the transgenic plants was thus 1.4 times that occurring in the wild type. It should be noted that overexpression of xyloglucanase accelerated the saccharification of wood and effectively increased the rate of cellulose hydrolysis that occurred during saccharification, as shown in poplar.[1] This effect could be harnessed into biotechnological developments aimed at improving the feedstock for cellulosic ethanol production by increasing the ease of saccharification.

It is well known that a decrease in lignin content results in an increase in the level of saccharification of plant cell walls[7] because lignin occurs in close association with cellulose microfibrils. In the present study, the removal of lignin unquestionably increased enzymatic hydrolysis in both

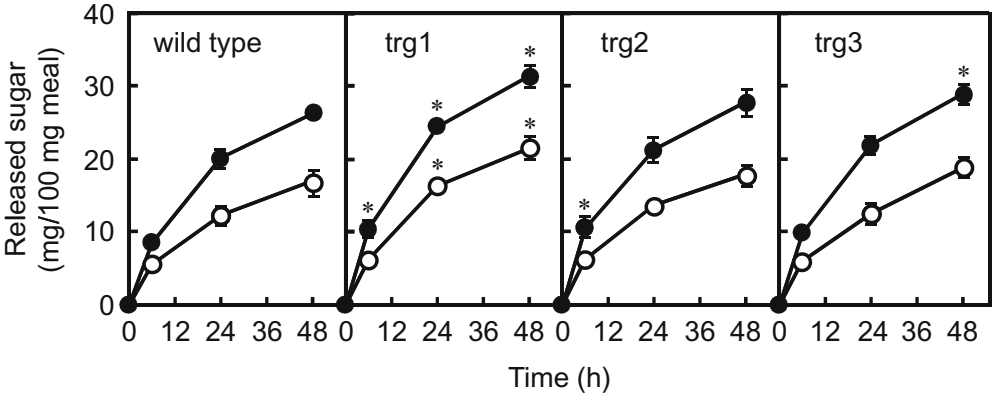

Fig. 3. Sugar released during enzymatic hydrolysis of woody meal from which lignin had been removed. *Closed circles*, reducing sugars; *open circles*, glucose. Individual values represent means ± SE of three independent experiments. *Asterisks* represent a statistically significant difference (Student's *t* test, $P < 0.05$) in comparison with the wild type

transgenic and wild-type plants. After delignification, however, the level of enzymatic hydrolysis was still higher in the transgenic plants than that in the wild type (Fig. 3). This result indicates that xyloglucan could be one of the components conferring recalcitrance to enzymatic hydrolysis.

We suggest xyloglucan is a key hemicellulose that tethers cellulose microfibrils in the secondary walls ever more tightly together. If these tethers could be loosened rather than tightened during growth, not only would trees placed horizontally be unable to bend upward,[8] but the cellulose microfibrils could also be highly hydrolyzed with cellulase preparation.[1] These predictions are supported by the finding that xyloglucan tightens the microfibrils in the secondary walls. Such technology could also be applied in the form of an in-fibril modification, similar to the reduction of lignin, or an in-planta modification, similar to postharvest autohydrolysis, rather than an in-wall modification. Because transgenic mangium xylem is still somewhat resistant to saccharification in spite of the improvements discussed here, it seems likely that mangium wood has other characteristics that also confer recalcitrance to enzyme hydrolysis.

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